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OSTEOPONTIN AND ANTI-OSTEOPONTIN ANTIBODIES AS POTENTIAL BIOMARKERS OF IMMUNE ACTIVATION IN PATIENTS WITH ALLERGIC DISEASES: A COMPREHENSIVE APPROACH

Cycle XXIX

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During my PhD, I had the precious opportunity to realize one of my greatest dreams: working as intern in a research laboratory and matching my clinical experience as Medical Doctor specialized in Allergology and Clinical Immunology with research learning and laboratory findings. Despite many moments of hard work and even frustration, I do feel that I have fullfilled my scientific expertise with a unique experience: I dedicated myself to an entire research project, from the protocol writing to the submission to Ethic Committee; from the patients recruitment to the analysis of their blood.

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1. Preface

The field of Allergology has grown up enormously in the last decades, due to the increasing prevalence of the allergic diseases in general, particularly respiratory allergies: it is estimated that by 2025, 1 out of 2 adults will be allergic. This phenomenon is associated with an important socio-economic burden, due to direct (i.e., costs of drugs, doctor visits, hospitalizations, access to emergency department) and indirect costs (i.e., days of working absence).

For these reasons, many research efforts are now focused on the study of molecular biomarkers, that allow to diagnose the patients at an early stage, to formulate a phenotypization and a prognosis for the patients, and to identify the proper treatment for each phenotypes, according to the principles of the "Precision Medicine".

At the Research Laboratory of Immunology, Health Science Department, of the University of Piemonte Orientale "Amedeo Avogadro" (Novara, Italy), Prof. Dianzani and his team found an association between genetic mutations and polimorphisms of genes involved in the regulation of inflammatory processes in autoimmune diseases: among these, the gene of a protein called osteopontin, that seems to represent a good biomarker of immune activation in autoinflammatory disorders.

Since in literature there are some preliminary and interesting data about osteopontin and allergic diseases, we decided to study this protein in a local cohort and to compare the results with those obtained from an ethnically different population.

2. Background

2.1 Introduction on allergic diseases

The immune system is represented by a complex network of molecules, cells, tissues and organs targeted to protect against different foreign organisms that are potentially harmful. Schematically, the functional organization of the immune system provides the maintenance of both a state of non-responsiveness to self molecules and a protective role against foreign antigens (not self). Besides the common peripheral tolerance mechanisms, such as anergy, idiotypic network, cellular apoptosis, anatomical segregation of self-antigens and clonal ignorance, the discovery of regulatory T cells (Treg) in active suppression of immune responses has been crucial. In 1995 Sakaguchi et al. discovered a population of $CD4^+$ T cells characterized by high surface expression of CD25 and ability to prevent the onset of autoimmune phenomena in a murine model. Multiple studies have been conducted afterwards, in order to characterize Treg cells about their immunophenotypic and functional aspects, in physiological and pathological conditions [1, 2].

Currently, it is believed that quantitative and qualitative impairment of immune regulatory functions play a major role in the genesis of both allergic and autoimmune disorders. The role of Treg cells in the onset of autoimmune diseases is confirmed by the immune effects of thymectomy in the neonatal period in experimental murine models [3].

The term "*atopy*" refers to the hereditary tendency to type 1 hypersensitivity manifestations (according to Gell and Coombs classification), with a prevalent involvement of IgE antibodies isotype. "*Allergy*" is a pathophysiological condition characterized by an abnormal reactivity to particular substances (haptens) that are generally harmless in healthy subjects. [4, 5].

Allergic diseases are characterized by the tendency, in atopic subjects, to develop immune responses, in different body districts, with a specific polarization towards type 2 T helper cells (Th2) and production of IgE antibodies against environmental antigens that are commonly harmless. Several studies have demonstrated that atopic patients show a statistically lower percentage of circulating Treg if compared with controls; in addition, Treg isolated from allergic subjects are less effective in suppressing the proliferation of $CD4^+CD25^-$ T cells induced by allergens. The deviation of naïve T lymphocytes towards Th2 rather than Th1 cytokine pattern (Th1 polarization is pathognomonic for autoimmune diseases) after exposure to allergens, may therefore be related to quantitative and/or qualitative defects of Treg cells.

Allergies and autoimmune diseases are both characterized by multifactorial etiology, with an interaction between a complex immunological network (consisting of cells, cytokines, chemokines, growth factors, receptors and soluble factors) and environmental causes, on the basis of a genetic predisposition linked to multiple genes [6, 7].

Although HLA molecules are the best studied among genetic factors at the moment, but also other genes codifying molecules commonly involved in the modulation of immune responses may be involved [8, 9, 10]. In particular, molecules implicated in the down-regulation of immune responses have recently gained a great interest.

In the Research Laboratory of Immunology, Department of Health Sciences, University of Piemonte Orientale "Amedeo Avogadro" (Novara, Italy), genetic mutations that may impair the process of the immune down-regulation in patients with autoimmune diseases such as systemic lupus erythemathosus (SLE), type 1 diabetes mellitus, systemic sclerosis (SS) and multiple sclerosis (MS) have been identified: among them, the gene coding for osteopontin [11-17].

2.2 Osteopontin and its role in immune diseases

Osteopontin (OPN), also known as bone sialoprotein I (BSP-1 or BNSP), early T lymphocyte activation (ETA-1) or secreted phosphoprotein 1 (SPP1), is a secreted phosphoprotein initially isolated from bone matrix in 1986. The prefix '*osteo*-' indicates that the protein is expressed in bone, even though it is also espressed in other tissues. The suffix '*-pontin*' derives from 'pons', a Latin word meaning 'bridge', and indicates OPN's role as a linking protein: in fact, OPN is an extracellular structural protein and an organic component of bone.

Nevertheless, OPN is also a pleomorphic and proinflammatory cytokine known to modulate both cell activation and cytokines production. The protein is secreted by a wide range of immune cells including activated macrophages, monocytes, dendritic cells, neutrophils, activated T and B cells, and is present at the sites of inflammation and in the extracellular fluids.

OPN mediates different biological functions such as bone remodelling, macrophage response, cell migration, adhesion and it is implicated in the pathogenesis of several diseases including lymphoproliferative diseases, MS, rheumatoid arthitis (RA), cancer and other chronic inflammatory diseases.

OPN is a member of the SIBLINGs (Small Integrin Binding Ligand N-linked Glycoproteins) protein family, mapped to the human chromosome 4, and synthetized as a native 32 kDa polipeptide. The molecular weight however ranges between 45 and 75 kDa due to extensive post-translational modifications: proteolytic processing, glycosylation, sulphuration and phosphorylation. The protein has two calcium binding sites, two putative heparin binding domains [18], and multiple adhesion motifs which allow the interaction with many cell types including osteogenic cells of the bone matrix, smooth muscle, endothelial and inflammatory cells. OPN has an RGD (arginine-glycine-aspartate) integrin binding domain that mediates OPN interactions with αvβ1, αvβ3, αvβ5, αvβ6, α8β1, and α5β1 integrins [18-22]. In the carboxy terminal, the protein has a cryptic SVVYGLR (SLAYGLR in

mice) sequence that becomes exposed upon thrombin cleavage (Arg169-Ser170) and mediates interactions with α9β1, α4β1, and α4β7 integrins [23-25]. During inflammation thrombin acts on the cleavage site of OPN and generate two OPN fragments, N- and C-terminal (**figure 1**). There are functional differences between the two fragments and the full length protein [26]. The N-terminal (OPN-N) produced by thrombin proteolysis, is found to have a cryptic binding site (RGD domain), which is specifically mediates binding to α9β1 and α4β1 and cell migration [27-28]. The C-terminal fragment (OPN-C) binds to CD44 isoforms containing the CD44 v6 and v7 domains, inhibits IL-10 secretion and plays a role in cell-cell adhesion [29]. Moreover, we know that CD44 variants are overexpressed in several cancer types [30] and OPN-CD44 interaction has been found to promote metastasis in a variety of malignancies [31].

OPN costimulates T cell proliferation and it is classified as a T helper (Th) cell 1 cytokine because of its ability to enhance IFN-γ and IL-12, and to decrease IL-10 production.

Particularly interesting is the high phosphorylation level of the protein. Some Authors studied OPN purified from human milk, using a combination of Edman degradation and mass spectrometry. They characterized the pattern of phosphorylation of the native human protein and found up to 36 phosphosites, whose 34 were phosphoserine and 2 were phosphothreonine residues. The phosphorylation status here described is higher than the one described in the rat bone OPN, this suggesting a tissue-specific phosphorylation and/or dephosphorylation, that is in line with the great variety of biological functions of OPN in different tissues and biological fluids [32].

Phosphorylations are particularly interesting since they seem to be correlated with OPN functional activity. For example: OPN phosphorylation has been shown to be essential for osteoclasts attachment and bone resorption [33] but also with hydroxyapatite formation [34].

Moreover, the interaction between OPN and certain cell receptors seems to be dependent on the posphorylation status, i.e. macrophages are stimulated

to express IL-12 through OPN-β3-integrin receptor interaction only if the N-terminal part of the protein is phosphorylated [35].

On a clinical level, dephosphorylation of circulating human OPN has been shown to correlate with severe valvular calcification in patients with calcific aortic disease and with vascular smooth muscle cell calcification [36, 37].

Independently on its phosphorylation status, OPN seems to be involved in many diseases. For instance, is highly expressed in several tumor types: after thrombin cleavage it can act as a pro-angiogenic factor [37-40] and the OPN-CD44 interaction promotes metastasis dissemination of various malignancies [41].

Thrombin cleaved N-terminal OPN had been shown at high level in the synovial fluid samples of RA patients and its high amount was correlated with the the degree of disease severity [42]. Data obtained in animal models as well as in humans strongly suggest that OPN may play a role in the pathogenesis of MS [43]. Patients with MS display a variable clinical course, at onset, approximately 15% of the patients have a primary progressive form (PP), whereas the remainder start out with a relapsing-remitting form (RR), and most of them switch to a secondary progressive form (SP) within 10-30 years. Both genetic and environmental factors are involved in the development/ progression of MS and several studies point to a complex picture characterized by interactions between different combinations of scenes that may influence the immune response [44, 45]. In the MS context, OPN transcript is abundant in plaques dissected from brains of MS patients, whereas it is absent in the control brain tissue [46]. This finding was confirmed in rat experimental autoimmune encephalomyelitis (EAE) by microarray cDNA analysis of spinal cord tissue. OPN protein levels had been evaluated in plasma, serum and cerebrospinal fluid (CSF) of MS patients and the results were correlated with different clinical forms of MS.

Genetic variants (single nucleotide polymorphisms, SNPs) of OPN determining a higher production of basal levels of the cytokine have been previously identified in the research laboratory of Immunology in Novara. Autoimmune lymphoproliferative syndrome (ALPS) and Dianzani autoimmune lymphoproliferative disease (DALD) patients present high serum levels of OPN [47] and tissue inhibitor of metalloproteinase-1 (TIMP-1) [48]. In the Research Laboratory of Immunology in Novara, Dianzani and his team demonstrated that OPN haplotypes B and C are associated with an increased OPN production, a predisposition to DALD and a major risk of ALPS development. High levels of OPN might cause the observed increase in TIMP-1, because OPN induces TIMP-1 secretion in monocytes. Since both molecules inhibit lymphocyte apoptosis *in vitro*, it seems likely that their high levels may contribute to ALPS and DALD development in subjects with hypofunctional FAS system. Moreover, OPN promotes T helper 1 (Th1)-mediated immunity and also plays a role in the development of T helper 17 (Th17) cells, which are pro-inflammatory Th cells characterized by the secretion of interleukin 17A (IL-17A) and interleukin 17F (IL-17F). These cytokines play a key role in host defence and inflammatory diseases [49].

Regarding the allergic diseases, OPN seems to have a role in the inflammation and in the systemic allergen sensitization process, since its expression was found in human eosinophils and increases after GM-CSF and IL-5 activation. Eosinophil-derived OPN contributes to eosinophil-induced angiogenesis while recombinant OPN promotes eosinophil chemotaxis *in vitro,* effect mediated by α4β1 integrin binding [50, 51]. There are some existing preliminary data, mainly regarding the asthma model [51-56].

OPN deficiency seems to have a protective role against bronchial hyperresponsiveness and tissue remodeling in an animal model [57-50]. In addition, it has been shown that OPN expression in the bronchial wall is more represented in patients with asthma than in healthy subjects and is associated with tissue remodeling, severity degree of the disease and decline of pulmonary function over the time [60-63].

Moreover, OPN levels have been observed to be significantly increased in sputum supernatants of smoking asthmatic patients [64, 66].

Interestingly, there are also some preliminary data regarding the association between OPN and asthma as comorbidity in patients with allergic rhinitis [67].

The only data regarding polimorphisms of SPP1, OPN gene, were found in a Puerto-Rican population, where some Authors found an association between some SNPs and the risk of asthma development [68].

With regard to allergic conditions other than asthma, scientific data are scanty. Contrasting results were found for allergic rhinitis where some Authors found that OPN expression in the nasal mucosa was comparable to that of healthy controls, and was not affected by allergen exposure [69].

OPN has been correlated with the onset and the chronicization of allergic contact dermatitis (that is a type 4, cell-mediated, hypersensitivity condition). [70-72].

OPN serum levels have been also reported to be increased in Hymenoptera venom immunotherapy, assuming a possible protective role against reactions after insect re-puncture [73, 74].

Interestingly, the suppression of OPN during H1-antihistamine (levocetirizine) assumption has been observed in a group of allergic subjects [75].

However, despite the existence of promising data in asthma, OPN in the other allergic conditions has not been extensively studied yet and the results are still not conclusive.

The study of anti-osteopontin autoantibodies (anti-OPN autoAbs) potentially provides several applications because of OPN ubiquity, structural and functional role. In general, autoantibodies play a key pathogenetic role in the autoimmune diseases even though also healthy subjects show a spontaneous production of autoAbs, including anti-cytokine antibodies, that are

physiologically involved in the regulation of immune responses [76]. Steinman et at. showed that the induction of experimental autoimmune encephalomyelitis (EAE) in a mouse model is correlated with the production of anti-OPN autoAbs and remission occurred when their titers peaked [77, 78]. Clemente et al. recently published the results of OPN serum levels detected by ELISA in 122 patients with MS collected cross-sectionally, and 50 patients with relapsing-remitting (RR) disease collected at diagnosis and followed longitudinally for 10 years. In the cross-sectional group, the anti-OPN autoAbs levels resulted to be higher in the RR patients than in the primary- and secondary-progressive MS and healthy control groups, and at the initial stages of the disease. In the longitudinal group, the levels at diagnosis directly correlated with the number of relapses during the following 10 years. Furthermore, in patients who underwent disease-modifying treatments, autoAbs levels were higher than in untreated patients and were significantly associated with low MS severity score. The autoAbs displayed a neutralizing effect and mainly recognized OPN-C rather than OPN-N fragments. To confirm the clinical effect of anti-OPN autoAbs *in vivo*, EAE was induced using myelin oligodendrocyte glycoprotein MOG35-55 in C57BL/6 mice pre-vaccinated with ovalbumin (OVA)-linked OPN or OVA alone. Vaccination significantly induced anti-OPN autoAbs production during EAE, decreased the degree of disease severity, and the protective effect was correlated with a reduced T cell secretion of IL-17 and IFN-γ *ex vivo*. The strongest effect was obtained with OPN-C, which induced significantly faster and more complete remission than other OPN vaccines. These preliminary data suggest that production of anti-OPN autoAbs may favor remission in both MS and EAE and new strategies boosting their levels, such as vaccination or passive immunization, may be studied as a future strategy in personalized MS therapy. [79].

