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EOSINOPHILIC INFLAMMATION IN NASAL POLYPOSIS: REGULATION OF INTERLEUKIN 5 AND INTERLEUKIN 5 RECEPTOR α ISOFORMS

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Thesis submitted as partial fulfilment of the requirements
for the degree of doctor in medical sciences
2004

Cover photo: Subepithelial eosinophilic inflammation in the mature polyp, EG2 stained (original magnification: 200X).

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ACKNOWLEDGEMENTS

In the beginning there was nothing … or just everything to make this thesis come true!

Paul van Cauwenberge, for giving me the opportunity to dedicate a part of my training to scientific research, for offering me full scope to develop my scientific and professional skills, for being proud as a father whenever I take a new step, for sharing his dreams with me ...
I truly hope to be your biggest fan. When can I get another signature?

Claus Bachert, for his research enthusiasm, for the countless stimulating discussions and laughs, for responding every e-mail and correcting any manuscript within 24 hours despite the continent he is traveling, but most off all for support and friendship!

Jan Tavernier, for his brilliant research ideas and interesting scientific visions, for his support in writing grant applications, for helping me to write manuscripts and to answer difficult questions of referees.

Claude Cuvelier, for teaching me the first principles of immunohistochemistry, for the hours we spend analyzing histological slides and making pictures, for his support in writing grant applications and manuscripts.

Gabriele Holtappels, for the laborious and accurate lab-work she performed for me. Her dedication and aim at perfection are striking. This thesis is largely the result of her work.

Jean-Baptiste Watelet, Thibaut van Zele, Sofie Claeys, and Peter Hellings, for the joyful discussions, their research enthusiasm and friendship.

Hubert Vermeersch, Ingeborg Dhooge, and Katrien Bonte, for the precious time they daily spend to my ENT-training, for giving me space to finish my thesis, for all their support and friendship.

All my colleagues: Benedicte Verhaeghe, Muriel van Kempen, Lore Deschrijver, Els De Leenheer, Sven Geukens, Nathalie Lagasse, Mieke Beel, Tom Vandenbussche, Philippe Heylbroeck, Yves Callier, Bruno Lantsoght and Catherine Dick.

I want to thank all members of the Examination Committee: **Eric Veys, Guy Brussele, Norbert Freayman, Paul van Cauwenberge, Claus Bachert, Jan Tavernier, Claude Cuvelier, Wytske Fokkens and Gunnar Johansson** for critically reading this thesis.

My sincere appreciation goes to the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) and to the Ghent University (BOF) for providing me with a mandate as research assistant.

I also whish to thank the European Academy of Allergy and Clinical Immunology for providing me the EAACI Exchange Research Fellowship for a collaboration with Gunnar Johansson and Lars Lundblad at the Karolinska Hospital in Stockholm, Sweden.

Gunnar Johansson, Cecilia Hellman, Joachim Lundahl, and Lars Lundblad, for the fantastic cooperation, hospitality, friendship and for the unforgettable time at Karolinska.

Lucie Fransen, Hugo Walter, and Stany Depraetere, from **Innogenetics NV** for their good collaboration.

Luc Klinkenborg, for his pleasant collaboration and **Schering-Plough Research Institute** for giving me the opportunity to test Reslizumab in nasal polyp patients.

All patients for their cooperation and punctuality during my studies!

Although it is not possible to name everyone individually without forgetting someone, I greatly extend my appreciation to various persons who directly or indirectly helped me in the preparation of this thesis: Rudi Van De Walle, Claudina Perez-Novo, Cindy Claeys, Marie-Chantal Herteleer, Gergory Clement, Vilmos Domjan, Wim Van Hecke, Bart Vinck, Geert Leroux-Roels, Jo Vandesompele, Frank Speleman, Jos Van Der Heyden, Lennart Zabeau, Jos Cleays, Geert De Vos, André Dierick, Dirk Boedts, Sabine Lepercque, Luc Van Bortel, Romain Lefebvre, Marc Bogaert, Peter De Paepe, Pieter Demetter, Marleen Preat, Georges Van Maele, Marc Mareel, Gernot Rohde, Bernard Gregory, Douglas Robinson, Peter Howarth…

To my family

At this occasion, I want to thank my parents-in law, Stephanie, Jan, Delphine and Pieter for their friendship, for being so warm and generous.

I want to thank my wonderful parents for my unforgettable and delightful youth and for their unconditional help and love. Also I want to thank my brother Lieven for the numerous lively discussions and for sponsoring my dinner.

Above all, I express thankfulness and love to Barbara, Bert and Lucas, who encouraged me to bring this thesis to a good end. Barbara, your patience, serenity, encouragement, and continuous support have helped me during this long and never-ending period. Though your full time practice, you managed to run the family and to be a loving mother for our sons.
Bert and Lucas, your playful enthusiasm and daily love have been the “motor of my life”.

Barbara, Bert and Lucas,

This work is dedicated to you.

LIST OF PUBLICATIONS

This thesis is based on the following articles submitted to, accepted or published in international peer reviewed journals.

Bachert C*, P. Gevaert*, G. Holtappels, SGO. Johansson, and P. Van Cauwenberge. 2001. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. *J.Allergy Clin.Immunol.* 107:607-614.

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Gevaert P., C. Bachert, G. Holtappels, C. P. Novo, J. Van der Heyden, L. Fransen, S. Depraetere, H. Walter, P. Van Cauwenberge, and J. Tavernier. 2003. Enhanced soluble interleukin-5 receptor alpha expression in nasal polyposis. *Allergy* 58:371-379.

Gevaert P., G. Holtappels, SGO. Johansson, C. Cuvelier, P. van Cauwenberge P, C. Bachert. Organisation of secondary lymphoid tissue and local IgE formation to S aureus enterotoxins in polyp tissue. *Allergy (In press 2004).*

Gevaert P, Hellman C, Lundblad L, Lundahl L, Holtappels G, Perez Novo C, Fransen L, van Cauwenberge P, Tavernier J, Bachert C. Regulated interleukin-5 receptor α isoform expression in blood and tissue eosinophils. *Submitted 2003.*

Gevaert P., Lang-Loidolt D., Stammberger H., Lackner A., Sacks H., Van Zele T., Holtappels G., Tavernier J., van Cauwenberge P., Bachert C. Nasal interleukin-5 levels determine the response to anti-interleukin-5 treatment in nasal polyp patients. *Submitted 2004.*

* Equal contribution

SUMMARY

As the vast majority of bilateral nasal polyps (NP) are associated with a prominent eosinophilic inflammation and as eosinophils are well recognised in eliciting tissue damage and subsequent re-modelling, we aimed to investigate the role of eosinophils in the pathogenesis of NP. Especially the regulation of interleukin-5 (IL-5) and the interleukin-5 receptor α (Rα) isoforms were studied with emphasis on future therapeutic strategies in NP.

Characterisation of NP suggests a central deposition of plasma proteins (albumin), regulated by the subepithelial, mainly eosinophilic inflammation, as pathogenetic principle of polyp formation and growth. The accumulation and activation of eosinophils is favoured by the low concentrations of TGF- β_1 and overproduction of IL-5 and eotaxin in NP tissue. Although elevated IgE levels are found in NP, total IgE and IgE antibodies in NP tissue was unrelated to skin prick tests, but correlated with the degree of eosinophilia. In addition, we demonstrated the organisation of secondary lymphoid tissue in NP tissue and a polyclonal hyper-immunoglobulinemia E associated with the presence of IgE specific to *S. aureus enterotoxins (*SAEs), colonization with *S. aureus,* and increased eosinophilic inflammation in a relevant subgroup of NP patients.

The ultimate way to test the role of IL-5 and eosinophils in the pathogenesis of nasal polyposis, is by antagonizing IL-5 in an interventional study in NP patients. However, eosinophils show varying IL-5 sensitivity due to a different expression of the IL-5Rα isoforms according to activation state, maturation and localization in the body. At the local tissue level, the membrane-anchored (TM) IL-5Rα isoform is down-regulated whereas the secreted (SOL) IL-5Rα variant is up-regulated in nasal polyp tissue, but eosinophils are still activated. Therefore, strategies to antagonize IL-5 may have to face unexpected difficulties.

We demonstrated shrinkage of nasal polyps in half of the verum-treated patients for up to four weeks after intravenous injection of a single dose of an anti-human IL-5 monoclonal antibody. When carefully analysing responders and non-responders, only those nasal polyps with elevated baseline levels of IL-5 in nasal secretions seemed to benefit from anti-IL-5 treatment. Remarkably, the degree of eosinophilia at baseline was not different between responders and non-responders. Our data show that at least in 50% of the nasal polyps, IL-5 and eosinophils play a key role (IL-5-dependent) in sustaining polyp size, whereas in the other group, eosinophilia may be more dependent on other factors (IL-5-independent).

Finally, these insights in the regulation of IL-5 and eosinophilia in NP, (re-)open therapeutic perspectives in nasal polyposis based on eosinophil-selective targets.

SAMENVATTING

Aangezien de meeste bilaterale neuspoliepen (NP) gekenmerkt zijn door een prominente eosinofiele ontsteking en omdat deze granulocyten kunnen bijdragen tot weefselschade en bijgevolg remodellering, wensten we de rol van eosinofielen te onderzoeken in de pathogenese van neuspoliepen. Meer bepaald de functie en regulatie van interleukine-5 (IL-5) en interleukine-5 receptor α (IL-5 Rα) zullen onderzocht worden voornamelijk in het kader van toekomstige therapeutische strategieën in NP.

Immunochemische karakterisering van NP toont een centrale depositie van plasma proteïnen (albumine), mogelijk geregeld door een eosinofiele ontsteking als pathogenetisch principe van poliepvorming en groei. De accumulatie en activering van eosinofielen wordt begunstigd door lage TGF- β_1 concentraties en een overmaat aan IL-5 en eotaxine in NP weefsel. Alhoewel we hoge IgE spiegels vonden in NP, was er geen relatie tussen huidallergietesten en totaal en specifiek IgE in NP weefsel. Daarentegen was lokaal IgE gecorreleerd aan de graad van eosinofilie in NP. Eveneens, toonden we een organisatie van secundair lymfoid weefsel in NP weefsel en een polyclonale hyper-immunoglobulinemie E die geassocieerd is met de aanwezigheid van specifiek IgE tegen *S. aureus enterotoxines (*SAEs), kolonisatie met *S. aureus* en een sterke eosinofiele ontsteking in een belangrijke subgroep van patiënten met NP.

De ultieme manier om de rol van IL-5 en eosinofielen in de pathogenese van NP te testen, is door de IL-5 functie te antagoniseren in een interventionele studie bij patiënten met NP. Eosinofielen tonen echter een wisselende sensitiviteit t.o.v. IL-5 door een verschillende expressie van de IL-5Rα isovormen naargelang de staat van activering, maturatie en lokalisatie in het lichaam. In het lokale weefsel compartiment vonden we een lage expressie van de membraangebonden (TM) IL-5Rα isovorm en een hoge expressie van de gesecreteerde (SOL) IL-5Rα variant in NP weefsel, ondanks het feit dat eosinofielen geactiveerd zijn. Het is daarom mogelijk dat strategieën, die de IL-5 functie antagoniseren, te kampen krijgen met onverwachte moeilijkheden.

Bij de helft van de behandelde patiënten vonden we kleinere NP tot vier weken na intraveneuze injectie van één enkele dosis van een anti-humaan IL-5 monoclonaal antilichaam. Bij zorgvuldige analyse van gevoelige en niet-gevoelige NP patiënten voor anti-IL-5 behandeling, bleek dat enkel NP met gestegen IL-5 in de neussecreties voordeel haalden uit deze behandeling. Opmerkelijk is dat de graad van eosinofilie in NP weefsel niet verschillend was tussen gevoelige en niet-gevoelige NP patiënten bij aanvang van de studie.

Onze data toonden dat IL-5 en eosinofielen een sleutelrol spelen in minstens 50% van de NP (IL-5 afhankelijk) in het onderhouden van de poliepgrootte, terwijl de eosinofiele ontsteking in de andere NP patiënten meer afhankelijk is van andere factoren (IL-5 onafhankelijk).
Uiteindelijk (her)-openen deze inzichten in de regulatie van IL-5 en eosinofilie in neuspoliepen, nieuwe therapeutische perspectieven gebaseerd op eosinofiel-selectieve behandelingen in neuspoliepen.

RESUME

Compte-tenu que la grande majorité des polypes naso-sinusiens bilatéraux (PNS) sont associés à une inflammation à prédominance éosinophilique et que les éosinophiles sont reconnus comme acteurs dans la dégradation tissulaire et le remodelage tissulaire conséquent, nous souhaitions investiguer le rôle joué par les éosinophiles dans la physiopathologie de la polypose naso-sinusienne. En particulier, la régulation de l'interleukine-5 (IL-5) et des isoformes du récepteur alpha de l'interleukine 5 (IL-5Rα) a été étudiée afin de traçer de nouvelles perspectives pour la stratégie thérapeutique des PNS.

La caractérisation des PNS repose sur une déposition de protéines plasmatiques (albumine), régulé par une inflammation subépithéliale, principalement éosinophilique, soutendant les principes physiopathologiques de formation et de croissance polypeuse. L'accumulation et l'activation éosinophilique sont favorisés par des concentrations basses en TGF-β_1 et une surproduction d'IL-5 et d'éotaxine dans le tissu polypeux. Malgré des taux élevés d'IgE trouvés au sein du tissu polypeux, les concentrations en anticorps IgE locaux n'étaient pas corrélées aux résultats des tests allergiques cutanés, mais bien au degré d'éosinophilie. De plus, nous avons mis en évidence une organisation du tissu lymphoïde secondaire au sein du tissu polypeux, une hyper-immunoglobulinémie E polyclonale associée à la présence d'IgE spécifique contre les entérotoxines de *Staphylococcus aureus (*SAEs), une colonisation par *S. aureus,* et une inflammation éosinophilique augmentée dans ces groupes.

La solution ultime pour tester le rôle de l'IL-5 et des eosinophils dans la pathogenèse de la polypose naso-sinusienne est l'antagonisme de l'IL-5 dans une étude interventionnelle chez des patients polypeux. Cependant, les éosinophiles montrent une sensibilité à l'IL-5 variable due à une expression différentes des isoformes IL-5Ra selon l'état d'activation, maturation et localisation dans le corps. Au niveau tissulaire local, l'isoforme transmembranaire (TM) de l'IL-5Rα est sous-régulé tandis que la variante soluble (SOL) de l'IL-5Rα est hyper-régulée mais les éosinophiles restent malgré tout activés. Ceci démontre que les stratégies d'antagonisation de l'IL-5 vont être confrontées à des difficultés inattendues.

Nous avons démontré une réduction de volume des polypes chez la moitié des patients jusqu'à 4 semaines après injection intra-veineuse d'une dose simple d'un anticorps monoclonal anti-IL-5 humain. Nous avons particulièrement analysé les répondeurs et les non-répondeurs: seuls les patients présentant des taux élevés d'IL-5 dans les sécrétions nasales avant injection semblent être les plus grands bénéficiaires du traitement anti-IL-5.