At the time of this writing, in literature there have been no published data regarding a possible association between anti-OPN autoAbs and allergic diseases.

3. Aims

In the present study, we aimed to verify if OPN may qualify as biomarker of an activated immune response in allergic patients belonging to two different ethnic groups: Caucasians and Asians.

A Southeast Asian population of allergic subjects has been previously studied at the Singapore Immunology Network (SIgN), within the Agency for Science, Technology and Research (A*STAR) in Singapore, in order to evaluate both allergic sensitization patterns and respiratory symptoms manifestations in a tropical urban environment [80]. This study demonstrated that two independent cohorts of 576 and 7373 ethnic Chinese subjects living in Singapore presented an allergic sensitization pattern that is almost exclusively directed to house dust mites (HDM), since a percentage of 80% of the individuals were HDM-sIgE positive. Of these, less than 30% had serum specific IgE (sIgE) for any other aeroallergens. In addition, HDM-sIgE titers were statistically higher in comparison to non-HDM allergen sIgE titers. Interestingly, the population of migrants from non-tropical countries presented low or non-detectable sIgE against HDM, but showed a time-dependent increase of HDM-sIgE levels. Moreover, subjects remaining for a prolonged time in Singapore presented also a significant and remarkable impairment of respiratory symptoms (allergic rhinitis and/or asthma), that was associated with the time-dependent, progressively higher HDM-sensitization.

These observations are in line with the "*Hygiene Hypothesis*": many scientific evidences nowadays support the hypothesis that in the last decades a decreased microbial exposure due to a life-style "Westernization", increased cleanliness and reduced family size, could explain the globally augmented prevalence of allergic diseases [81].

The main purposes of the present study were the following:

1. to create a case record of subjects phenotypically compatible with allergic diseases diagnosed through officially recognized methods;

2. to create a bio-bank of DNA and biological samples representing two genetically different allergic populations: one of Caucasian origins and the other one from Southeast Asia;

3. to identify new serological markers (such as OPN and IgG anti-OPN autoAbs) that may be correlated with immune activation in allergic diseases;

4. to validate and compare molecular risk profiles for allergic diseases in Caucasian and Southeast Asian cohorts;

5. to evaluate and compare molecular risk profiles for allergic diseases in different subsets of patients (e.g. gender, age, ethnicity, etc.).

4. Materials and methods

4.1 Study design and patients

This is a cross-sectional study aimed to investigate OPN and IgG anti-OPN AutoAbs as possible emerging biomarkers of the immune response in common allergic diseases (i.e., allergic asthma, allergic rhinitis, allergic contact dermatitis, IgE – sensitization to beta-lactams, Hymenoptera venom allergy, food allergy). The trial was previously approved by the Institutional Ethical Committee of "Maggiore della Carità" University Hospital, Novara (NO), (protocol number: 986/CE; study number: CE 140/14).

Adults who met the diagnostic criteria established by international guidelines for the allergic diseases mentioned above, were identified both retrospectively and prospectively.

For the retrospective recruitment, subjects potentially suffering from allergies were identified on the basis of their clinical history from the Allergology and Clinical Immunology Unit, "Maggiore della Carità" University Hospital, Novara. At the time of informed consent and enrollment, patients answered a questionnaire regarding demographics, medical history (family and personal) and specific information on their allergic diseases. Patients data and blood samples were immediately made anonymous after their collection and were identified by a code (with deleted sensitive data, such as name and surname, date of birth, address etc.). All the data useful to re-identify patients were stored in a separate archive.

Inclusion criteria were the following:

- male or female patients, aged 18 years and over, suffering from allergic diseases (allergic asthma, allergic rhinitis, allergic contact dermatitis, allergic sensitization to beta-lactams, Hymenoptera venom allergy, food allergy) diagnosed according to criteria and methods established by official guidelines;
- signed and dated informed consent.

Subjects with the following conditions were excluded:

- patients under 18 years of age;
- inflammatory conditions and/or infectious episodes during the last month;
- immunodeficiency/immunosuppression;
- systemic autoimmune diseases;
- malignancies.

About the Singaporean cohort, ethnic Chinese subjects were recruited at the National University of Singapore (NUS) as a part of an ongoing national study on the prevalence of allergic diseases. Volunteers were then classified as individuals with allergic rhinitis, asthma, and healthy controls using a questionnaire collecting information on demographics and medical history, and based on the Allergic Rhinitis Impact on Asthma (ARIA) [82] and International Study of Asthma and Allergies in Childhood (ISAAC) guidelines [83]. The volunteers also underwent a skin prick test using a panel of allergens common in Singapore (including Dermatophagoides pteronyssinus, Blomia tropicalis, Elaeis guineensis, and Curvularia lunata). The initial discovery cohort consisted of a total of 576 ethnic chinese volunteers.

4.2 DNA extraction

Genomic DNA was extracted from an aliquot of whole blood using "Gentra reagents PureGene" (Qiagen). Biological samples (serum/plasma) and DNA were stored at -80° C for further investigations: i.e., genomic DNA polymorphisms (+1239A>C and -156G>GG) of OPN gene, that were previously associated with various autoimmune diseases, might be of interest.

4.3 Molecular Biomarkers and ELISA Assay

Presence and levels of serum biomarkers (OPN and IgG anti-OPN autoAbs) in the Caucasian cohort were assessed by enzyme-linked immunosorbent assay test (ELISA), according to the protocol provided by the manufacturer (Human Osteopontin Duoset, R&D Systems, Minneapolis, MN, USA, for OPN assessment; 'in house' ELISA kit for IgG anti-OPN AutoAbs detection). The optical density was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA). The I-smart program was used to create a regression curve. The precision of the test is measured as a coefficient of variation (CV) from the mean value.

Both intra-assay and inter-assay precision should always be considered. Intra-assay precision is the reproducibility between ELISA test wells within an assay. This allows to run multiple replicates of the same sample simultaneously on one plate and get similar results. Inter-assay precision is the reproducibility between assays, using multiple kits over a period of time. R&D Systems Quantikine Immunoassays have CV values less than 10% across the standard curve for both intra- and inter-assay precision, according technical datasheet.

In the Singaporean cohort, OPN plasma values were assessed by ELISA (same kit used in Novara, R&D Systems) and by Luminex test (Milliplex Map Bone Metabolism Multiplex Assay, Merck Millipore).

4.4 Western blot

Western blots were carried out using commercial gradient gels and rabbit polyclonal anti-OPN IgG antibody (ab8448, Abcam) as primary antibody, while the secondary antibody was an anti-rabbit horseradish peroxidase (HRP) conjugated from Cell Signaling Technology (Cat. No. 7074P).

N.=49 plasma samples were randomly selected for Western blot analysis from the 80 plasma samples previously processed with ELISA test for OPN levels detection.

4.5 Phos-Tag™ gels

Phos-Tag™ (Wako) is a novel phosphate-binding tag at physiological pH [84-89]. Phos-Tag™ SDS-PAGE is an electrophoresis technique capable of separating phosphorylated and non-phosphorylated proteins based on phosphorylation levels. Western blot can be employed after electrophoresis. This technique can be also useful in detecting new phosphosites. In the Phos-Tag™, two metallic ions (manganese or zinc) trap phosphoproteins during migration: the higher amount of phosphorylation, the slower the migration velocity. Separation occurs based on the phosphorylation levels.

We carried out several experiments with Phos-Tag™ and a positive control, bovine casein (Sigma), treated or not with calf intestinal alkaline phosphatase (Promega) at different timepoints and in different buffers; subsequently, we applied the same protocols for OPN.

4.6 Bioinformatics analysis

In a primary data analysis, a case/control approach was used comparing the results obtained in cases with respect to controls. In a secondary analysis,

all the data obtained were compared among the various groups of patients (i.e., subjects with different allergic diseases) to investigate a possible trend of differences in the groups.

Serological data were compared using the nonparametric Mann-Whitney U test or Kruskal-Wallis test.

The approximation of population distribution to normality was investigated by using statistics for kurtosis and symmetry. As the results were asymmetrically distributed, they were presented as median values and percentiles.

All P-values are 2-tailed and the significance cut-off was $P < 0.05$.

GraphPadPrism and TIBCO Spotfire softwares were used to perform biostatistics analysis and related graphs.

ImageJ software was used to quantify, in arbitrary units (pixels), the thickness of full lenght OPN and OPN fragments bands.

5. Results

In the Italian cohort, data related to a series of 121 adult patients, 57 males and 64 females (mean age: 48 years; median age: 49 years) with different allergic diseases, were studied: 40 patients (33%) had allergic rhinitis, 25 (21%) allergic asthma, 21 (17%) Hymenoptera venom allergy, 19 (16%) food allergy, 13 (11%) allergic contact dermatitis, and 3 (2%) IgE-mediated hypersensitivity to beta-lactams (**figure 2**). 116 healthy subjects with similar demographic characteristics and no history of allergic symptoms/autoimmune diseases/chronic inflammatory diseases/malignancies served as controls. Data were analyzed comparing cases to controls, as well as looking for subgroup differences within the group of allergic patients.

OPN prevalence and serum levels were statistically higher in cases with respect to controls (median: 10330.8 pg/ml; interquartile range: 25^{th} 5712.49 pg/ml, 75^{th} 16476.21 vs median 6099.12 pg/ ml; interquartile range 25^{th} 3122.57 pg/ml, 75^{th} 14519.91 pg/ml; P = 0.001 by the Mann-Whitney test).

OPN levels were statistically higher in patients with asthma (median: 13083.83 pg/ml; P = 0.0269), followed by those, less significant, observed in the food allergy group (median: 10204.33 pg/ml; P = 0.046), in comparison to controls. Not significantly different OPN levels were detected in patients with Hymenoptera venom allergy (median: 13289.95 pg/ml; P = 0.0624), allergic rhinitis (median: 9431.2504 pg/ml; P = 0.1277) and allergic contact dermatitis (median: 6380.44 pg/ml; P =0.39). Patients with IgE-mediated sensitization to beta-lactams had heterogeneous values, not statistically different in comparison to controls (P = 0.47) (**figure 3**).

No statistical differences were found between males and females in cases (P = 0.07 by the Mann-Whitney test), whereas in the healthy controls males

presented higher OPN serum values with respect to females (P = 0.01182). Made exception for allergic rhinitis and Hymenoptera venom allergy groups, males show higher OPN serum levels in comparison to females, as general trend, even though the P value does not reach the statistical significance (P < 0.05). No male patients with CD were recruited, probably due to the low representation of the male gender in the disease epidemiology (**figure 4**).

Regarding serum IgG anti-OPN autoantibodies, both prevalence and titers resulted to be statistically lower in allergic patients with respect to controls (median: 0.179 pg/ml; interquantile range: 25^{th} 0.060 pg/ml, 75^{th} 0.227 pg/ml vs median 0.250 pg/ml: interquantile range: 25^{th} 0.152 pg/ml, 75^{th} 0.367 pg/ml; P < 0.0001).

Significantly lower levels of anti-OPN autoantibodies versus controls were found in patients with Hymenoptera venom allergy (median: 0.067 pg/ml; P < 0.0001), allergic rhinitis (median: 0.107 pg/ml; P = 0.0009), allergic contact dermatitis (median: 0.078 pg/ml; P = 0.0011) and asthma (median: 0.123 pg/ml; P = 0.0013); conversely, the statistical correlation for the food allergy group seems to be less significant (median: 0.185 pg/ml; P = 0.0575). Patients with IgE-mediated sensitization to beta-lactams presented heterogeneous results, not statistically different with respect to controls (P= 0.175) (**figure 5**).

In parallel, OPN levels were analyzed and compared also in both asthmatic cases ("lifetime asthma") and healthy controls of a Singaporean cross-sectional cohort.

The two groups did not present significant differences of OPN plasma levels (P = 0.597) (**figure 6**) detected by Luminex in 74 patients with lifetime asthma and 257 healthy subjects.

However, comparing OPN plasma levels in males and females, a remarkably strong gender effect was shown. In the control group, for instance, a statistical difference was found between 131 males and 111 females (P <

0.0001), with OPN plasma values higher in males versus females. The gender effect was confirmed in the asthmatics group (45 males and 26 females), where males presented significantly higher OPN plasma levels in comparison to females ($P < 0.0001$) (**figure 7**).

Due to the deep discrepancies observed about OPN levels between the Italian and the Singaporean cohorts, OPN plasma levels were revalued through ELISA assay in a subgroup of 80 samples belonging to the same Singaporean cohort previously analyzed by Luminex.

In this new dataset, a strongly significant difference was recorded both in asthma cases (6 males and 4 females) and healthy controls (15 males and 22 females) between males and females ($P < 0.0001$) (**figure 8**).

Considering the strong gender effect, the mRNA expression levels of SPP1 gene were checked in whole blood of males and females of the Singaporean cohort with Illumina chips (2 probes), and no difference was found (**figure 9**). Consequently, we hypothesized that antibody recognition, in ELISA and Luminex assays, could have been affected by qualitative differences of OPN between males and females.

We carried out several experiments to verify if possible post-transcriptional/post-translational modifications of OPN could interfere with antibody detection, thus leading to possible gender differences. Consequently, we set up several experiments, mainly represented by Western Blots with commercial gradient gels, potentially showing possible qualitative differences, (in terms of the thickness of the bands or in the number of the bands) related to OPN isoforms, between males and females.

At a first glance, the full length (FL) OPN isoform presents two significant and different bands: the upper one (FL1) is thicker, the smallest one (FL2) is slightly fainter. We interpreted this finding as a result of the protein

metabolism, since OPN is cleaved in two equal fragments by thrombin enzyme for its functional activation. Other lower bands are quite clearly visible in the Western blots, and correspond to the fragments of OPN after its cleavage by other enzymes, i.e. carboxypeptidase B and metalloproteinases.

While a correlation between FL2 OPN and fragments bands could not be shown ($r = 0.03248$), FL1 OPN and fragments bands seemed to be slightly correlated ($r = 0.23$), thus demonstrating a possible metabolic association between FL OPN and the fragments production after OPN enzymatic cleavage.

Actually no qualitative/quantitative differences in the Western Blot bands were found between males and females, asthmatics and healthy controls, in terms of thickness of the bands related to both full lenght OPN and OPN fragments. A trend towards increased thickness of the upper FL OPN bands, however, was found in males versus females, not reaching the statistical significance though ($P = 0.0966$) (**figures 10, 11**).

We wondered also if post-translational modifications, such as phosphorylation, could interfere with antibody recognition in the tests used (Luminex, ELISA assays). Consequently, several experiments with Phos-Tag gels were set up to investigate if any possible difference in OPN phosphosites could have been detectable. However, whilst the Phos-Tag positive control represented by bovine casein, another phosphoprotein, is properly represented in its different bands, corresponding to different phosporylation status, the experiments resulted to be technically difficult and not conclusive for OPN, also probably due to the low levels of the protein normally found in plasma (**figures 12, 13, 14**).

6. Discussion and conclusion

This is the first study investigating and comparing OPN and IgG anti-OPN autoAbs systemic levels in the most common allergic diseases. OPN serum values were found to be statistically higher in allergic asthma and food allergy groups in a Caucasian population. OPN seems to be a new potentially useful biomarker in active, current allergic asthma – and not in "lifetime asthma" -, as its high proinflammatory role reflects the inflammatory condition in the patients that are affected by the disease. In the asthmatic Caucasian cohort, all the subjects were clinically stable, taking long-term treatment with inhaled corticosteroids (ICS) and only one patient presented severe asthma.

OPN serum levels were statistically significant also in the food allergy group: this is an interesting result, since no valid biomarkers for diagnosing and monitoring food allergy have been identified yet. Nevertheless, large-scale population-based studies are needed in order to confirm these data.

The Singaporean population did not show a statistically significant association between allergic asthma and OPN systemic levels. This observation can be mainly explained by the fact that the asthmatic cases of the Singaporean cohort were recruited based on a validated ARIA/ISAAC questionnaire and were not regularly followed up by a medical doctor. ISAAC, (International Study of Asthma and Allergies in Childhood) is a unique worldwide epidemiological research programme established in 1991 to investigate the prevalence of asthma, rhinitis and eczema due to considerable concern that these conditions were increasing in Western as well as in developing countries. Current data on the clinical conditions or functional test (such as spirometry test, fractional exhaled nitric oxide, etc.) of the enrolled subjects were not available; through the questionnaire tool, it is known that they presented asthmatic symptoms at least once in their life, condition defined as "lifetime asthma". At the moment of this writing, it is believed that this

observation represents the main reason explaining the different results on OPN systemic levels between the two, ethnically different, asthmatic groups. Despite the bias, the study of these two cohorts allowed us to state that OPN cannot be considered as an universally acceptable marker of asthma; on the contrary, it can represent a promising biomarker of allergic inflammation in current, active asthma.

The gender effect observed within the Singaporean population, both in cases and controls, is quite remarkable. The same gender effect, even though less evident, was observed in the Italian control group. Assuming that no differences of SPP1 mRNA expression levels, between males and females, were observed in whole blood, it can be hypothesized that some post-transcriptional/post-translational modifications of the protein possibly may lead to the gender difference observed, with males presenting higher detectable levels of OPN with respect to females.

OPN as nascent protein can go through a proteolytic processing and be activated by different enzymes (i.e., thrombin, carboxypeptidase B and metalloproteinases). These various structural isoforms theoretically could influence the overall OPN detection through common assays (e.g., ELISA, Luminex, Western blot, etc.).

It is commonly known that another post-translational modification like phosphorylation is an important regulatory mechanism that occurs in both prokaryotic and eukaryotic organisms, since reversible phosphorylation results in activation or deactivation of enzymes or receptors. OPN is highly phosphorylated in serine, threonine and tyrosine amino acidic residues (36 phosphorylation sites globally). Phosphate groups, that are essential for OPN role in biomineralization, can lead to a change in the conformational status of the molecule itself and, consequently, be involved in the pathogenesis of immune diseases and in the immune senescence phenomenon as well.

Currently, there are several technical difficulties to assess if post-translational protein modifications may interfere with antibody recognition through Western Blot technique. This phenomenon is probably due to the intrinsically disordered structure of OPN, its complex (and still unclear) metabolism, and its low expression levels in whole blood (**figure 15**). The laboratory tests that are currently available in commerce present different limitations: i.e., the antibody used in the ELISA assay does not recognize OPN fragments but only the full lenght protein; however, the same antibody can recognize also the fragments if used for Western blot. Furthermore, it is unclear why can we detect phosphosites in casein and we can dephosphorylate it, while for OPN is not possible yet, at the optimal conditions requested.

Concerning the autoAbs detection, different articles show discrepancies data about both prevalence and titers of autoAbs directed against cytokines. One of the problems is to establish whether these differences are due to intrinsic heterogeneity of the the population in analysis or to the method used to detect the autoAbs. Usually the most common assays used by the Authors are represented by ELISA test and Western blot. Both of them use recombinant cytokines that are immobilized in plastic wells (ELISA) or in Polyvinylidene Fluoride (PVDF) or nitrocellulose membrane (WB); the cytokines are bound by the autoAbs present in serum samples and then are revealed by horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies.

In general, anti-human IgG polyclonal antibodies are employed to investigate the memory responses and avoid the aspecific binding of IgM autoAbs. One problem is represented by the formulation of the recombinant protein considered, since its conformational status can change and important epitopes displayed in the wild-type protein may be lost in the assay. Secondarily, the detection of autoAbs can be difficult for the presence of either immune complexes - between autoAbs and endogenous cytokines - and multispecific heterophilic antibodies giving false positive signals. Technical

bias should be carefully avoided in setting up a reliable immunological test for autoAbs.

In the present study, IgG anti-OPN autoAbs serum levels were mostly decreased in Italian patients affected with Hymenoptera venom allergy, that is the most pure IgE-mediated allergy type with no underlying inflammation. IgG anti-OPN autoAbs were statistically lower also in patients with allergic rhinitis, asthma and allergic contact dermatitis, with respect to controls. On the contrary, anti-OPN autoAbs were not significantly different in the food allergy group in comparison to healthy controls. A further analysis of IgA anti-OPN autoAbs could be of great interest to investigate the production of anti-OPN autoAbs in the digestive tract and to clarify this scientific observation.

7. Future perspectives

Osteopontin seems to represent a good biomarker of asthmatic inflammation in case of active, current respiratory symptoms. In order to make this observation more robust, our future direction will be to study a greater number of Italian asthmatics, from Novara and Vercelli hospital Allergy Units, and also a cohort of Singaporean subjects with doctor-diagnosed asthma (the recruitment carried out with a questionnaire may represents a scientific bias). Hopefully it will be possible to correlate OPN levels with other markers of inflammation (i.e., exhaled nitric oxide, systemic periostin levels, etc.), and assess whether OPN can be also recognized as an organ (lung, in this case)-specific biomarker.

In parallel, evaluating a major number of individuals with food allergy would be desirable, since the significance for OPN was statistically borderline. This would be particularly interesting since, so far, there are no biomarkers for food allergy officially recognized. In addition, would be also interesting to study how other anti-OPN autoAbs behave, in particular IgA isotype class.

The importance of these future developments is based on the constant search for reliable biomarkers for allergic diseases. At the moment, in fact, there are no systemic biomarkers that can be helpful to quantify the allergic inflammation. The only promising data regards periostin, another protein from the bone tissue, that seems to be strongly associated with airways eosinophilia and Th-2 driven, severe asthma [90].

The discovery of specific biomarkers would be also very useful in order to determine the phenotype of the patients, evaluate their clinical prognosis and tailor the right treatment for each phenotype.

Appendix

Figures

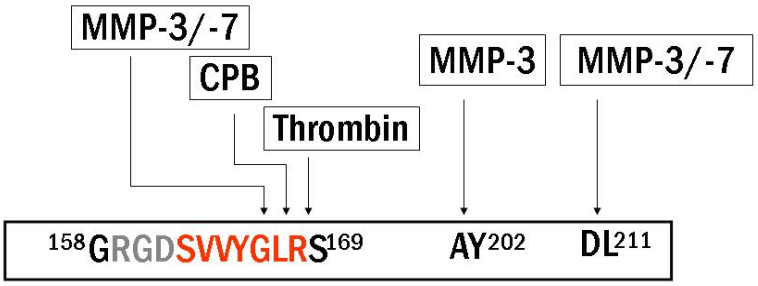

Figure 1

Osteopontin isoforms and proteolytic processing.

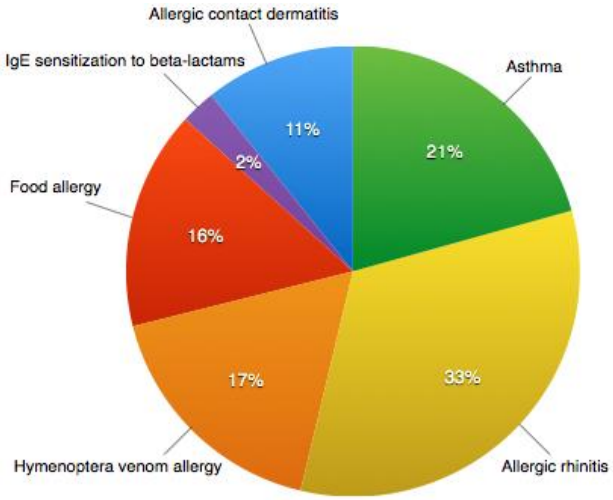

Figure 2

Clinical characteristics of the 121 allergic patients recruited in Novara, Italy, sorted for allergic disease: 57 males and 64 females >18 y.o.. Mean age: 48 years; median age: 49 years.

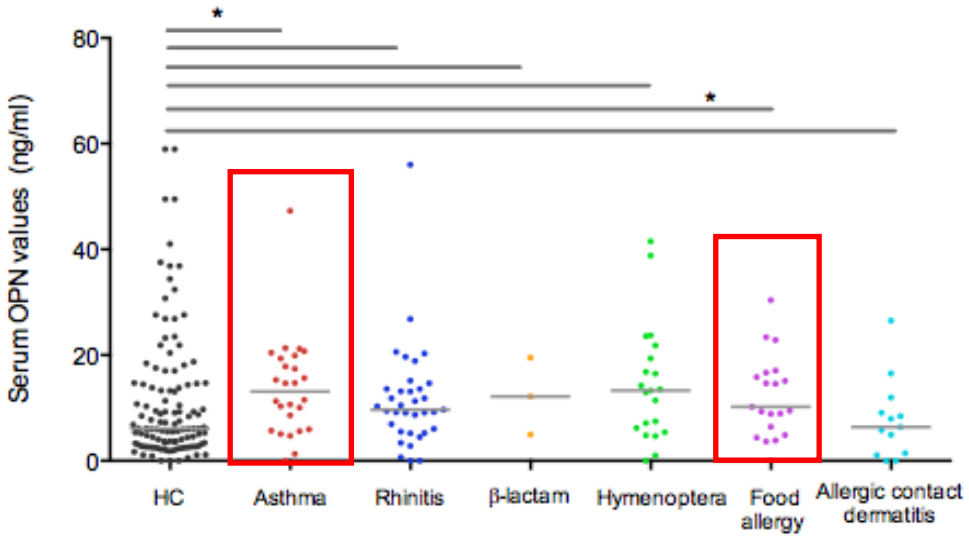

Figure 3

OPN serum values in cases and healthy controls (HC). OPN serum levels were significantly higher in cases versus controls (P = 0.001). Statistically higher levels were found in asthma (P = 0.0269) and food allergy groups (P = 0.046) in comparison to HC.

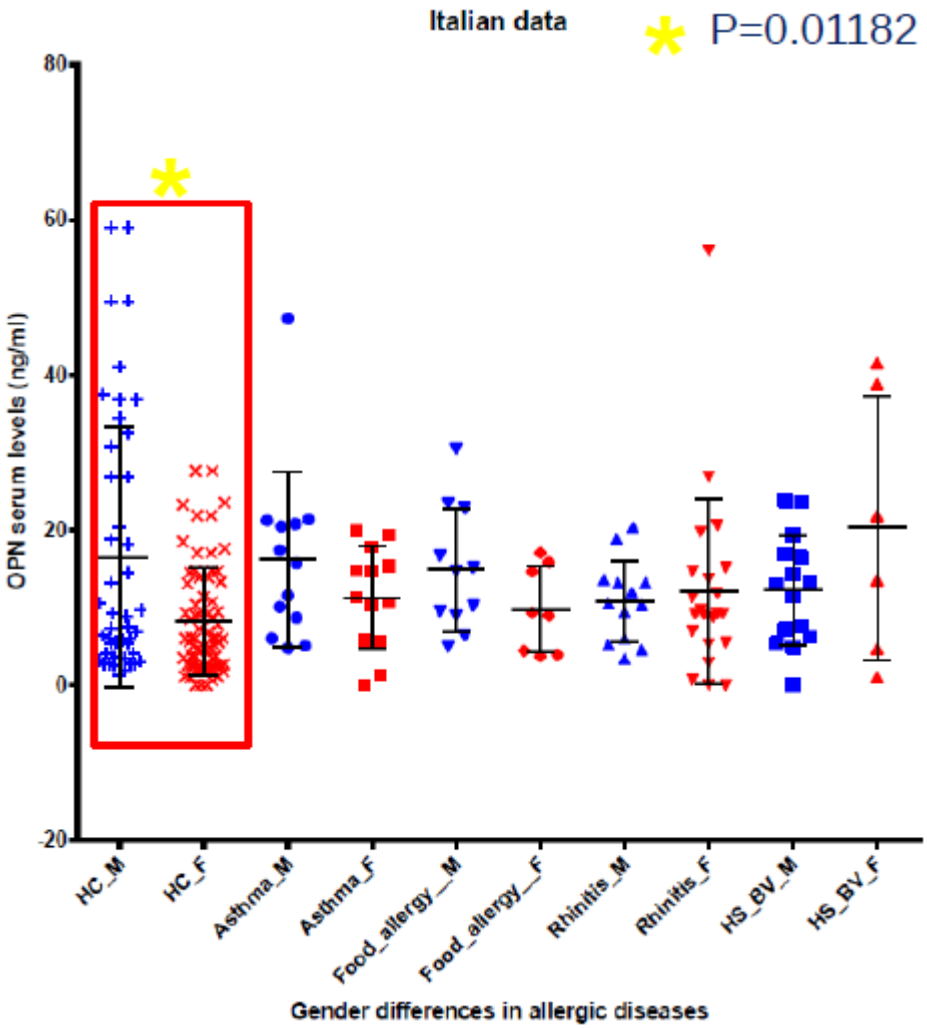

Figure 4

Gender effect on OPN plasma levels in asthmatics and in healthy controls (HC) in the Italian cohort. No difference was found between males and females in the allergic groups, while a significant difference was recorded in the healthy controls (P = 0.01182). HS_BV = hypersensitivity to bee and vespids venom.

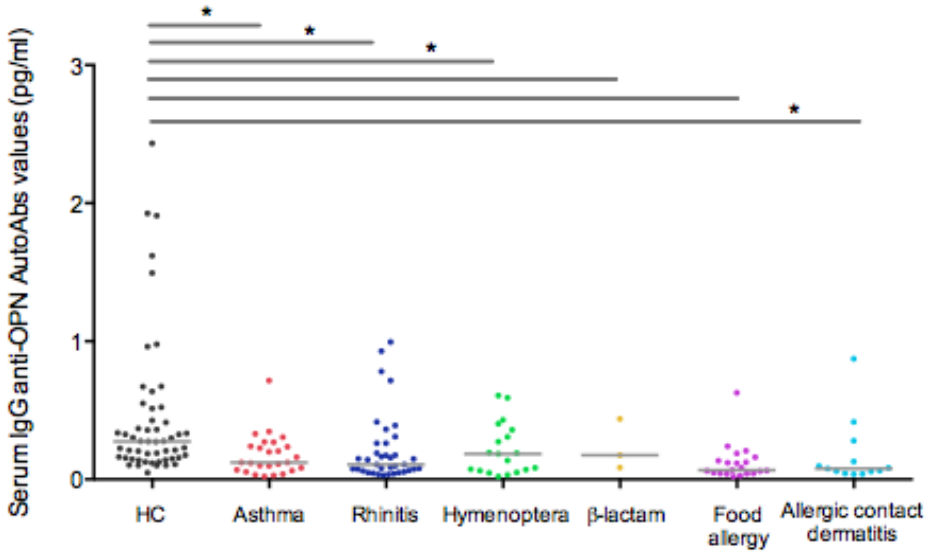

Figure 5

IgG anti-OPN autoAbs serum values in cases and healthy controls (HC). Prevalence and titers of serum IgG anti-OPN autoAbs were significantly lower in cases with respect to controls ($P < 0.0001$). Lower levels of autoAbs versus controls were found in patients with Hymenoptera venom allergy ($P < 0.0001$), allergic rhinitis ($P = 0.0009$), allergic contact dermatitis ($P = 0.0011$) and asthma ($P = 0.0013$), but not in the food allergy group ($P = 0.0575$). β-lactam = IgE-mediated hypersensitivity to β-lactams. Hymenoptera = Hymenoptera venom allergy.