D’une manière remarquable, le degré d’éosinophilie avant injection n’était pas différent entre les groupes de répondeurs et non-répondeurs. Nos résultats ont démontré qu’au moins dans 50 % des PNS, l’IL-5 et les éosinophiles jouent un rôle (IL-5-dépendant) dans la formation polypeuses, alors que dans l’autre groupe, l’eosinophilie semble plus dépendante d’autres facteurs (IL-5-indépendant).

Enfin, ces données sur la régulation de l’IL-5 et de l’éosinophilie dans la polypose naso-sinusienne, (re-)ouvrent des perspectives thérapeutiques sélectives basées sur un ciblage éosinophilique dans la polypose naso-sinusienne.

CHAPTER 1

NASAL POLYPOSIS:
AN EOSINOPHIL MEDIATED DISEASE?

NASAL POLYPOSIS:
AN EOSINOPHIL-MEDIATED DISEASE?

CLINICAL ASPECTS OF NASAL POLYPOSIS

Nasal polyps represent edematous semitranslucent masses in the nasal and paranasal cavities, mostly originating from the mucosal linings of the sinuses and prolapsing into the nasal cavities. The preferred site of origination of nasal polyps was found to be the mucosa of the outlets of the sinuses: frontal recess, ethmoidal cell cleft and sinus ostia [1;2]. Sometimes, the proper diagnosis can only be made during surgery, when polyps are present in the sinuses only. Nasal polyps cause long-term symptoms, in particular nasal obstruction and reduced sense of smell, the latter being indicative for polyps but not specific[3]. The typical history is a common cold like symptomatolgy that persists over month and years, leading to nasal obstruction and discharge as the most prominent symptoms[4]. Over time, hyposmia or anosmia develop, and additional complaint such as the sensation of a "full head" are present [5;6]. Interestingly, whereas chronic sinusitis is sometimes associated with a kind of headache and facial pain, nasal polyposis itself rarely causes pain despite the fact that most of the sinuses including the frontal sinus are opacified.

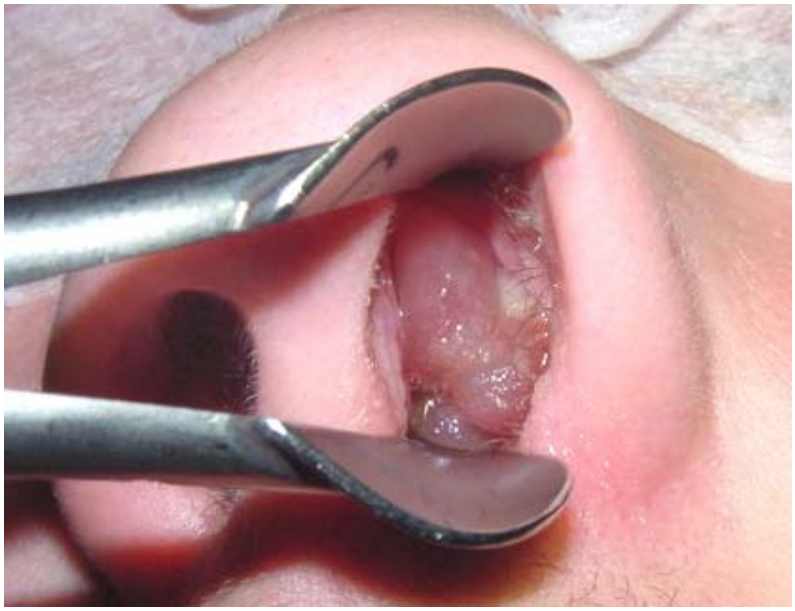

Figure 1: Massive nasal polyps in left nostril

Diagnosis

Anterior rhinoscopy only is not considered to be sufficient to diagnose or exclude polyps, and especially for the differential diagnosis, an endoscopic investigation of the nose after topical decongestion is necessary. To investigate the extent of disease within the sinuses, a computer tomography (CT scan) is performed. A diagnosis based exclusively on the case history is not reliable. The prevalence figures of NP depend upon the methods chosen: questionnaire, endoscopy, CT-scan imaging. With the introduction of rigid or flexible endoscopes into daily practice, nasal polyps are discovered earlier today than 15 years ago.

Staging

To determine the extension of disease within the nose and the sinuses, endoscopy and CT-scan based staging systems have been proposed and partially validated [7]. These systems may proof useful for medical communication and for the evaluation of therapeutic response. The endoscopic staging system is mainly based on the assumption that polyp growth starts from the middle nasal meatus and then extends in a two-dimensional way towards the floor of the nose. However, the fact that the nasal cavity is three-dimensional may sometimes have a negative impact on the repeatability of this technique by different investigators. In this dissertation we used an endoscopic staging system adapted from the Davos score (Table1)[8]. The radiologic staging system includes all sinuses and the ostiomeatal complex bilaterally (table 2).

Table 1: Adapted endoscopic staging system

Polyp Score	Polyp Size
0	No polyps.
1	Small polyps in the middle meatus not reaching below the inferior border of the middle concha.
2	Polyps reaching below the lower border of the middle turbinate.
3	Large polyps reaching the lower border of the inferior turbinate or polyps medial to the middle concha.
4	Large polyps causing complete congestion/obstruction of the inferior meatus.

Table 2: The radiologic staging system modified after Lund and Mackay[7]

0: no abnormalities; 1: partial opacification; 2: total opacification;

* Ostiomeatal complex: 0: not occluded; 2: occluded

	Right	*Left*
Maxillar sinus (0,1,2)		
Anterior Ethmoid (0,1,2)		
Posterior Ethmoid (0,1,2)		
Sphenoid (0,1,2)		
Frontal sinus (0,1,2)		
Ostiomeatal Complex (0,2) *		
Total		

Classification

Nasal polyposis is not a consistent disease; on clinical grounds and based on etiology, histopathology [9] and recently also mediator content [10], it may currently be subdivided into different groups, with impact on prognosis and treatment. In this dissertation, only bilateral nasal polyps were studied.

Table 3: Classification of nasal polyps [4]

1) The antro-choanal polyp, mostly arising from the maxillary sinus and prolapsing into the choana, a commonly large isolated unilateral cyst-like non-eosinophilic formation
2) Idiopathic unilateral or bilateral, mostly eosinophilic polyps without involvement of the lower airways
3) Bilateral eosinophilic polyposis with concomitant asthma and/or aspirin sensitivity
4) Polyposis with underlying systemic disease such as cystic fibrosis, primary ciliary dyskinesia, Churg-Strauss-syndrome, Kartagener-syndrome etc.

EPIDEMIOLOGY AND NATURAL HISTORY

The exact prevalence of nasal polyposis in the general population is not known, because there are few epidemiological studies and their results depend upon the selection of the study population and the diagnostic methods used. A postal questionnaire survey of a population-based random sample of 4300 adult women and men aged 18-65 years was recently performed in southern Finland [4;11]. The prevalence of nasal polyposis was 4.3%, and nasal polyposis and aspirin sensitivity were associated with an increased risk of asthma. The

prevalence of doctor-diagnosed aspirin sensitivity was 5.7%. The incidence is higher in men than in women and significantly increases after the age of 40 years [12;13]. Nasal polyps occur more frequently in subgroups of patients such as asthmatics, aspirin sensitive and cystic fibrosis patients [14]. When polyps occur in children and adolescents, cystic fibrosis should always be considered [14;15]. Other conditions associated with nasal polyps are Churg-Strauss Syndrome and Kartagener's syndrome.