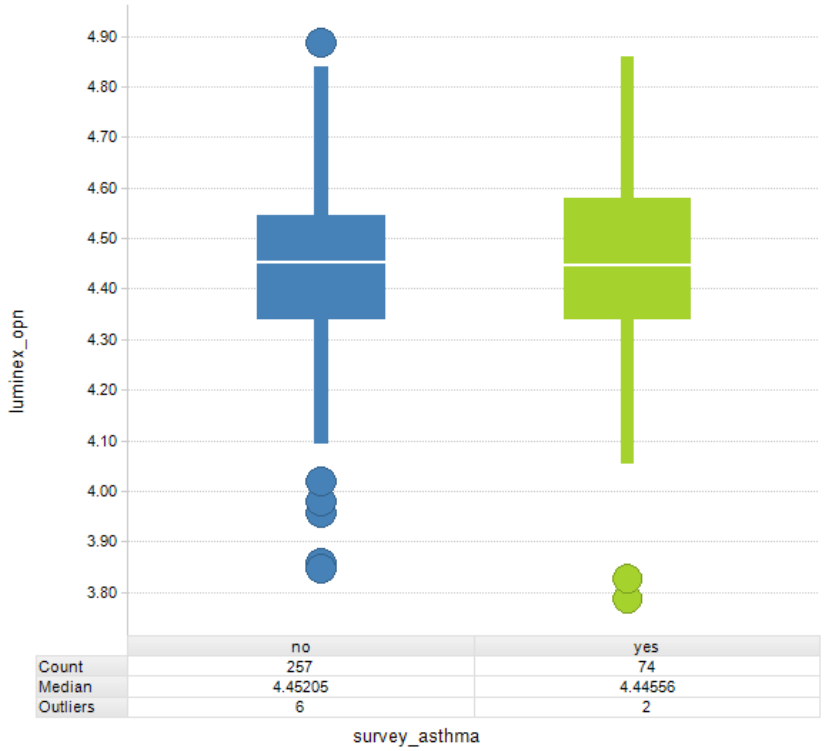

Figure 6

OPN plasma levels, evaluated by Luminex assay, in asthmatics and in healthy controls in the Singaporean cohort. No significant difference was recorded between the two groups (P = 0.597).

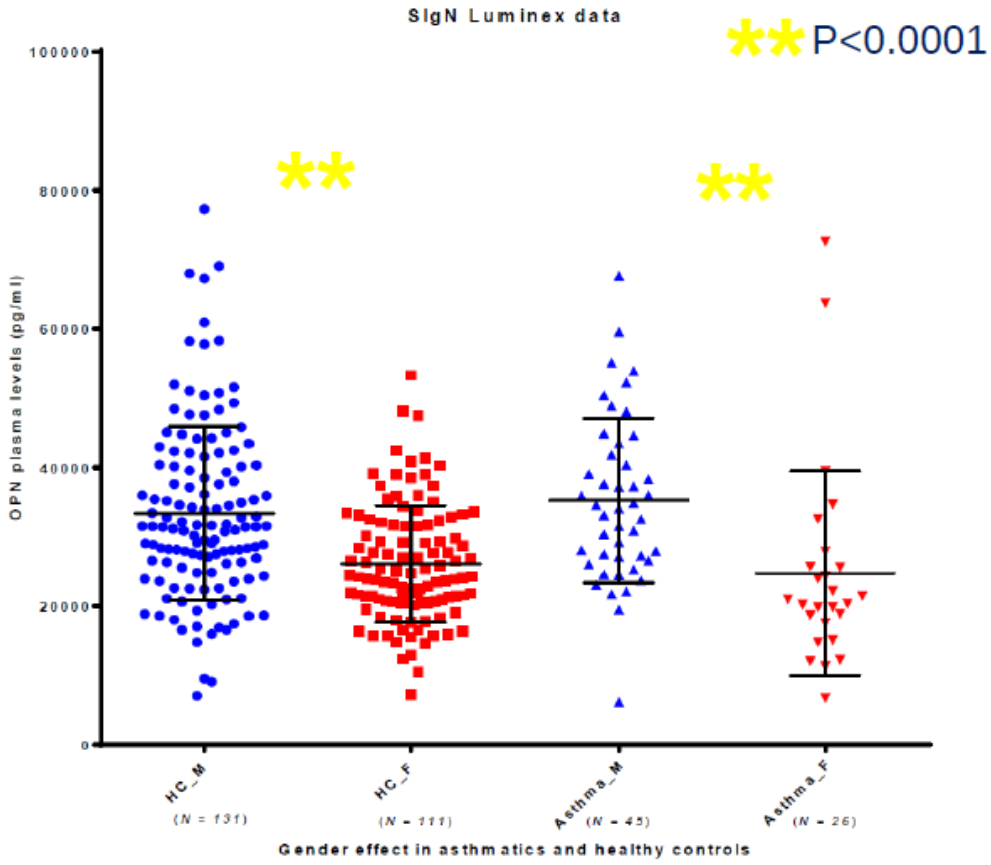

Figure 7

Gender effect on OPN plasma levels in asthmatics and healthy controls (HC) of the Singaporean population (SIgN). A statistically strong difference was found between males and females in both groups ($P < 0.0001$).

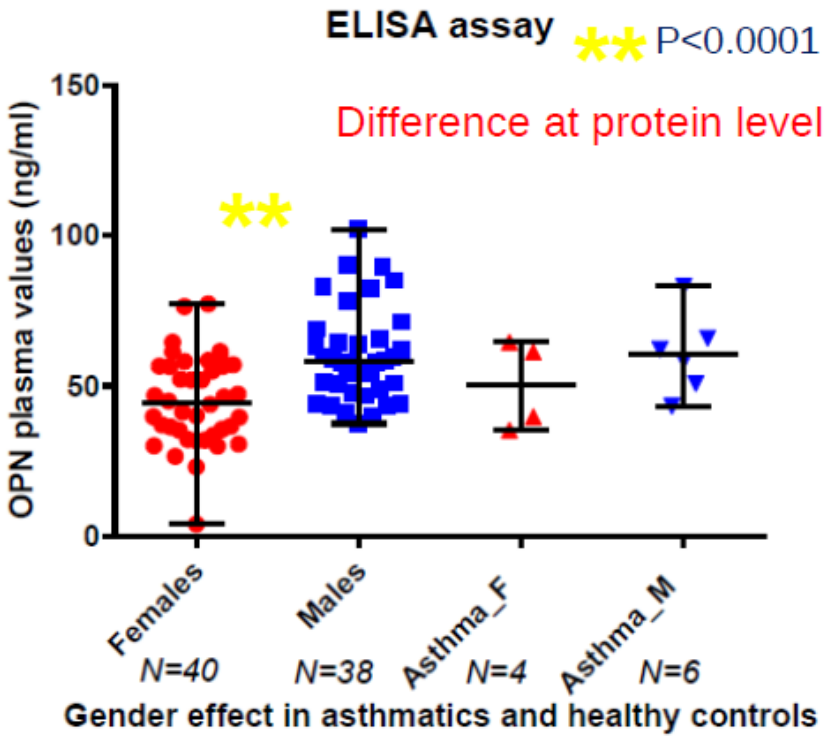

Figure 8

Gender effect on OPN plasma levels detected by ELISA assay in asthmatics and in healthy controls in the Singaporean cohort. A statistical difference between males and females was found in healthy controls and in the asthmatics ($P < 0.0001$). A gender difference was confirmed at protein level.

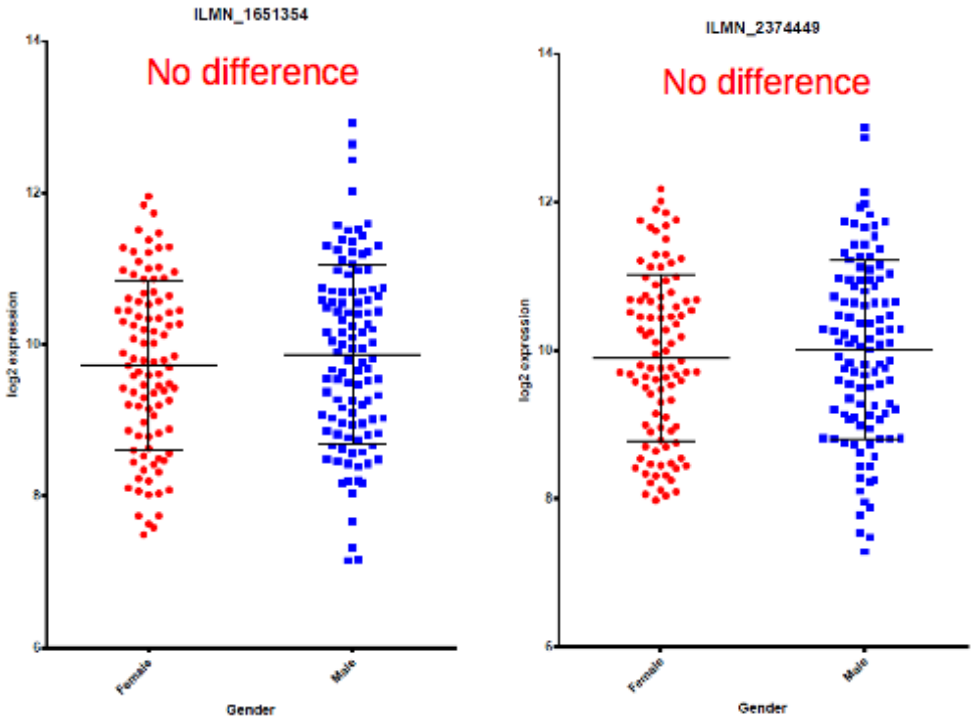

Figure 9

Gender effect on SPP1 mRNA expression levels in whole blood detected by Illumina chips in the Singaporean cohort. No significant difference was detectable between males and females.

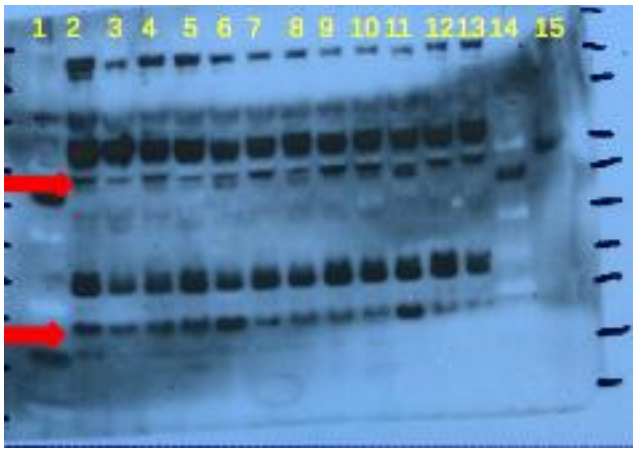

Figure 10

OPN isoforms detected by Western Blot in plasma samples from the Singaporean cohort. Lanes 1 and 14: marker/protein ladder 4 µL; lanes 2-13: plasma samples 30 µL; lane 15: hrOPN 1 µL of 1:100 (stock concentration: 100 µg/mL). The upper red arrow indicates the full length OPN (FL), with upper band (FL1) and lower band (FL2) (molecular weight: ~65 kDa). The lower red arrow indicates OPN fragments (molecular weight: ~23 kDa).

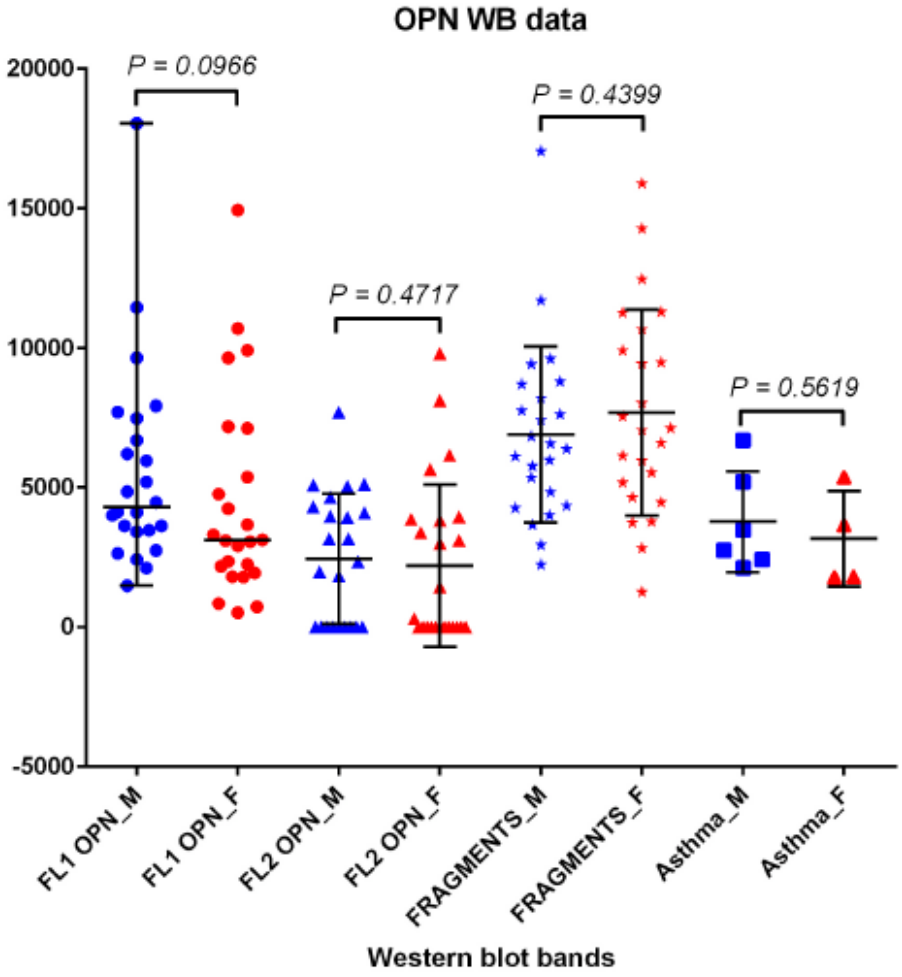

Figure 11

Gender difference of OPN isoforms detected in plasma samples from the Singaporean cohort. The results are based on no. 4 Western Blots with 48 plasma samples previously analyzed by ELISA test. No significant difference between males and females was detectable in terms of thickness of the bands (expressed in arbitrary units, pixels) analyzed through ImageJ software.

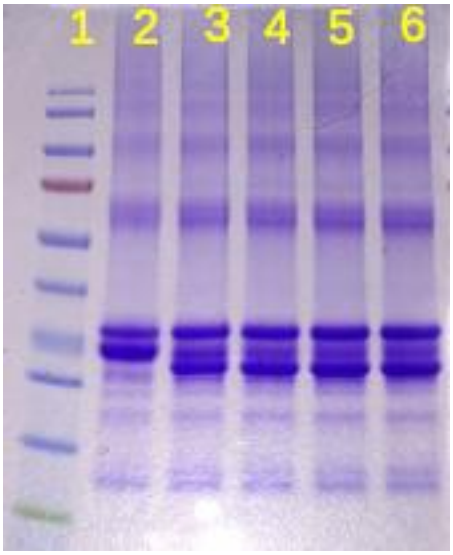

Figure 12

Commercial gradient gel stained with Coomassie solution. A saline solution of bovine casein (Sigma), treated or not treated with alkaline phosphatase (AP, Promega) was used as positive control to study the differential electrophoretic migration of the phospo-bands in the gel. Numbered lanes are the following:

1: Page Ruler, 5 µL; 2: casein without AP, 5 µL; 3: casein + AP at 30', 5 µL; 4: casein + AP at 60', 5 µL; 5: casein + AP at 120', 5 µL; 6: casein + AP at 180', 5 µL. Casein final concentration: 3.3 µg/µL (loaded amount: 16.50 µg).

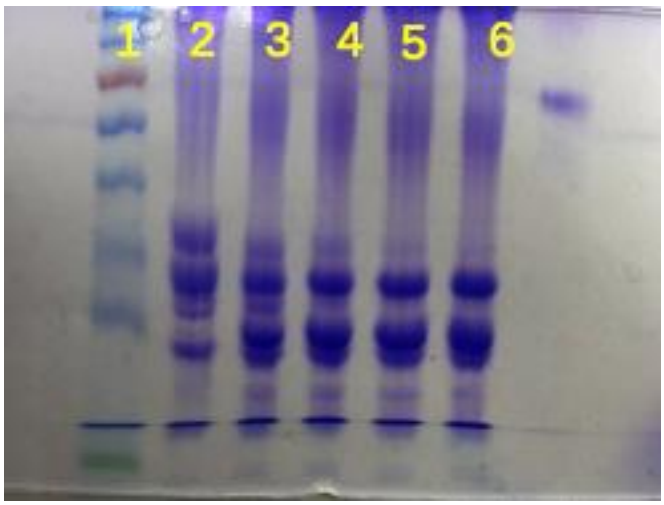

Figure 13

Phos-Tag gel stained with Coomassie solution. A saline solution of bovine casein (Sigma), treated or not treated with alkaline phosphatase (AP, Promega) was used as positive control to study the differential electrophoretic migration of the phospo-bands in the gel. Numbered lanes are the following: 1: Page Ruler, 5 µL; 2: casein w/o AP, 5 µL; 3: casein + AP at 30', 5 µL; 4: casein + AP at 60', 5 µL; 5: casein + AP at 120', 5 µL; 6: casein + AP at 180', 5 µL. Casein final concentration: 3.3 µg/µL (loaded amount: 16.50 µg).

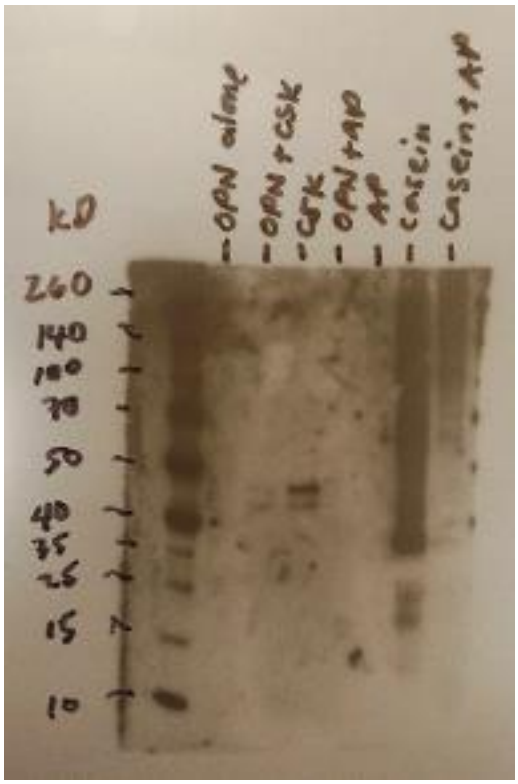

Figure 14

Western Blot with anti-phosphoserine antibodies. OPN: osteopontin; CSK: casein kinase I; AP: alkaline phosphatase. While for casein the difference in the phospho-sites (before and after treatment with AP) is quite remarkable in terms of different bands with different molecular weight, for osteopontin such a difference is not detectable through this technique.

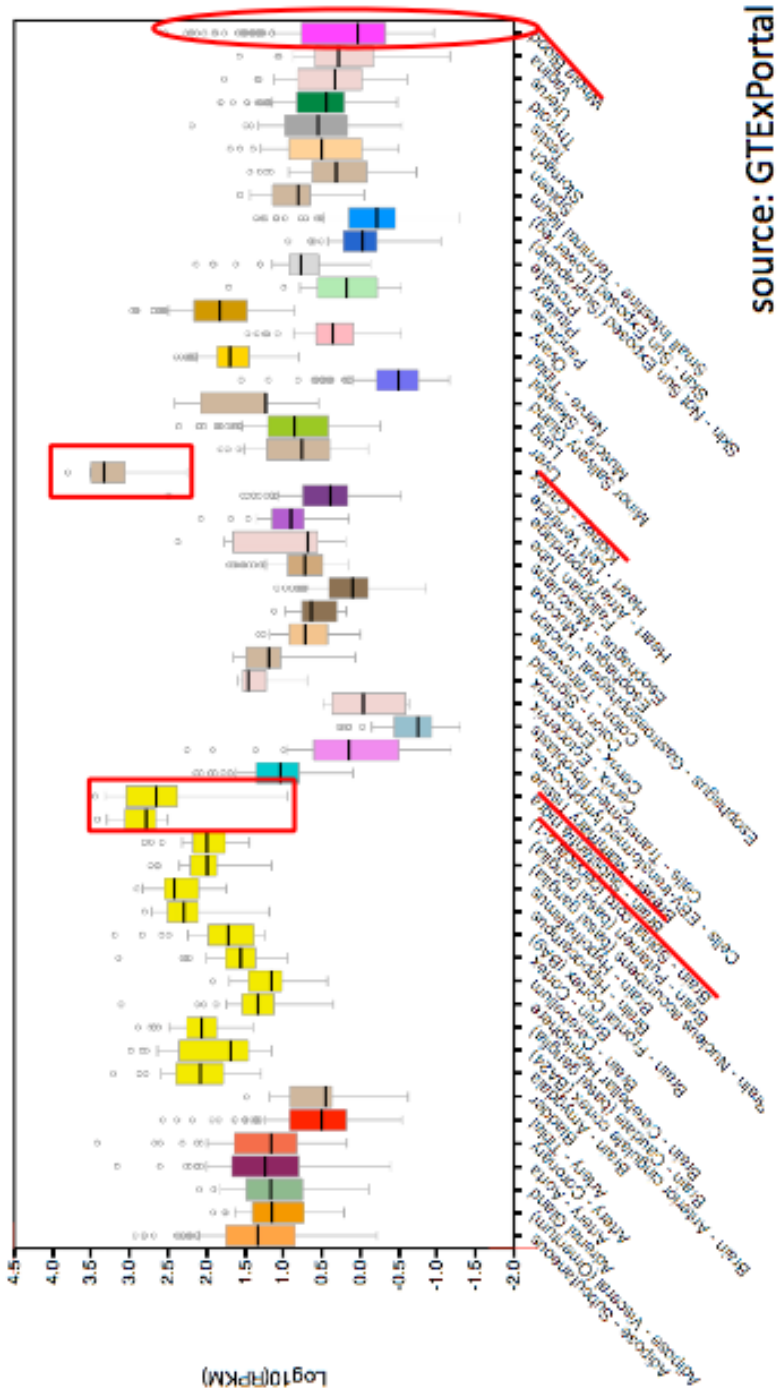

Figure 15

SPP1 expression in various tissues: high in the brain and at renal level, while in the whole blood the levels are less represented.

List of publications

1. Bommarito L, Zisa G, Riccobono F, Villa E, D'Antonio C, Calamari Ambra M, Poppa M, Moschella A, Di Pietrantonj C, Galimberti M. Avoidance of nonsteroidal anti-inflammatory drugs after negative provocation tests in urticaria/angioedema reactions: Real-world experience. *Allergy Asthma Proc*. 35:1–4, 2014.

2. Elisa Villa, Rosalba Minisini, Olaf Rötzschke, Kia Joo Puan, Boris Buenbrazo San Luis, Anand Andiappan, Bernett Lee, Elena Boggio, Luca Gigliotti, Nausicaa Clemente, Annalisa Chiocchetti, Umberto Dianzani, Mario Pirisi. "**Evaluation Of Serum Levels Of Osteopontin And IgG Anti-Osteopontin Autoantibodies As Potential Biomarkers Of Immune Activation In Patients With Allergic Diseases". *Journal of Allergy and Clinical Immunology* (*JACI*). 2016 February. 137(2), AB394. Congress abstract, American Academy of Allergy Asthma Immunology (AAAAI), 2016 meeting, Los Angeles - California (USA).**

3. Lombardi C, Passalacqua G; AAITO Italian Smoke and Allergy Group AISAG. Collaborators: Arena A, Beghi G, Billeri L, Borrelli P, Conte ME, Cortellini G, Crivellaro M, Della Torre F, Di Marco G, Quercia O, Emiliani F, Stefanini F, Galdi E, Gani F, Guarnieri G, Liccardi G, Musicco E, Randazzo S, Ridolo E, Olivieri E, Bonzato L, Montagni M, Savi E, Peveri S, Tomsic M, Vicentini L, Boccafogli A, Zisa G, Villa E. Italian Multicenter Cross-Sectional Study (AISAG) on light smoking and allergic diseases in adults. *Eur Ann Allergy Clin Immunol*. 2016 Mar;48(2):49-54.

4. Elisa Villa, Rosalba Minisini, Olaf Rotzschke, Anand Andiappan, Elena Boggio, Luca Gigliotti, Nausicaa Clemente, Annalisa Chiocchetti, Umberto

Dianzani, Mario Pirisi. Evaluation of serum levels of Osteopontin as a potential biomarker of immune activation in patients with allergic diseases. *World Allergy Organization Journal*. 2016; 9(Suppl 1):A2. Congress abstract, World Allergy Organization Congress (WAC) 2015, Seoul - South Korea.

5. Villa E, Patrucco F, Malerba M. Potential role of hematological parameters in patients with chronic obstructive pulmonary disease: current point of view. *Pol Arch Intern Med*. 2018 Mar 29;128(3):143-144.

In preparation:

1) International guidelines on "*Testing for IgE Associated Conditions and Interpreting Results*" - a World Allergy Organization paper (as selected member of the World Allergy Organization committee on 'Allergy Diagnosis and Molecular Allergy'). Authors: Ignacio J Ansotegui*, G. Walter Canonica*, Elisa Villa*, Gianni Passalacqua, Tari Haahtela, Martti Antila, Jean Bousquet, Luis Caraballo, Victoria Cardona, Chiang Wen Chin, Pascal Demoly, Lawrence DuBuske, Motohiro Ebisawa, Marta Ferrer, Roy Gerth van Wijk, Maximiliano Gómez, Sandra Gonzalez-Diaz, Edgardo Jares, Erika Jensen-Jarolim, Luciana Kasse Tanno, Marek L. Kowalski, Dennis Ledford, Olga Luengo, Giovanni Melioli, John Oppenheimer, Oliver Pfaar, Lars K Poulsen, Ruby Pawankar, Harald Renz, Antonino Romano, Nelson Rosario, Lanny Rosenwasser, Mario Sanchez-Borges, Enrico Scala, Gian-Enrico Senna, Juan Carlos Sisul, Mimi Tang, Rudolf Valenta, Robert Wood, Torsten Zuberbier. *These authors equally contributed to this manuscript.

2) Revision of international guidelines on molecular-based allergy diagnostics (as selected member of the World Allergy Organization committee on 'Allergy Diagnosis and Molecular Allergy'): "*A WAO - ARIA - GA^{2}LEN*

consensus document on molecular-based allergy diagnostics", World Allergy Organization Journal.

Avoidance of nonsteroidal anti-inflammatory drugs after negative provocation tests in urticaria/angioedema reactions: Real-world experience

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ABSTRACT

Drug provocation tests (DPTs) are the gold standard in diagnosing nonsteroidal anti-inflammatory drug (NSAID) hypersensitivity; however, only few data about follow-up of patients with negative DPTs are actually available. The aim of this study was to assess patients' behavior in taking NSAIDs again and to evaluate NSAID tolerability after negative allergological workup. This is a follow-up study involving patients evaluated for history of cutaneous reactions (urticaria and or angioedema) after NSAID intake and with negative DPTs with the suspected NSAID. Patients were asked during a phone interview about the intake of NSAIDs, tolerance, or reasons of avoidance. The negative predictive value (NPV) of NSAIDs DPTs was calculated. One hundred eleven of 142 patients were successfully contacted; 46/111 (41.44%) took the same NSAID previously tested with two adverse reactions reported (4.34%). Fifty-three of 111 (47.74%) patients did not take the same NSAID, but 34 of them took at least another strong cyclooxygenase (COX) 1 inhibitor, with 1 adverse reaction (2.94%) and 19 of them took only weak COX-1 inhibitors. Twelve of 111 patients (10.8%) did not take any NSAID. Reasons for drug avoidance were mainly fear of reactions (70.8%) and no need (29.2%). NPV, overall, was 96.97% (95% confidence interval, 91–99%). Although NSAID hypersensitivity diagnosis was ruled out by oral provocation test, the majority of patients with a history of urticaria/angioedema avoided the intake of the tested NSAIDs for fear of new reactions, particularly when strong COX-1 inhibitor NSAIDs were involved. The high NPV value of DPT resulting from this study should reassure NSAID intake.

(Allergy Asthma Proc 35:303–306, 2014; doi: 10.2500/aap.2014.35.3765)

Nonsteroidal anti-inflammatory drugs (NSAIDs) may induce cutaneous manifestations in 0.3% of the general population,[1,2] and the prevalence of NSAIDs hypersensitivity reaches 27–35% in patients with chronic urticaria.[3] In recent studies NSAIDs are proven to be the medications most frequently involved in hypersensitivity drug reactions.[4] The occurrence of urticaria and/or angioedema as the sole manifestation of NSAID hypersensitivity is frequently observed as an immediate reaction in subjects with no history of preexisting chronic urticaria; cross-intolerance to cyclooxygenase (COX) 1 inhibitors of various chemical groups (multiple-NSAID induced) or selective mechanisms (single-drug induced) can be involved.[5,6]

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Presented (preliminary data) at the Drug Hypersensitivity Meeting (DHMS), Munich, Germany, April 11–14, 2012

The authors have no conflicts of interest to declare pertaining to this article

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Drug provocation tests (DPTs) with NSAIDs (with the suspected molecule or with an alternative strong COX-1 inhibitor one) are actually the gold standard in diagnosing NSAIDs hypersensitivity, because standardized cutaneous or *in vitro* tests are available only for a limited number of NSAIDs and are indicated when selective mechanisms are suspected; DPTs are recommended by the European Network for Drug Allergy to establish a definite diagnosis.[6] DPTs have to be performed in hospital centers and require specialized staff and are time-consuming.