Table 4: Prevalence of nasal polyposis in population subgroups (adapted from Settipane)[16]

Aspirin hypersensitivity	36-72%
Adult asthma	7%
IgE-mediated	5%
Non- IgE-mediated	13%
Chronic sinusitis	2%
IgE-mediated	1%
Non- IgE-mediated	5%
Childhood asthma/sinusitis	0,1%
Cystic fibrosis	
Children	10%
Adults	50%
Allergic fungal sinusitis	66-100%

Nasal polyps and allergy

At the beginning of the last century, an allergic aetiology of nasal polyps has been presumed, but never firmly demonstrated. The major features pointing to an allergic cause of nasal polyps include clinical symptoms similar to allergic rhinitis, the association with late-onset asthma and an elevated local IgE in polyp fluid as well as a pronounced tissue eosinophilia. However, polyps were found in only 0.5% of 3000 consecutive atopic patients examined by Caplin et al [17]. Until today, an increased risk for allergic subjects to develop nasal polyps could not be demonstrated. In contrast, in a retrospective study by Settipane and Chafee [18], polyps were present in 2.8% of atopic patients, but in 5.2% of non-atopic subjects. In another study, there was a positive association between the blood eosinophil count and the presence of asthma with the number of polypectomies, but not with positive skin tests for different allergens [19]. Seasonal allergen exposure in patients with nasal polyps also did not enhance symptoms or

markers of eosinophilic inflammation such as eosinophil percentage or eosinophil cationic protein concentration in nasal secretions [20]. Although elevated total IgE was found in polyp fluid, there was no difference between polyps from allergic and non-allergic subjects [5]. However, it was noted that total IgE was higher in polyp fluid than the corresponding serum in both allergic and non-allergic polyp subjects. Local specific IgE production could also be demonstrated in nasal polyps associated with negative skin tests and serum RAST [21].

Nasal polyps and asthma

Nasal polyposis is frequently found in association with lower respiratory tract disorders, such as asthma and non-specific bronchial hyperreactivity [22]. In studies involving large series of patients with nasal polyposis, asthma was found in 20% to 70% [23]. Furthermore, non-allergic asthma is significantly more frequently linked to polyps compared to allergic asthma [3;16]. A long-term follow-up study confirmed that the incidence of subsequent clinically significant bronchial asthma in patients with NP was much higher than in the general population [3;16]. Interestingly, patients with nasal polyposis and asymptomatic bronchial hyperreactivity have an eosinophilic bronchial inflammation similar to that observed in asthmatic patients with nasal polyposis, whereas patients with nasal polyposis without bronchial hyperreactivity do not feature eosinophilic lower airway inflammation[22].

Aspirin sensitivity

About 15% of polyp patients have aspirin sensitivity whereas about 40 to 80% of patients with aspirin sensitivity suffer from polyposis. In 1922, Widal described a symptom triad consisting of aspirin sensitivity, steroid-dependent asthma and nasal polyposis (rhinosinusitis), which was made known by Samter and Beers later [24]. Out of 500 patients registered at the European Network on Aspirin-Induced Asthma (AIANE), almost 80% complained of nasal blockage accompanied by rhinorrhea [25]. Asthma and rhinitis attacks are caused by ingestion of aspirin and other non-steroidal anti-inflammatory drugs that share the possibility to inhibit cyclooxygenase enzymes. About 15% of patients with aspirin-inducible asthma and rhinitis are unaware of aspirin sensitivity, indicating that aspirin challenge is necessary to fully diagnose the disease. About 50% of patients need systemic steroid treatment on top of inhaled corticosteroids, emphasizing the severity of the disease in the upper and lower respiratory tract. Interestingly, the course of disease is independent from aspirin intake, indicating that the disease is driven by a so far unknown agent and with few exceptions, aspirin sensitivity remains life-long[4].

The full clinical picture of aspirin-sensitive rhinosinusitis (ASRS) is characterized by raised blood eosinophil counts, increased eosinophils in the nasal and bronchial mucosa, and elevated cys-leucotriene concentrations in the tissue and urine, which further raise due to aspirin exposure [4;25;26]. Although ASRS often is associated with allergy and highly elevated local IgE levels [4], an IgE-mediated mechanism could not be demonstrated, and atopy does not seem to influence the risk to develop aspirin sensitivity [25].

Cystic fibrosis

Sinus disease is common in cystic fibrosis with 90 per cent of patients having radiological evidence of sinus disease. The association between nasal polyposis and CF is well documented. Hadield *et al*. studied 211 adults with CF and found nasal polyps in 37 per cent [14] and other studies report nasal polyps in between 10 and 32 per cent of CF patients[15]. Fifty percent of the children between 4 and 16 years of age presenting with nasal polyps have CF [15].

Fungal disease

The syndrome of nasal polyposis combined with positive *Aspergillus* cultures from the paranasal sinuses was recognized by Safirstein in 1976 [27]. Further clinical reports supported this finding and noted its similarities with allergic bronchopulmonary aspergillosis. After the recognition that species other than *Aspergillus* were associated with the disease the term 'allergic fungal sinusitis' (AFS) was introduced. The sinus mucosa shows a characteristic eosinophilic inflammation, with allergic mucin filling the sinuses [28;29]. AFS is found unilateral in more than 50% of the cases, but may involve several sinuses bilaterally, and bone erosion and extra-sinus extension have been reported. It was recently suggested that almost all chronic sinusitis cases would be due to fungus involvement [30]. Fungal cultures of nasal secretions were positive in 96% of 210 consecutive chronic rhinosinusitis patients, without an IgE-mediated hypersensitivity to fungal allergens in the majority of subjects[30]. However, fungus may be cultured from healthy subjects in the same magnitude using the same technique, and no pathophysiologic pathway was demonstrated to relate fungal presence to nasal disease. The presence of fungi alone is insufficient to implicate it as the pathogen in chronic sinusitis, as fungal DNA was found in 42% of control subjects and 40% of patients with chronic sinusitis by PCR analysis [31], while standard cultures were positive in 7% and 0% in this study.

HISTOPATHOLOGY OF NASAL POLYPS

Histomorphological characterisation of polyp tissue reveals frequent epithelial damage, a thickened basement membrane, and an oedematous to sometimes fibrotic stromal tissue, with a reduced number of vessels and glands, but virtually no neural structure [3;32].

Most of the polyp surface is covered by a ciliated pseudostratified epithelium, but a transitional and squamous epithelium is also found, especially in anterior polyps, influenced by the inhaled air currents [3;33]. Epithelial defects have apparently also been described in NP [34], but when polyps are removed carefully and gentle methods are used for fixation, dehydration and cutting, the polyp epithelium appears well preserved without defects on scanning electron microscopy [35].

The sensory nerves and the autonomic vasomotor and secretory nerves invariably found in normal and abnormal nasal mucosa cannot be identified within the stroma of NP, either in the vicinity of the epithelial basement membrane or within the walls of blood vessels or glands. A few nerve fibres can be seen in the stalk of some NP [36;37]. It is therefore assumed that denervation of NP causes a decrease in secretory activity of the glands and induces an abnormal vascular permeability, leading to an irreversible tissue oedema. NP develop in areas where the lining of the nasal cavity joins that of the sinuses, and these marginal zones contain thin nerve fascicles[36;37] which may be more sensitive to damage from, for example, eosinophil derived proteins[3].