After a negative oral DPT rules out the diagnosis of NSAIDs hypersensitivity, little is known about follow-up of patients and their resultant behavior about drug intake. On the other hand, missing cofactors, such as infections, comedications, and physical exercise, may lead to false negative DPT results and patients may experience a new adverse reaction after drug intake. The aims of this study were to assess patients' behavior in taking NSAIDs again after a negative allergological workup and to evaluate the subsequent NSAIDs tolerability.

MATERIALS AND METHODS

This is a follow-up study involving patients evaluated in the allergy centers in Novara and Domodossola

Table 1 **Patient interview questionnaire**

1. Have you taken the drug tested in the hospital after negative challenge?
2. If yes, have you had adverse reactions? If yes, what kind of reaction?
3. If no, what were the reasons for avoidance?
4. Have you taken other types of NSAID? Which ones?*
5. If yes, have you had adverse reactions? If yes, what kind of reaction?
6. Have you adopted alternative methods to control pain/inflammation?

**To facilitate patients, a list of NSAIDs brand names was presented.*
NSAIDs = nonsteroidal anti-inflammatory drugs.

Hospitals between July 2004 and June 2010, with a history of immediate cutaneous reactions (urticaria and/or angioedema) after NSAID intake. The Ethics Committee of our Hospital approved the study protocol.

Exclusion criteria were chronic urticaria, NSAID-induced respiratory symptoms, anaphylaxis, severe delayed cutaneous reactions, and clinical contraindications to DPT. All of the patients underwent single-blind placebo-controlled DPT with the suspected offending NSAID as previously described.[7] All of the patients with a negative DPT with the suspected culprit NSAID were contacted by phone not <11 months after the provocation test and were asked to answer to the questions shown in Table 1. Patients were considered dropouts if not answering after five phone calls were made on different days. The brand names of the NSAIDs taken after the DPT were collected and categorized in "strong" and "weak" COX-1 inhibitors and "selective COX-2" inhibitors according to COX inhibition capability.

Statistical Analysis

The frequency of adverse reactions in patients taking the drug previously tested and the reasons why patients did not assume the drug tested despite a negative DPT were recorded and statistically analyzed. Comparisons between groups were made using chi-square test or Student's *t*-test. The negative predictive value (NPV) with its 95% confidence interval (CI) was calculated. Data were analyzed with the statistical SPSS software package (Version 17; SPSS, Inc., Chicago, IL).

RESULTS

One hundred fifty-nine patients were evaluated for suspected NSAID hypersensitivity and the 142 patients who showed negative results to the DPT with the suspected NSAID were included in the study; 111 of them (78.17%) were successfully contacted and 31 (21.8%) dropped out (10 not answering, 20 changed telephone number, and 1 deceased). Mean time between DPTs and phone contact was 29 months (SD ± 13.5 months).

Among the 111 subjects included, 39 were male and 72 female patients. One DPT was performed in 91 patients, 2 DPTs in 19 individuals, and 3 DPTs in 1 patient, with a total number of 132 DPTs performed. In Table 2, the different types of NSAIDs tested in DPTs are described according to their COX-1 inhibition capability.

Ninety-nine patients took an NSAID at least once again; 46 of them took the suspected culprit NSAID after the negative test; 34 took an alternative NSAID, similar to the drug tested for pharmacologic efficacy (a strong COX-1 inhibitor); and 19 subjects took a weaker alternative COX-1 inhibitor NSAID. The majority of these patients (88/99) took more than an NSAID on different occasions. Twelve of 111 patients did not take any NSAID, 2 only using nonconventional therapies, such as homeopathy or acupuncture.

In Table 3, characteristics of the patients in relation to NSAID use or avoidance are described. No significant differences about sex were recorded; concerning age, subjects who did not take the suspected culprit drug after the negative test were of a higher mean age than the patients who took the same NSAID (42.35 ± 16.5 years versus 33.6 ± 16.8 years; $p = 0.007$). Reasons for avoiding the suspected culprit NSAID despite a negative DPT were reported as "no need to use" in 19/65 patients (29.2%) and "fear of a new potential reaction" in 46/65 subjects (70.8%; Table 3). Patients chose a different NSAID than the one tested based on the advice of the pharmacist or the general practitioner. No patients developed further chronic urticaria.

Considering COX-1 inhibition of the NSAIDs tested (Table 2), we found that the weak COX-1 inhibitors tested were taken again in 28/46 cases; meanwhile, strong COX-1 inhibitors were taken in 28/85 cases (60.9% versus 32.9%; $p < 0.01$). In particular, paracetamol was taken in 24/35 cases (68.6%) and acetylsalicylic acid (ASA) only in 11/43 cases (25.6%; Table 2).

Among 99/111 patients taking the same drug or at least a different NSAID, 3 of them (3.03%) experienced an adverse reaction. ASA and paracetamol, tolerated in previous DPTs (cumulative dose, 560 and 675 mg, respectively), were involved in triggering urticaria in two patients at a lower than tested dose, during upper airways viral infection; a subject tolerating ASA developed urticaria after ketoprofen intake for flu. The three observed adverse reactions consisted of mild urticaria and occurred >1 hour after the first intake and controlled at home with antihistaminic therapy. None of the patients agreed to further allergological reevalua-

Table 2 **Types of NSAIDs tested in DPTs**

COX-1 Inhibition	NSAID	NSAIDs Used in DPTs	Took DPT (−) NSAID
Weak COX-1 inhibitors (n = 46)	Paracetamol	35	24 (68.6%)
Strong COX-1 inhibitors (n = 85)	Nimesulide	11	4 (36.4%)
	ASA	43	11 (25.6%)
	Ibuprofen	9	6 (66.7%)
	Ketoprofen	12	7 (58.3%)
	Naproxen	1	0
	Flurbiprofen	1	0
	Diclofenac	8	1 (12.5%)
	Indomethacin	1	1
	Morniflumate	1	1
	Metamizole	4	1 (25%)
	Piroxicam	1	0
Selective COX-2 inhibitors (n = 1)	Celecoxib	1	0

COX-1 = cyclooxygenase 1; NSAID = nonsteroidal anti-inflammatory drugs; DPTs = drug provocation tests; ASA = acetylsalicylic acid.

Table 3 **General characteristics and answers to questionnaire in subjects who used the suspected culprit NSAID after a negative DPT and subjects who did not take the DPT (−) NSAID**

	Took DPT (−) NSAID (n = 46)	Did not take DPT (−) NSAID (n = 65)	Total (n = 111)
Gender M/F	17/29	22/43	39/72
Mean age ± SD (yr)	33.6 ± 16.8*	42.3 ± 16.5	38.8 ± 16.9
Another strong COX-1 inhibitor intake, n (%)	26 (56.5)	34 (52.3)	60 (54)
Another weak COX-1 inhibitor intake, n (%)	16 (34.8)	35 (53.4)	51 (45.9)
Patients who took strong and weak COX-1 inhibitor NSAIDs, n (%)	9 (19.5)	14 (21.5)	23 (20.7)
NSAIDs avoidance, n (%)		12 (18.4)	12 (10.8)
Reactions, n (%)	2 (4.3)	1 (1.5)	3 (2.7)
Reasons for avoiding the same NSAIDs, n (%):			
No need		19 (29.3)	
Fear of a potential reaction		46 (70.7)	

*$p < 0.05$.
COX-1 = cyclooxygenase 1; NSAID = nonsteroidal anti-inflammatory drugs; DPTs = drug provocation tests.

tion. No significant differences in frequency of reactions were observed between patients taking the NSAID previously tested and those subjects who took another NSAID (2/46 versus 1/53; $p = 0.59$).

Considering the three reactions in patients who took at least an NSAID again (99), an overall NPV of 96.97% (95% CI, 91–99%) was calculated. If we limit our analysis to those patients who took the same NSAID or another as strong COX-1 inhibitor (80) an NPV of 96.25% (95% CI, 89.55–99.52%) was calculated; considering only the two reactions occurred in those patients who took the same drug as the one previously negatively tested (46), the NPV was 96.65% (95% CI, 93.99–99.3%).

DISCUSSION

Our study involved 111 patients who experienced an urticaria and/or angioedema reaction after NSAIDs intake and resulted in a negative DPT with the suspected drug. Among them, 46 subjects took the suspected culprit NSAID, and 53 used at least another NSAID; the total number of adverse reactions was 3/99, with an NPV of 96.97%.

A negative DPT performed in hospital settings should reassure patients, ruling out a diagnosis of drug hypersensitivity. However it is well known that a patient who experienced a suspected drug reaction could be anxious about taking again the same drug again, structurally correlated molecules, or even drugs of dif-

ferent categories. Sometimes this behavior may lead to complete avoidance of specific pharmacologic therapies. In real life, prospective studies show that a consistent number of subjects with a negative DPT report avoiding the same tested drug (50–57%) and the main reasons are no need, a different prescription by the general practitioner, and fear of a potential reaction.[8,9] In our study 99 patients took at least an NSAID again (89%); this result is significantly different from some data already available in literature (percentages closer to 50%)[8,10] but is consistent with another follow-up study about NSAIDs,[9] probably because of the type of drug tested, because NSAIDs are commonly used also for common cold, fever, and as pain relievers. However, 58.56% of patients did not take the tested tolerated NSAID, and 10.8% of them did not take any NSAIDs, two only using nonconventional therapies, such as homeopathy or acupuncture. There was no significant difference between male and female patients taking the same NSAID and subjects avoiding the tolerated drug. Moreover, no significant correlation between age and patients' behavior was found, even though a statistical difference was observed.

It is interesting to notice that a negative DPT with paracetamol is cited by a higher intake of the drug (68.6% in our study and 73% in a study by Waton *et al.*) in comparison with other NSAIDs. In our study, many patients were afraid to use strong NSAIDs again, such as ASA, despite a negative DPT. We suppose that Italian general practitioners and pharmacists (for drugs without prescription) could recommend this behavior, because paracetamol is seen as low-risk molecule and it is widely used.

We did not find a statistically significant difference in the number of reactions between patients who have taken the same NSAID and those who have taken a different NSAID. Although none of these patients agreed to undergo a further oral DPT after the described reactions, we think that the reason for these adverse reactions was the presence of a concomitant viral infection.

Previous studies have focused on the high NPV of NSAIDs DPTs (89–97.8%).[8,9,11] Waton *et al.* found an NPV of 89% in patients who experienced cutaneous drug reactions with NSAIDs.[8]

In a cohort study, Defrance *et al.* observed a high NPV (97.8%) in 139/279 patients who took the same NSAIDs tested before. Their study design was different, because it was testing patients with clinical manifestation of respiratory symptoms and anaphylactic shock/anaphylaxis as well as cutaneous symptoms.[9]

Limitations of this study are likely a selection bias, even if characteristics of the contacted persons were comparable from the population first tested through DPTs and the small size sample considered.

In conclusion, we observed a poor compliance to the indications provided to the patients themselves that continued to avoid the NSAID tested intake, mainly for fear of new adverse reactions, particularly when strong COX-1 inhibitor NSAIDs were involved. On the other hand, we observed a small percentage of reactions both in patients who took the same NSAID and in those who took a different NSAID. Hence, it is important to have effective communication between patients and physicians in explaining the meaning of the results of allergological tests (including its limits), to reassure the patients and their general practitioner and to avoid unjustified therapies limitation.

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L16 Immune Phenotype in Children with Mitochrondial Disease

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RATIONALE: Mitochondria contributes to metabolic processes important for cellular growth and function. Defects in mitochrondrial function might negatively impact immune development and responses. Interestingly, there have only been a few publications reporting on increased rate of infections in certain patients with mitochrondial disease. In this clinical retrospective study, we performed immune analysis on 70 pediatric patients diagnosed with mitochrondial disease defined by definitive Walker criteria. The majority of patients lack a history of life-threatening infections.

METHODS: From our mitochrondia cohort, we selected all patients diagnosed as definitive based on the Walker criteria. Seventy patients were identified, 16 with Leigh, 6 with depletion, 2 with SANDO, 1 with NARP, 1 with MELAS and the rest with unknown syndrome. We performed a laboratory retrospective review, documenting all commercial immune results.

RESULTS: Immunoglobulin and IgG subclass levels were within normal range for >90% of patients. Lymphocyte subset data was present for 44 patients. Although the CD45RO absolute count was within the age-specific normal range, the vast majority (65/71, 92%) of the values were in the lower third of the normal range. However, the %CD45RO was below the lower threshold for normal values (n=60, 85%). Conversely, the CD45RA values were on the upper threshold of normal. Most patients have protective titers to tetanus, diphtheria and pneumococcus.

CONCLUSIONS: Most patients with mitochondrial disease do not have perturbed immune development except for reduced CD45RO memory lymphocytes. The clinical significant of this result is unclear, but it suggests that mitochondrial function might be necessary for optimal immune memory development.

L17 Evaluation of Serum Levels of Osteopontin and IgG Anti-Osteopontin Autoantibodies As Potential Biomarkers of Immune Activation in Patients with Allergic Diseases

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RATIONALE: Osteopontin (OPN) is a pleomorphic cytokine known to influence a wide range of immune cells; high OPN and IgG anti-OPN autoantibodies (AutoAbs) levels are associated with an increased risk of autoimmune lymphoproliferative syndrome, multiple sclerosis and systemic lupus erythematosus. We aimed to verify if serum levels of OPN and IgG anti-OPN AutoAbs may qualify as biomarkers of an activated immune response also in allergic patients.

METHODS: Serum OPN levels were measured by ELISA test (Human Osteopontin Duoset, R&D Systems, for OPN detection; "in-house" kit for anti-OPN AutoAbs). A series of 121 adult patients affected by asthma, allergic rhinitis (AR), Hymenoptera venom allergy (HVA), food allergy (FA), allergic contact dermatitis (ACD) and IgE-mediated hypersensitivity to beta-lactams (IEHB) was studied. 116 healthy subjects served as controls.

RESULTS: OPN serum levels were significantly higher in cases in comparison to controls ($p=0.0010$ by the Mann-Whitney test). Statistically higher levels were found in asthma ($p=0.0269$) and FA ($p=0.046$) groups in comparison to controls. Prevalence and titers of serum IgG anti-OPN AutoAbs were significantly lower in cases with respect to controls ($p<0.0001$). Lower levels of AutoAbs versus controls were found in patients with HVA ($p<0.0001$), AR ($p=0.0009$), ACD ($p=0.0011$) and asthma ($p=0.0013$), but not in FA group ($p=0.0575$). Patients with IEHB presented heterogeneous results for OPN and anti-OPN AutoAbs.

CONCLUSIONS: Serum OPN levels may represent a novel, potentially useful biomarker for allergic asthma and, interestingly, for food allergy.