The vascularity of NP is sparse compared with normal nasal mucosa [36], and neither venous sinusoids nor arteriovenous anastomoses are encountered. The venules of the NP show unusual organisation with respect to their endothelial cell junctions and the basement membrane. Many cell junctions have the appearance of a web of villous processes and are incompletely sealed, while others are wide open [3;37]. The release of ECP, histamine and other inflammatory mediators (see below) may be an important factor in causing microvascular plasma exudation, which is highly characteristic NP. The vascular exudation of plasma suggests that the lamina propria, the basement membrane, the airway epithelium, and the mucosal surface are furnished with potent plasma derived peptides and proteins, and that the mucosal macromolecular milieu in nasal polyposis is therefore dramatically different from that of the normal nasal mucosa [3;38]. It is of particular interest that the process of microvascular exudation of plasma may participate in the chronic generation of oedema fluid in NP. The stroma of bilateral NP is mainly characterised by its oedematous nature and consists of supporting fibroblasts and infiltrating inflammatory cells, localized around "empty" pseudocyst formations. Amongst the inflammatory cells, EG2 (activated) eosinophils

are a prominent and characteristic feature in about 80% of polyps [39;40], whereas lymphocytes and neutrophils as predominant cells occur in cystic fibrosis and in primary ciliary dyskinesia[14;15]. Eosinophils are localised around the vessels, glands, and directly beneath the mucosal epithelium [32]. The neutrophilic inflammation predominates in 7% of all cases. The latter is often found in cases associated with CF, primary ciliary dyskinesia syndrome, or Young's syndrome [9]. Mast cells, with most being degranulated, are found in increased numbers within the stroma of nasal polyps [5;26]. Increased numbers of plasma cells and lymphocytes, have been found at the cellular level [5;25]. Fokkens et al reported that more lymphocytes occurred in the lamina propria than in the epithelium, and that T lymphocytes were more numerous than B lymphocytes [41]. The majority of the lymphocytes occurred as single cells, whereas small aggregates were seen relatively often and large aggregates rarely[41]. Furthermore, more $CD8^+$ cells (suppressor/cytotoxic T cells) than $CD4^+$ cells (helper/inducer T cells) were found in the polyps [39;41].

To summarize, the stroma of NP is cell-poor, and no marked characteristics other than tissue eosinophilia can be found. The abundance of eosinophils in NP is the first key question to address in order to understand this disease. It may be explained in two different ways: first, by an increased migration of eosinophils into the tissue; second, by a prolonged survival of these cells, or - most likely - by a combination of both. The second key question concerns the precise pathomechanism by which eosinophils may contribute to the tissue damage, inflammation and polyp formation.

CYTOKINES, CHEMOKINES AND ADHESION MOLECULES IN NASAL POLYPS

Early studies by Denburg et al [42]demonstrated that conditioned medium derived from cultured nasal polyp epithelial cells contained potent eosinophil colony-stimulating activities, as well as an interleukin-3-like activity. The authors suggested that accumulation of eosinophils in polyps may partly be a result of differentiation of progenitor cells stimulated by soluble hemopoietic factors derived from mucosal cell populations. An increased synthesis of GM-CSF by epithelial cells, fibroblasts, monocytes, and eosinophils was suggested later [42-44]. According to Hamilos et al [45], polyp tissue samples from patients with or without allergy contained different cytokine profiles. They found by *in situ* hybridization studies that patients with “allergic” polyps had higher tissue densities of GM-CSF, IL-3, IL-4, and IL-5 transcripts than controls, whereas patients with non-allergic polyps had higher tissue densities of GM-CSF, IL-3, and IFN-gamma transcripts. From these results, distinct pathomechanisms for allergic versus non-allergic polyps were suggested[45]. However, other studies involving protein

measurements in tissue homogenates could not support these findings [10;46]. In contrast, IL-3 and GM-CSF protein concentration were found in only a small number of polyp and control turbinate samples. However, IL-5 was found to be significantly increased in nasal polyps, compared to healthy controls, and the concentration of IL-5 was independent of the atopic status of the patient. Actually, the highest concentrations of IL-5 were found in subjects with non-allergic asthma and aspirin sensitivity[10;46]. Furthermore, eosinophils were positively stained for IL-5, suggesting a possible autocrine role for this cytokine in the activation of eosinophils. A strong correlation between concentrations of IL-5 protein and eosinophilic cationic protein (ECP) was demonstrated later [4]. The key role of IL-5 was supported by the finding that treatment of eosinophil-infiltrated polyp tissue with neutralizing anti-IL-5 monoclonal antibody (mAB), but not anti-IL-3 or anti-GM-CSF mAbs in vitro, resulted in eosinophil apoptosis and decreased tissue eosinophilia [47]. Collectively, these studies suggest that increased production of IL-5 is likely to influence the predominance and activation of eosinophils in nasal polyps independent from atopy. The lack of difference in the amounts of cytokines detected in polyps from allergic and non-allergic patients was meanwhile supported by several studies [48;49].

The eosinophilic inflammation in polyps is orchestrated by T-cells, which have been characterized as activated [50]. They represent a mixed population, consisting of CD4+ and CD8+ cells, and show a mixed Th1/Th2 profile. However, also inflammatory cells such as eosinophils, macrophages or mast cells may contribute to the release of cytokines, as was especially shown for eosinophils, contributing IL-4 and IL-5 in an autocrine manner.

Recent studies have shown that NP also express high levels of RANTES and eotaxin, the predominantly recognised eosinophil chemoattractants. Bartels and colleagues [51] demonstrated that expression of eotaxin- and RANTES mRNA, but not MCP-3 mRNA, was elevated in non-atopic and atopic NP, when compared to normal nasal mucosa. Similarly, Jahnsen et al. [52] demonstrated an increased mRNA expression for eotaxin, eotaxin-2, and MCP-4. The expression of eotaxin-2, a novel CCR3-specific chemokine, was found to be the most prominent of the three chemokines investigated. This is in accordance with the findings of a recent extensive study of about 950 non-allergic or allergic polyp patients, which has also suggested that NP eosinophilic infiltration and activation may correlate mainly with increased eotaxin gene expression, rather than with RANTES expression [53].

Studies of cell adhesion molecules are relatively scarce. Early studies by Symon et al. [54] demonstrated that ICAM-1, E-selectin and P-selectin were well expressed by NP endothelium, whereas VCAM-1 expression was weak or absent. In a study by Jahnsen et al [55],

employing three-colour immunofluorescence staining, has however demonstrated that both the number of eosinophils and the proportion of vessels positive for VCAM-1 were significantly increased in NP compared with the turbinate mucosa of the same patients. Moreover, treatment with topical glucocorticosteroids decreases the eosinophils density and the expression of VCAM-1 in NP [56]. The interaction between VLA-4 on eosinophils and VCAM-1 on endothelial cells may be of importance in priming eosinophils by modifying the activation and effector functions [57].

Consistent with the current knowledge on the pathophysiology of nasal polyposis, new therapeutic approaches could focus on eosinophilic inflammation, eosinophil recruitment, the T-cell as orchestrating cell, IgE antibodies, as well as on tissue destruction and remodeling processes.

MANAGEMENT OF NASAL POLYPOSIS

The management of patients with nasal polyps is one of the major problems otorhinolaryngologists have to face in daily practice. Especially since little evidence based or satisfying treatment approaches are available to help these patients. The primary goal of treatment is the relief of symptoms, the primary symptoms being nasal blockage, congestion, hypo- or anosmia and secretion. Other symptoms include post-nasal drainage, facial pain, headache, sleep disturbance and diminished quality of life [4]. Secondary goals of treatment include a decrease in the frequency of infections and disease recurrences, an improvement in associated lower airway symptoms, and the prevention of complications such as mucoceles and orbital involvement [4].

Hippocrates removed polyps with a snare and this remained the only treatment available until recently[3]. However, there have been two remarkable therapeutic innovations in the last two decades: first, the introduction of topically active corticosteroids, and second, endoscopic sinus surgery [58].

Endoscopic polypectomy and sinus surgery

The acronym “FESS” (Functional endoscopic sinus surgery) is frequently applied to any sinus surgery undertaken with endoscopic control, but it is clear that the removal of polyps is rarely functional in the sense in which the term was originally conceived. The extent of surgery varies according to the extent of disease, surgeon‘s individual practice and ranges from removal of polyps within the middle meatus, perhaps combined with uncinectomy, middle meatal antrostomy and opening of the bulla to a complete “nasalization” of all sinuses with

exenteration of all sinus mucosa. Nasal polyp tissue for research purposes was obtained within the middle meatus and was immediately processed.

Extensive postoperative care and follow-up is required to preserve the postoperative results and prevent re-growth of polyps. Nevertheless, nasal polyposis is a chronic disease with a high rate of recurrences even after careful medical and surgical treatment. In a 20-year follow-up study of 41 patients with nasal polyps, 85% of patients still suffered from the disease with anosmia present in 61% [59]; 8 subjects, including 7 with aspirin sensitivity, had undergone 11 or more surgical operations during the 20-year period. This study, as well as others showing the high recurrence rate in nasal polyps, clearly indicate the chronicity of the disease especially in this subgroup of patients and suggest a conservative surgical approach. Instead, a combined strategy involving surgical and medical treatment is recommended for long-term control of the disease [60;61].

The medical or surgical treatment of nasal polyposis may have impact on the control of asthma. A study involving 205 patients with asthma and aspirin sensitivity indicated that surgery improves asthma for relatively long periods of time [62]. However, there are no data concerning the evolution of asymptomatic bronchial hyperreactivity in patients with nasal polyposis, and individual patients may develop asthma symptoms after surgery. This development may represent the natural course of the disease rather than a true shift from upper to lower airway disease.

Topical and systemic glucocorticosteroids (GCS)

As nasal polyposis represents an eosinophilic inflammation with consecutive tissue changes, topical and systemic corticosteroids are the first choice treatment approaches[4]. Systemic application affects all polyp tissue within the nose and sinuses, but has the disadvantage of systemic side effects, when used for long-term treatment. Topical application of corticosteroids significantly reduces side effects, but does not impact polyps within the sinuses. In fact, the distribution of the drug within the nasal cavity, partially or completely obstructed by polyp tissue, already presents a notable problem to treatment success[4].

Glucocorticosteroids can suppress many phases of the inflammatory process [63;64], which may explain their strong effect on inflammation. T-cells are highly sensitive to treatment with GCS, reducing the number of T-cells in a dose dependent way, and the expression of mRNA and protein for IL-3, IL-4, IL-5 and IL-13 and their receptors [65]. The secretion of numerous other cytokines and chemokines is also reduced, among them IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α, IFN-γ, GM-CSF, RANTES, and eotaxin [66]. This affects recruitment, localization,

activation, protein synthesis and survival of inflammatory cells such as eosinophils. The recruitment of inflammatory cells is also diminished by the inhibition of expression of adhesion molecules such as ICAM-1 and VCAM-1, which affects the influx of basophils and mast cells in the epithelial layers of the nasal mucosa. GCS may also reduce the release of preformed and newly-generated mediators, such as histamine, prostanoids and leukotrienes[4;65]. However, this action may be partly due to the reduction of inflammatory cells in the mucosa. Finally, GCS can also act on IgE production [67] and may affect plasma protein retention. However, macrophages and neutrophils do not seem to be influenced [68], which might explain why local GCS do not have a negative effect on the immunity to bacterial infections.

The symptomatic efficacy of intranasal corticosteroids in patients with nasal polyps is well documented [69]. Symptoms such as nasal blockage, rhinorrhea and occasionally hyposmia are reduced during the period of treatment, especially in obstructive polyposis [70;71]. However, recurrence of symptoms or polyp growth, monitored by symptoms, endoscopy or rhinomanometry, occurs within weeks to months [72]. After surgery, topical corticosteroids may also reduce the incidence of polyp recurrences or prolong the symptom-free time interval [73;74]. Nevertheless, topical corticosteroids may be insufficient in severe bilateral polyps [4;58;75], and polyp growth may be observed despite treatment in these patients.

Systemic oral GCS are indicated to start off or enforce conservative local treatment. So far, to our knowledge, not a single placebo-controlled, randomized trial with oral GCS has been published. However, oral GCS treatment has been studied in an uncontrolled study, showing a significant effect for some months with an improvement of symptoms in 72 % of the patients and a reduction of polyp size and of sinus opacification in the CT-scan in 52 % [76]. In those subjects responding, recurrences mostly occurred within 5 months. Thus, oral GCS may be indicated to delay surgery or to facilitate surgery. However, there is no evidence so far that the natural course of the disease might be influenced by short- or long-term low-dose treatment regimes.

The use of topical corticosteroids, taken on a daily basis for several months to years, is considered first line therapy in small to medium sized nasal polyps to reduce symptoms, and to avoid surgery and relapse [4]. Topical treatment may be enforced by oral application of GCS, for which controlled studies are clearly needed, or combined with other treatment approaches. However, surgery needs to be considered in case of failure, side effects or unwillingness of the patient to comply with drug treatment, as well as in case of complications [4].

Antileukotrienes

Changes in the arachidonic acid metabolism have been suggested to be involved in the pathomechanism of nasal polyposis especially in aspirin sensitive subjects. Theoretically, the use of antileukotrienes especially in aspirin sensitive nasal polyp patients could be appropriate [77]. However, large-scale controlled trials in clearly characterized patients – with or without aspirin sensitivity - are lacking so far.

Parnes treated 40 patients diagnosed with sinonasal polyposis and sinusitis with either zileuton or zafirlukast on top of the classical treatment. Outcome measures included subjective interviews and questionnaire responses, as well as office endoscopic examinations and chart reviews[78;79]. Overall, 26 patients (72%) experienced subjective improvement of symptoms after starting the medication, and four patients (11%) discontinued the medication because of side effects [78;79].

Montelukast, a leukotriene D4 receptor antagonist, was studied as an add-on therapy to topical and inhaled corticosteroids in patients with or without aspirin sensitivity, both with nasal polyposis and asthma [80]. Clinical subjective improvement in nasal polyposis occurred in 64% aspirin tolerant and 50% of sensitive patients, asthma improvement in 87% and 61% respectively. However, acoustic rhinometry, nasal inspiratory peak flow and nitric oxide levels did not change significantly in any group, and improvement on montelukast therapy was not associated with aspirin sensitivity [80]. The findings are consistent with a subgroup of nasal polyps/asthma patients in whom leukotriene receptor antagonists may be effective, however unrelated to aspirin sensitivity [4].

Antihistamines

Antihistamines may be indicated in subjects with nasal polyposis and allergic rhinitis complaints; however, their use in patients with polyps only has not been extensively studied. Furthermore, even subjects with ragweed positive skin tests did not show enhanced symptoms or an increase in markers of eosinophilic inflammation (eosinophil percentage or ECP concentrations in nasal secretions) due to seasonal exposure [20]. In a clinical study including 45 patients with residual or recurrent nasal polyposis after ethmoidectomy, treatment with either cetirizine at twice the daily dose recommended (20 mg) or placebo for three months was performed [81]. Although the number and size of polyps remained unchanged during the study period, the active treatment reduced sneezing and rhinorrhoea effectively, and also had a late effect on nasal obstruction [81].