L18 Patient-Reported Outcomes (PROs) in Patients Receiving Omalizumab (OMB): A Systematic Literature Review

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RATIONALE: To summarize clinical trial and real-world evidence describing the magnitude and duration of impact of OMB as add-on therapy on PROs in patients with moderate to severe allergic asthma.

METHODS: Systematic literature review (MEDLINE/EMBASE) was conducted to identify studies of OMB in pediatric/adolescent/adult patients with moderate to severe allergic asthma. Outcomes of interest included measures of self-reported asthma control, asthma-specific and general quality of life assessments/questionnaires, and patient symptom reports.

RESULTS: 25 randomized controlled trials (RCTs) and 34 non-randomized studies (NRSs) were included. Among 8 RCTS reporting the Asthma Quality of Life Questionnaire (AQLQ) overall score, statistically significant improvements favoring OMB versus placebo/control, were documented in 5 studies; at 52 weeks, mean/median changes from baseline in domain and overall scores ranged from 1.01-1.33 for OMB and from 0.8-0.98 for placebo ($P<0.01$). At 20–52 weeks, proportions of patients with a minimally important difference (MID) in AQLQ improvement (≥0.5 points from baseline) ranged from 57.5%–78.8% with OMB and from 22.2%–69.8% with placebo/control. Statistically significant improvements in mean Asthma Control Test (ACT) scores from baseline to post study were found in 12 of 22 NRSs, ranging from 9.4-17.28 at baseline to 17.4-22.5 at 8 months to 6 years. Seventeen of 22 NRSs reported achievement of a MID in ACT (≥3 points from baseline) for patients treated with OMB.

CONCLUSIONS: Results from this systematic literature review confirm that OMB-treated patients with moderate to severe asthma achieve clinically meaningful improvements in PROs, which are observed across both RCTs and observational studies.

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Italian Multicenter Cross-Sectional Study (AISAG) on light smoking and allergic diseases in adults

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Key words

Smoking; allergic respiratory diseases; food allergy; allergic dermatitis

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Summary

Allergic rhinitis, allergic dermatitis, and food allergy are extremely common diseases and are frequently associated to each other and to asthma. Smoking is a potential risk factor for these conditions, but so far, results from individual studies have been conflicting. On the basis of these contradictory data in the literature we have carried out a multicenter cross-sectional study to evaluate the relationship between some allergic conditions and exposure or not to active light smoking. The study was carried out between May 2013 and November 2013 in 22 different Italian hospitals. Patients with respiratory and/or food allergy, and aged 18 years and over, visited at Allergy Outpatient Clinics, were invited to participate. A total of 1586 allergic patients (21.6% smokers) with a mean age of 39.2 years (standard deviation, SD = 15.1) were included. We demonstrated that the prevalence of tobacco smoking was higher in patients with food allergy and in asthmatic patients in stage III-IV. But no other statistical differences were found at univariate analysis. The sensitization patterns of non-smokers and smokers were similar. Furthermore, tobacco smoking was associated with higher risk of food allergy and lower risk of asthma. Moreover, tobacco smoking was an independent risk factor for persistent respect to intermittent rhinitis, and for asthma GINA stage III-IV with respect to stage I-II.

Introduction

Population-based studies appear to show a relationship between smoking and bronchial hyperresponsiveness. However, the presence of asthma in adults has generally been unrelated to smoking history, possibly reflecting a false opinion about the tendency for asthmatics not to become regular smokers or to smoke less than their non-asthmatic counterparts (1). Several studies have demonstrated that active smoking increases the risk for developing asthma (2-5).

But there are also scattered studies that seem to cast doubt on the relationship between exposure to cigarette smoke and asthma / allergies. For example, it was reported that IgE levels in smokers showed a moderate inverse correlation with the degree of smoking and that the mean IgE level in ex-smokers was much lower than in current light smokers but was still higher in nonsmokers (6). It was also demonstrated that cigarette smoking is associated with high prevalence of chronic rhinitis and low prevalence of allergic rhinitis in men (7). On the basis of these contradictory data in the literature we have carried out a multicenter cross-sectional study to evaluate the relationship between some allergic conditions and exposure or not to active light smoking.

Methods

The study was carried out between May 2013 and November 2013 in 22 different Italian hospitals. Patients with respiratory and/or food allergy, and aged 18 years and over, visited at Allergy Outpatient Clinics, were invited to participate. Patients were asked about their smoking habit; non-smokers and light smokers (defined as 5-10 cigarettes per day for 5-10 years) were included in the study. The local Ethics Committee approved the study design and protocol and patients gave written informed consent.

For each subject, we collected data on age, gender, smoking habit, allergic symptoms, the pattern of respiratory sensitization, the presence of food allergy and atopic dermatitis. Asthma severity and control, and rhinitis severity were scored according to the Global Initiative for Asthma (GINA) and the Allergic Rhinitis and its Impact on Asthma (ARIA) Guidelines, respectively.

Skin prick tests (STPs) were done using a panel of standardized commercial extracts of allergens of the most common ones responsible for respiratory symptoms in Italy: pollens (*Graminaceae mix 5:* grass; *Compositae mix*; *Parietaria mix*: pellitory; *Betula pendula*: birch; hazelnut; olive, cypress), house dust mites (HDM: *Dermaptophagoides pteronyssinus* and *D. farinae*), animal danders (dog, cat), feathers mix, moulds (*Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillum mix*).

Serum specific IgEs were detected by currently available commercial laboratory methods (RAST and ImmunoCAP; Pharmacia AB, Uppsala, Sweden, then Phadia AB, now Thermo Fischer Scientific).

Patients were divided into two groups according to tobacco smoking: non-smokers and light smokers. Common statistical methods were used for the analysis of proportions and associations between tobacco smoking and demographic and clinical features. Furthermore, multivariate logistic regression models, adjusted for covariates, were used to investigate associations of tobacco smoking (independent variable) with allergic symptoms, food allergy, atopic dermatitis and the pattern of respiratory sensitization (dependents variable). Results are presented as odds ratios (OR) and 95% confidence intervals. The selection of variables for fitting the most parsimonious model was performed using a backward stepwise procedure, with p = 0.10 for retaining each variable in the model.

Statistical analysis

For statistical tests, *P* values lower than 0.05 were considered significant in two-tailed tests. All statistical analysis were carried out using STATA, version 12.0, software (STATA Statistics/Data Analysis 12.0 - STATA Corporation, College Station, TX, USA).

Results

A total of 1586 allergic patients (21.6% smokers) with a mean age of 39.2 years (standard deviation, SD = 15.1) were included. The majority of them were aged 35 years or less. Asthma was present in 72.2% of subjects, rhinitis in 79.4% and United Airways Disease (rhinitis plus asthma) in 47.6%. Most of asthmatic were in GINA stage I and II (89.9%), whereas among patients with rhinitis, 57.6% had intermittent symptoms. The most common respiratory allergenic sensitizations were, in decreasing order: grass (62.9%), HDM (53.3%), Betula (29.6%) and Parietaria (25.0%).

The demographic and clinical features according to smoking habits are shown in **table 1**. The proportion of tobacco smokers was significantly higher in males, subjects younger than 45 years, non-asthmatic patients, and those with persistent rhinitis. In addition, the prevalence of tobacco smoking was higher in patients with food allergy and in asthmatic patients in stage III-IV, next to statistical significance threshold. No other statistical differences were found at univariate analysis. The sensitization patterns of non-smokers and smokers were similar, as shown in **figure 1**; this was confirmed when we restricted the analysis to monosensitized patients (**figure 2**).

After adjusting for demographic and clinical features, tobacco smoking was associated with higher risk of food allergy (OR = 1.46, CI 95%: 0.97-2.19; p = 0.069) and lower risk of asthma (OR = 0.75, CI 95%: 0.57-0.99; p = 0.042). Moreover, tobacco smoking was an independent risk factor for persistent respect to intermittent rhinitis (OR = 1.51, CI 95%: 1.16-1.96; p = 0.002), and for asthma GINA stage III-IV respect to stage I-II (OR = 1.73, CI 95%: 1.09-2.74; p = 0.021). No associations were observed between tobacco smoking and other clinical characteristics in multivariate logistic regression models.

***Table 1** - Demographic and clinical characteristics according to tobacco smoking.*

Variables	Categories	Non-smokers n (%)[1]	Smokers n (%)[1]	Total n (%)[1]	P value[2]
Total		1243 (78.4)	343 (21.6)	1586	
Gender	Male	687 (55.3)	143 (41.7)	830 (52.3)	< 0.001
	Female	556 (44.7)	200 (58.3)	756 (47.7)	
Age (years)	32 ≤	428 (34.4)	128 (37.3)	556 (35.1)	0.010
	33-44	375 (30.2)	123 (35.9)	498 (31.4)	
	≥ 45	440 (35.4)	92 (26.8)	532 (33.5)	
Family history of allergy	No	877 (71.9)	241 (70.9)	1118 (71.7)	NS
	Yes	342 (28.1)	99 (29.1)	441 (28.3)	
Asthma	No	326 (26.3)	111 (32.4)	437 (27.6)	0.025
	Yes	915 (73.7)	232 (67.6)	1147 (72.4)	
GINA criteria (restricted to patients with asthma)	I-II	801 (90.7)	197 (86.8)	998 (89.9)	*0.080*
	III-IV	82 (9.3)	30 (13.2)	112 (10.1)	
Rhinitis	No	967 (77.6)	282 (22.4)	1258 (79.4)	NS
	Yes	267 (81.6)	60 (18.4)	327 (20.6)	
ARIA classification (restricted to patients with rhinitis)	Intermittent	581 (59.6)	143 (50.9)	724 (57.6)	0.009
	Persistent	394 (40.4)	138 (49.1)	532 (42.4)	
Polysensitization	No	405 (32.6)	112 (32.7)	517 (32.6)	NS
	Yes	838 (67.4)	231 (67.3)	1069 (67.4)	
United airways disease	No	646 (52.0)	183 (53.7)	829 (52.4)	NS
	Yes	596 (48.0)	158 (46.3)	754 (47.6)	
Atopic dermatitis	No	1164 (93.9)	319 (93.5)	1483 (93.8)	NS
	Yes	76 (6.1)	22 (6.5)	98 (6.2)	
Food allergy	No	1136 (91.6)	302 (88.6)	1438 (91.0)	0.082
	Yes	104 (8.4)	39 (11.4)	143 (9.0)	

[1]Column percentage,

[2]Chi square test. NS: Not statistical significant

***Figure 1** - Pattern of the sensitizations (skin test positive) between non-smokers and smokers.*

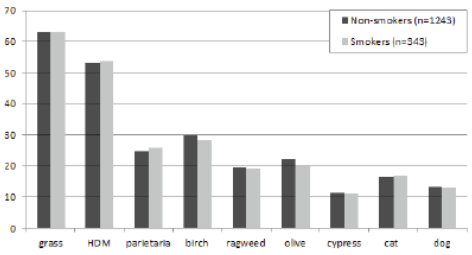
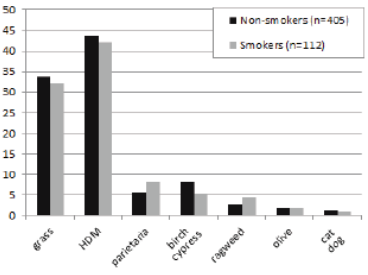


***Figure 2** - Pattern of the sensitizations (skin test positive) between monosensitized non-smokers and smokers.*



Discussion

Allergic rhinitis, allergic dermatitis, and food allergy are extremely common diseases and are frequently associated to each other and to asthma. Smoking is a potential risk factor for these conditions, but so far, results from individual studies have been conflicting. Prevalence rates for smoking in asthma are relatively close to those found in the general population and several studies have demonstrated that active smoking increases the risk for developing asthma (8-10). A 1996 longitudinal study of 5801 people born in 1958 who were part of a national British cohort has implicated smoking in the development of wheeze and asthma in young adults (8). Subjects were followed up at the ages of 7, 11, 16, 23, and 33 years. Active smoking was strongly associated with the incidence of asthma and wheezing illnesses between the ages of 18 and 33 (OR = 4.42, 95% CI 3.31-5.92) after controlling for a variety of factors, including gender, maternal age, birth order, gestational age, hay fever, eczema, father's social class, and maternal smoking. In addition, among the 880 children who developed asthma or wheezy bronchitis by age seven, relapse at age 33 after prolonged remission of childhood wheezing was more common among current smokers. A study of adolescents found that those who smoked ≥ 300 cigarettes per year had a relative risk of 3.9 for developing asthma, compared to their non-smoking peers (9).

There is also a growing body of evidence that secondhand smoke exposure is associated with the development of asthma in early life (10). Maternal smoking is the most important cause of secondhand smoke exposure, because of the greater exposure of the child to the mother than the father (11-13).

In adults, data on the effects of environmental tobacco exposure on nonmalignant lung disease are sparse. The association between passive exposure to tobacco smoke and respiratory symptoms was studied in a sample of 4197 non-smoking adults as part of the Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA Study) (14). Passive exposure to tobacco smoke was associated with increases in the risks of doctor-diagnosed asthma (odds ratio = 1.39), wheezing, bronchitis, and dyspnea.

Prenatal exposure to smoking may also be important, being associated with reduced pulmonary function in the infant. One study, for example, evaluated the effect of prenatal maternal cigarette smoking on the pulmonary function of 80 healthy infants shortly after birth (15). Maternal smoking was assessed by questionnaire reports and urine cotinine concentration at each prenatal visit. Pulmonary function (assessed as flow at FRC) was lower in infants whose mothers smoked compared to those whose mothers did not smoke. Another report evaluated the effect of early levels of lung function on the subsequent occurrence of a wheezing lower respiratory tract illness in the first year of life (16). Reduced pulmonary function early in life increases the risk for wheezing and subsequently for asthma later in life. It has been proposed that prenatal smoking exposes the fetus to the growth-retarding effects of tobacco and enhances airway-parenchymal dysanapsis (disproportionately small airways compared to the size of the pulmonary parenchyma). These changes may contribute to the postnatal expression of increased airway responsiveness and asthma (17).

Two other studies have examined the effects of prenatal and postnatal exposure to smoking on asthma and wheezing in children (18-19). The first study used a broad case definition to identify 620 schoolchildren aged seven to nine years in Cape Town with current asthma or wheeze in the last 12 months (18). In bivariate analyses, maternal smoking, whether defined as ever smoking (OR = 1.80), smoking during pregnancy (OR = 1.97), smoking during the first year of the child's life (OR = 1.70), or

current smoking (OR = 1.70) was significantly associated with current asthma/wheeze among the children. The number of cigarettes smoked daily by the mother and the number of household smokers were also related to current asthma/wheeze. Further strengthening these findings, the children's cotinine-creatinine ratio was significantly associated with current asthma/wheeze (OR = 1.61 for the highest quartile versus the lowest quartile). In a multivariate logistic regression model controlling for a variety of known risk factors, maternal smoking during pregnancy (OR = 1.87, 95% CI 1.25-2.81) and the number of household smokers (OR = 1.15, 1.01, 1.30) remained significantly associated with current asthma/wheeze. The second study examined the relationship between current and past exposure to maternal, paternal, and non-parental environmental tobacco smoke in the home and several measures of asthma and wheeze in a large sample of school-aged children (11,534 children) from 24 communities in the US and Canada (19). Asthma was identified based on either an active diagnosis of asthma or use of medication for asthma. Wheeze outcomes were: any wheezing, wheezing with a cold, wheezing without a cold, persistent wheeze, shortness of breath with wheeze, awakening at night by wheezing, wheezing with exercise, medication for wheeze, emergency department visit for wheeze, and hospitalization for wheeze. Children who were currently exposed had a significantly increased risk of reported wheeze with a cold (OR = 1.65), emergency department visit for wheeze (OR = 1.63), persistent wheeze (OR = 1.42), shortness of breath with wheeze (OR = 1.35), wheeze with exercise (OR = 1.24), and medication for wheeze (OR = 1.23) in past year. For most of the wheeze outcomes, there was an increasing risk associated with increasing number of smokers in the home and number of cigarettes smoked in the home per day. Active asthma was significantly associated with expo-sure to environmental tobacco smoke in pregnancy only (OR = 2.70, 95% CI 1.13-6.45), and no significant association was found for currently exposed children.