Anti-IgE

Considering the marked local production of IgE-antibodies in nasal polyps and its relation to severity of disease, it appears that local IgE is functional and involved in the regulation of chronic inflammation. Additionally, some studies have shown that anti-IgE therapy reduces circulating eosinophil numbers, but this is unlikely to be due to a direct effect because eosinophils have little, if any, IgE and FcεRI on their surface [82]. Thus, strategies to antagonize IgE antibodies could be of relevance. Treatment of allergic asthma and rhinitis with omalizumab, a humanized monoclonal anti-IgE antibody, causes a marked reduction in circulating free IgE levels [83-89]. Treatment has been shown to reduce symptoms and exacerbations and decrease the need for other medication in patients with these allergic diseases. No studies in NP have been performed yet to answer the question, whether high concentrations of IgE-antibodies within the NP tissue can be targeted with success.

Anti-CCR3, anti-eotaxin

CC-chemokine receptor 3 (CCR3)-stimulating chemokines are likely to have an important *in vivo* role in the regulation of eosinophil, basophil, and potentially T helper type 2 and mast cell recruitment. Several CC chemokines including eotaxin and RANTES are potent eosinophil chemotactic and activating peptides acting through CCR3. As eosinophils have also been implicated in the pathogenesis of nasal polyposis, and RANTES and eotaxin have been identified as eosinophil chemoattractants in polyp tissue, antagonism of CCR3 could have a therapeutic role in this disease. The development of small non-peptide molecule CCR3 antagonists currently offers advantages over anti-chemokine (i.e. eotaxin) antibodies in terms of a broader approach, affecting several chemokines and effector cells, and a favorable pharmacokinetic, targeting the receptors on peripheral blood cell surfaces and being independent from tissue penetration.

Recently anti-eotaxin antibodies were administered intranasally in grass pollen sensitive subjects. After nasal challenge pretreatment with anti-eotaxin reduces nasal obstruction, eosinophil influx and mast cells compared to placebo pretreatment [90]. Studies in nasal polyps have not been reported yet.

Conclusion

The treatment of nasal polyps with systemic and topical corticoids and with surgery is common clinical use. However no medical or surgical treatment guarantees cure. Management of patients with severe nasal polyposis is still unsatisfactory and recurrences

remain a problem with a general rate of recurrence of more than 40% after 5 years. Future progress in therapy seems to be closely linked to a better understanding of the mechanisms underlying the migration, activation and maintenance of eosinophils in nasal polyp tissue. In ***chapter 2*** we focus on the role of IL-5 and IL-5 receptor in eosinophilic inflammation, with emphasis on strategies to interfere with IL-5 function especially in nasal polyposis.

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CHAPTER 2

EOSINOPHILS AND INTERLEUKIN-5 IN NASAL POLYPS: TARGETS FOR THERAPY?

EOSINOPHILS AND INTERLEUKIN-5 IN NASAL POLYPS: TARGETS FOR THERAPY?

INTRODUCTION

For more than 100 years, eosinophils have been readily recognized by microscopic examination of blood, bone marrow and other tissues. Maturated and differentiated in the bone marrow, eosinophils are released at a low rate into the blood circulation, and their levels only represent 1-2% of the total peripheral leukocytes in healthy subjects. Eosinophils circulate in the blood with a half-life of 18 hours, before entering the tissue, which serves as the primary site for the cells[1]. Traditionally viewed as killer-effector cells in helminth parasitic infections they are more recently viewed as pro-inflammatory cells in allergic diseases. Eosinophils are known to be attracted from the peripheral blood circulation towards inflamed tissues where they can modulate the inflammatory process by releasing a range of toxic basic proteins, lipid mediators, cytokines and superoxide anions. One of the most characteristic features of eosinophils is the membrane-bound specific granule containing the cytotoxic proteins including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO)[1]. It was the characteristic staining of the granules with acidic dye eosin that rendered the cell its name when P. Ehrlich identified it in 1879 [1]. Since eosinophils are one of the most cytotoxic cells in the body, mobilization and activation is under the tight control of cytokines and chemokines. Eosinophils leave the bone marrow with a so-called “non-primed” phenotype that is refractory to activation. Upon interaction with cytokines and/or chemokines these cells become prone for activation by physiologically relevant activators. This process is referred to as “priming”. Virtually all eosinophil responses are under control of this priming mechanism which acts as a safety lock preventing specific activation of this highly cytotoxic cell.

Interleukin 5 (IL-5) is the key cytokine in an eosinophil’s life span: it supports eosinophilopoiesis and eosinophil differentiation, contributes to eosinophil migration, tissue localisation and function, and prevents eosinophil apoptosis. Given the likely role of eosinophils in nasal polyps, this chapter reviews IL-5 and IL-5 receptor research, with emphasis on strategies to interfere with IL-5 function especially in nasal polyposis.

BIOLOGY OF INTERLEUKIN-5

Cellular source of IL-5

The human IL-5 gene, composed of four exons and three introns, has been mapped to the long arm of chromosome 5, in close proximity to the cytokines IL-3, IL-4 and GM-CSF [2]. Expression of IL-5 appears to be predominantly regulated at the transcriptional level [1].

Within lymphocyte lineages, the major source of IL-5 is a subset of helper T-cells [3]. Among the myeloid lineages, mast cells and eosinophils are the major producers of IL-5. T- cells are involved in the control of growth and effector functions of cell types that contribute to allergic inflammatory responses [1;3]. They do not store IL-5, but upon appropriate stimulation, synthesize and immediately release this cytokine. Immuno-histochemical staining revealed that besides in T-cells, IL-5 can also be found in mast cells and eosinophils. However, regulation of its production is different in these latter cell types, which rapidly release preformed IL-5 from storage granules upon cellular activation [4;5]. Thus, in allergic diseases eosinophils could maintain the inflammation by autocrine stimulation. It is of note that sensitized mast cell-deficient mice are fully capable of producing high amounts of IL-5 [6], whereas T cell-deficient mice lack the ability to produce a substantial IL-5 driven cellular response [7]. As a third source of IL-5, human bronchial and nasal epithelial cells grown in culture constitutively express IL-5 mRNA and protein, which is up-regulated on stimulation with tumor necrosis factor (TNF)-alpha[8]. Finally, malignant and virally transformed cells have been identified to produce IL-5. Reed-Sternberg cells in patients with Hodgkin's disease express mRNA and proteins of various cytokines and growth factors, including IL-5 [9]. Similarly, cell supernatants from Epstein-Barr virus transformed B-cells contain large amounts of IL-5 [10].

The role of interleukin-5 for eosinophilia

Due to the restricted expression pattern of its receptor, human IL-5 selectively functions on eosinophils and basophils, two predominant effector cell types in allergic inflammation [11]. IL-5 has numerous effects on precursor of eosinophils and on the mature eosinophils. (a) It stimulates growth and differentiation of myelocytes of the eosinophil lineage in the bone marrow [1]. (b) It regulates the pro-inflammatory function of eosinophils by promoting the release of the eosinophil granular proteins: major basic protein, eosinophil cationic protein and eosinophil peroxidase. Eosinophils incubated for four days with IL-5 released up to 60% of their granule proteins [12]. (c) IL-5 is weakly chemotactic on mature eosinophils, mostly by priming the cells for enhanced responsiveness to other chemotactic mediators, like IL-8,

RANTES or platelet-activating factor [12]. IL-5 up-regulates the adhesion of eosinophils to endothelium, thereby further increasing eosinophilic accumulation [13]. (d) Eosinophils present at sites of inflammation, persist because of IL-5-induced inhibition of programmed cell death or apoptosis [14;15]. Apart from its actions on eosinophils, IL-5 also increases histamine and leukotriene production in basophils [16]. Furthermore, Rizzo *et al* recently demonstrated an exaggerated contraction of IL-5-treated, isolated normal human bronchus smooth muscle cells in response to acetylcholine [17]. This cholinergic contractility was blocked by neutralizing anti-IL-5 and anti-IL-5 receptor antibodies, and occurred independently of an eosinophil influx. They further showed a clear expression of the IL-5 receptor in bronchus muscle, and to a lesser extent in saphenous vein and atrial muscle.