Cigarette smoking and asthma interact to induce important adverse effects on clinical, prognostic and therapeutic outcomes (20-25). Active smokers, particularly females, are at risk of developing asthma. Smokers with asthma experience worse asthma control than nonsmokers with asthma. Mechanisms for the adverse effects of smoking in asthma include altered airway inflammation and corticosteroid insensitivity. Finally, in a recent systematic review and meta-analysis, it were observed very modest associations between smoking and some allergic diseases among adults (26). Among children and adolescents, both active and passive exposure to second hand smoke were associated with a modest increased risk for allergic diseases, and passive smoking was associated with an increased risk for food allergy. In our study we demonstrated that the prevalence of tobacco smoking was higher in patients with food allergy and in asthmatic patients in stage III-IV. But no other statistical differences were found at univariate analysis. The sensitization patterns of non-smokers and smokers were similar. Furthermore, tobacco smoking was associated with higher risk of food allergy and lower risk of asthma. Moreover, tobacco smoking was an independent risk factor for persistent respect to intermittent rhinitis, and for asthma GINA stage III-IV respect to stage I-II. Additional studies with detailed measurement of exposure and better case definition are needed to further explore the role of smoking in allergic diseases. In conclusion, quitting smoking can improve symptoms and lung function, but the low rates of smoking cessation highlights the need for improved strategies for managing these patients. Clinical trials assessing new therapies for asthma need to enroll smokers to identify treatments that are effective in the asthma smoking phenotype.

Limits of the study

We are aware that our research may have an important limitation. The association between tobacco smoking and asthma could be affected by reverse causality bias: subjects with asthma are less inclined to start smoking than not asthmatic subjects, and probably smokers tend to quit smoking at the onset of asthmatic symptoms. A prospective cohort study of subjects without asthma and/or allergy should be appropriate to disentangle this topic, though it requires large sample and long times of observation. Finally, we have not considered in the study the problem of "secondhand" smoke during pregnancy or in early childhood and the and its potential consequences in adulthood.

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In 0~3 and 45~59 years old group, the positive rate of sIgG antibody of different categories of food were not statistically different in gender, in 4~6 years old group, the positive rate of cereals, fruits, vegetables of male are higher than female (x^2=7.068, p=.003; x^2=4.850, p=.031;x^2=4.135, p=.042), in 7~16 years old group, the positive rates of vegetables and crustaceans of male are higher than female (x^2=5.011, p=.026; x^2=5.491, p=.019), in 17~44 years old group, the positive rates of vegetables of female are higher than male (x^2=8.445, p=.004), in ≥60years old group, the positive rates of cereals, nuts and oilseeds, vegetables, milks and dairy products, fishes of male are higher than female (x^2=3.902, p=.048; x^2=6.836, p=.006; x^2=16.228, p=.000x^2=6.163, p=.011;x^2=8.123, p=.003).
A lot of food sIgG antibody levels are associated, there are 51 couples of food highly correlated (r_s=0.8), including 34 couples of vegetables, 2 couples of fruit and vegetable.
Conclusion
There are certain distribution characteristics of 90 kinds of food sIgG antibody in different age and gender, clinical diagnosis should be combined with sex,dietary habits and other factors to make guidance more reasonable for the patient's diet adjustment.

A375

Evaluation of Serum Levels of Osteopontin As a Potential Biomarker of Immune Activation in Patients with Allergic Diseases

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A) Background: Osteopontin (OPN) is a pleomorphic cytokine known to influence a range of immune cells, including macrophages, neutrophils, dendritic cells, T and B cells. High OPN levels are associated with a significantly increased risk of autoimmune lymphoproliferative syndrome, multiple sclerosis and systemic lupus erythematosus, suggesting that OPN is a candidate biomarker of these conditions. In the present cross-sectional study, we aimed to verify if serum levels of OPN may qualify as a biomarker of an activated immune response in allergic patients.
B) Method: Serum OPN levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Human Osteopontin Duoset, R&D Systems). A series of 77 adult patients (median age females: 49 years; males: 47 years) with different allergic diseases, was studied: 34 patients (44%) had allergic rhinoconjunctivitis, 15 (19%) asthma, 17 (22%) hymenoptera venom allergy, 5 (6%) allergic contact dermatitis, 3 (4%) food allergy and 3 (4%) IgE-mediated hypersensitivity to beta-lactams. 116 healthy subjects with similar demographic characteristics served as controls. Data were analyzed comparing cases to controls, as well as looking for subgroup differences within the group of allergic patients.
C) Results: OPN serum levels were significantly higher in cases in comparison to controls (median 12181 pg/ml, interquartile range 6953 – 19359 pg/ml vs 6099 pg/ml, interquartile range 3122 – 14520 pg/ml; p = 0.0010 by the Mann-Whitney test). The highest serum OPN levels were observed among patients with asthma (median: 15668 pg/ml; p = 0.0156) followed by those observed in the hymenoptera venom allergy group (median: 14239 pg/ml; p = 0.0080). Lower values of OPN were detected in the group of patients with rhinoconjunctivitis (median: 10291 pg/ml; p = 0.0436), allergic contact dermatitis (median: 9088 pg/ml) and food allergy (median: 4386 pg/ml). Patients with IgE-mediated sensitization to beta-lactams had heterogeneous values, not statistically different in comparison to controls.
D) Conclusions: Serum OPN levels may represent a novel, potentially useful biomarker of allergic respiratory diseases and hymenoptera venom allergy. Consideration should be given to explore clinical correlates of high OPN levels in these conditions.

A376

Prevalence of Allergic Rhinitis in 3-6-Year-Old (preschool) Children in Chiba City (urban area), Japan

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Background
The sequential development of allergic diseases (beginning with food allergy and atopic dermatitis followed by asthma and allergic rhinitis (AR)) during early childhood is often referred to as the allergy march. Recently, the number of school-age children with AR has shown to increase in Japan. But early onset of AR is poorly described, and it remains unknown about the prevalence of allergic rhinitis in young children.
Objective
We aim to evaluate the prevalence, clinical characteristics, and treatment of AR in a population of 3-6-year-old (preschool) children in Chiba city (urban area), Japan.
Method
A total of 13,963 children aged 3-6 years in all 84 kindergartens of Chiba city, Japan were surveyed. Prevalence of symptoms of allergic rhinitis was assessed using a modified version of the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire.
Results
A total of 9,822 (70.3%) questionnaires were returned for evaluation (sex: Male 50.5%, Female 49.5%; age 3y = 2.3%, 4y = 31.7%, 5y = 35.1%, 6y = 30.9%). The prevalence of lifetime, current and physician-diagnosed allergic rhinitis were 54.1%, 50.7% and 37.3%, respectively. The prevalence of AR was higher in males than that in females (cf. physician-diagnosed AR; 40.6% vs. 33.6%, P < 0.05) and increased with age (cf. physician-diagnosed AR; 3y = 18.5%, 4y = 28.3%, 5y = 37.3%, 6y = 46.7%). Many children showed AR symptoms during September and April, especially in February and March (cedar pollen allergy season). About 70 % of children with AR visited clinic or hospital, but more than half of them were dissatisfied with their treatment.
Conclusion
The prevalence of AR symptoms was high and starting early in life.

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Comparative Efficacy of Combination Nebulized Salbutamol and Fluticasone Propionate and Nebulized Salbutamol in Children with Mild Moderate Asthma Attack

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Background: Short acting beta-2 agonist (SABA), systemic corticosteroids and oxygen are the primary therapy in asthma attack. Repeated use of systemic corticosteroid is at risk of systemic side effects. Inhaled corticosteroid offer potential benefit because of direct effect on the airways and lower systemic side effects.
Objective: To compare the efficacy between combination of nebulized salbutamol and fluticasone propionate and nebulized salbutamol in children with mild and moderate asthma attack.
Methods: Thirty children (age 5-14 years) with mild and moderate asthma attacks in outpatient clinic and emergency department of Soetomo hospital were investigated in an open label randomized controlled trial study. Subjects are divided into treatment group (combination of salbutamol and fluticasone propionate) and control group (salbutamol), each of the 15 children. Pulmonary score and side effects (tachycardia and tremor) was evaluated at before and after nebulized at 20, 50 and 60 minutes. The statistical analysis used were t test, Mann Whitney test and the Wilcoxon signed rank test according to the type of data.

EDITORIAL

Potential role of hematological parameters in patients with chronic obstructive pulmonary disease: current point of view

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The article by Kalemci et al[1] published in the current issue of *Polish Archives of Internal Medicine* (*Pol Arch Intern Med*) deals with a topic of recent scientific interest. Chronic obstructive pulmonary disease (COPD) is recognized as one of the most important lung diseases leading to disability and death. For many years, COPD has been considered an exclusive pulmonary disease, characterized by a well-defined respiratory pattern; only recently, it has been reevaluated as a systemic chronic inflammatory condition. In particular, according to the first scientific evidence, increased airway inflammation in COPD exacerbations seems to represent an optimal prothrombotic stimulus.[2,3]

In the above article, the authors aimed to analyze the relationship between platelet indices, including the platelet-to-lymphocyte ratio (PLR), white blood cell count to mean platelet volume ratio (WMR), and red cell distribution width (RDW) and the severity degree of COPD. A retrospective cohort, with a total of 153 patients admitted between March 2014 and March 2015, was studied. The diagnosis of COPD was established according to the Global Initiative for Chronic Obstructive Lung Disease criteria, and patients were divided into 4 groups depending on disease severity: group A (mild), group B (mild to moderate), group C (moderate to severe), and group D (severe).[4] The authors found a significant increase in platelet distribution width (PDW), mean platelet volume (MPV), plateletcrit (PCT), and RDW values and a decrease in WMR and PLR values as the severity of COPD increased from groups A to D. Patients with severe COPD, belonging to groups C and D, were older, mainly male, presented higher RDW, PDW, MPV, PCT, PLR, and NLR values but had lower hemoglobin levels, lymphocyte count, and WMR compared with the mild COPD groups (A and B). In particular, the PDW was shown to be significantly higher in each COPD group in an increasing manner from group A to group D. The RDW value also significantly increased with disease severity, except for the difference between groups A and B. Moreover, the RDW (adjusted odds ratio [OR], 3.668; 95% confidence interval [CI], 1.234–11.75) and the PDW (adjusted OR, 2.454; 95% CI, 1.036–5.811) were found to be independently associated with the presence of severe COPD (groups C and D). To determine the cut-off values for the RDW and PDW for severe COPD, a receiver operating characteristic (ROC) curve analysis was performed. Using the cut-off level of 14.85, the PDW was correlated with the presence of severe COPD with a sensitivity of 85% and specificity of 86% (area under the ROC curve [AUC], 0.946; 95% CI, 0.911–0.980; P <0.001); in addition, an RDW value above 14.45 was associated with severe COPD with a sensitivity of 90% and a specificity of 87% (AUC, 0.948; 95% CI, 0.916–0.981; P <0.001). Platelet indices such as the MPV, PDW, PCT, PLR, RDW, and neutrophil-to-lymphocyte ratio showed an increasing trend, while the lymphocyte count and WMR tended to decrease with the increasing severity of COPD. Furthermore, the authors found that the RDW and PDW parameters were independently associated with severe COPD besides the effects related to age.

Platelets play an important role in several systemic inflammatory conditions by secreting different cytokines and mediators that regulate activation of immune cells and their adhesion to the endothelial barrier, thus presenting an active role in the modulation of inflammatory immune responses. In our previous study, we hypothesized that an increase in PDW with increasing severity of COPD could be correlated with elevated atherothrombotic risk and/or major systemic inflammation.[5] Increased MPV is a marker of platelet activation and an acute-phase reactant in inflammatory conditions depending on the severity of systemic inflammation; patients with more

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severe COPD tend to present higher MPV values. PCT depends on the number of platelets in blood, which is associated with the risk of cardiovascular events, including thrombosis and worse outcomes in acute coronary syndrome. The low lymphocyte count is correlated with systemic inflammation; if considered together with the platelet count, the PLR reflects the inflammatory status in a more sensitive manner.

The RDW parameter represents a quantitative indicator of both complete blood count and anisocytosis. It is usually increased in conditions associated with ineffective red cell production and increased red cell destruction. Seyhan et al[6] and Ozgul et al[7] found a significant relationship between the RDW and increased mortality of patients with stable COPD. An increased RDW reflects an impaired regulation of erythrocyte homeostasis, including abnormal erythropoiesis and red blood cell survival, which are due to a variety of underlying metabolic abnormalities, such as shortening of telomere length, oxidative stress, chronic inflammation, poor nutritional status, dyslipidemia, hypertension, erythrocyte fragmentation, and deregulation of erythropoietin function. The RDW was also found to be a useful diagnostic tool in patients with suspected acute pulmonary embolism.[8] In conclusion, PDW and RDW values could be used as indicators of hypoxemia, underlying inflammation, and oxidative stress in patients with COPD.

Among the limitations of the study, we must note that the authors did not include healthy controls to compare with the study group. It would be also interesting to study a possible correlation between platelets and blood cell parameters with other markers of inflammation[9,10] and endothelial involvement, such as interleukin 6, von Willebrand factor, D-dimer, prothrombin, and plasminogen activator inhibitor 1, as well as with functional measurement of endothelial dysfunction such as noninvasive peripheral arterial tonometry.[11] Also the correlation with spirometry and other functional data, both in active smokers and nonsmokers with COPD, would be of great interest. Moreover, it would be important to confirm the data in larger population-scale studies to obtain more statistically powerful results.

Despite the above limitations, the study by Kalemci et al[1] has significantly contributed to confirming the role of platelets in the systemic inflammatory process in patients with COPD and the correlation between the severity of COPD and PDW, since so far there have been paucity of literature data on this topic. Hopefully, these preliminary data will soon be useful for monitoring systemic inflammation levels and for finding prevention measures and biologic treatments for atherothrombotic complications of COPD in selected patients.

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Awards

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2. Selected member of the World Allergy Organization (WAO) Committee on *'Allergy Diagnosis and Molecular Allergy'* for the term 2016 - 2018.

3. Selected member of the American Academy of Allergy Asthma Immunology (AAAAI) Committee on *'Immunotherapy, Allergen Standardization and Allergy Diagnostics'* for the term 2017 - 2018.

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