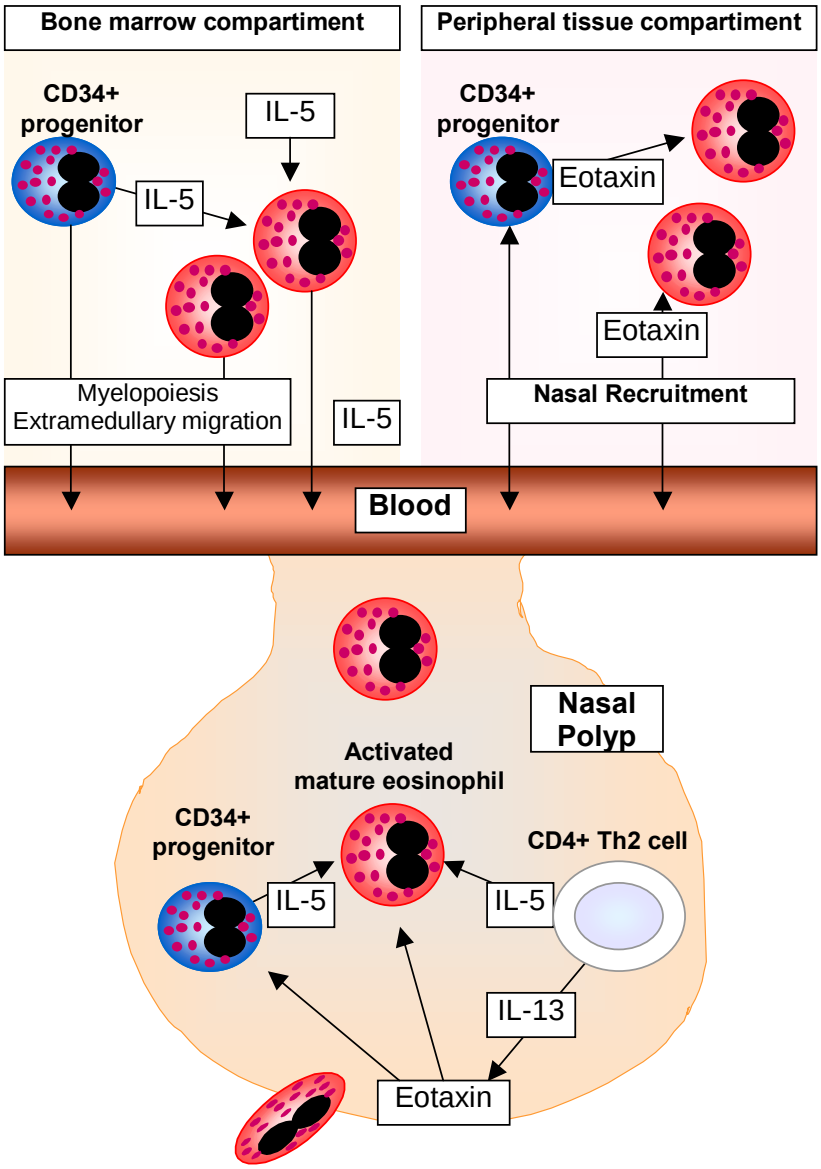

***Figure 1**: Model of Eosinophil recruitment in nasal polyposis*

Generation of mice lacking the IL-5 gene has demonstrated the obligatory role of IL-5 in eosinophil development [18]. IL-5$^{-/-}$ mice have normal baseline eosinophil and immunoglobulin levels and generally exhibit normal T- and B-cell development. However, IL-5$^{-/-}$ mice do not develop eosinophilia in response to helminth and nematode infections[19;20]. It is of note that in the case of IL-5 deficiency, IL-3 or GM-CSF, which can also act on eosinophils *in vitro*, cannot take over the function of IL-5 *in vivo*[21]. This might suggest the existence of a strictly IL-5-dependent eosinophilic progenitor. Nevertheless, low background levels of eosinophils were always present in knockout mice, to leave a small number of IL-5-independent eosinophils. Recently, Foster and coworkers have shown that local chemokine networks in the allergic lung regulate eosinophil accumulation independently of IL-5, and that this mechanism plays an important role in disease processes[22]. In the absence of IL-5 and eotaxin, tissue eosinophilia is abolished in BALB/c mice and so is AHR[18;23]. Furthermore, inhibition of eotaxin alone does not abolish eosinophilia and AHR, thus targeting both pathways is required[23]. These studies indicate that pathways operated by local chemokine systems (in particular those which involve CCR3, the eotaxin receptor) play an important role in regulating the recruitment of eosinophils into tissues independently of IL-5, and that this mechanism is linked to the induction of disease. Importantly, this mechanism also operates in the absence of blood eosinophilia[22].

IL-5 has a variety of functions on the murine immune system ranging from stimulation of immunoglobulin release to enhancing B-cell growth and differentiation [24]. Although there is a well-known association between eosinophilia and IgE levels, IL-5 does not appear to be involved in the IgE response, where IL-4 is the major controlling cytokine. Although IL-5 is able to induce *in vitro* IgA and IgM production in human B-cells, a physiologically functional role in controlling antibody production in the human immune system is yet to be determined [25].

The IL-5 protein

The hIL-5 precursor is 134 residues long, and includes a signal peptide of 19 amino acids [26]. Mature hIL-5 has a calculated molecular mass of 13,149 kDa, which is in accordance with the observation that the *E. coli*-derived protein migrates as a single 13-kDa band on SDS-PAGE gels in the presence of reducing agents. However, under non-reducing conditions, IL-5 is detected as a molecule of 27 kDa. It has been shown that IL-5 exists as a cross-linked homodimer [27]. Mutagenesis of cysteine residues on position 44 and 86 identified both residues as essential in the generation of S-S bridges, and showed that both monomers are arranged in an anti-parallel (i.e. head-to-tail) manner [28]. Reduction and alkylation of these two residues in

recombinant IL-5 lead to a biologically inactive monomer, illustrating that dimer formation via disulfide bonds is essential for biological activity [27-29].

PROPERTIES OF THE INTERLEUKIN 5 RECEPTOR (IL-5R) SYSTEM

IL-5 mediates its biological effects upon binding and activation of a membrane bound receptor (IL-5R). This receptor is composed of two subunits: a ligand-specific α-chain (IL-5Rα) and a shared β-chain [30]. The use of this latter receptor is shared with the receptors for IL-3 and GM-CSF, which both also have their own specific α-chains. For this reason, the β-subunit is often referred to as β common or β_c. This observation provides the molecular basis for the overlapping biological activities of these three cytokines. The α-chain binds to the cytokine with low affinity and when the β-chain associates to any of the α-chins, a high affinity receptor is formed [31-34]. All three receptors are expressed on eosinophils and the IL-5R is specifically expressed on eosinophils and their precursors.

The ligand-specific IL-5Rα-chain

The nucleotide sequence of the human IL-5Rα (hIL-5Rα) cDNA predicts a polypeptide of 420 residues [31-34]. It contains a 20 residue-long signal peptide, an extracellular domain of 322 amino acids, a 20 residue-long transmembrane domain, and a cytoplasmic tail of 58 amino acids. The predicted molecular mass for the α-chain is 45.5kDa, indicating that N-linked glycosylation of one or more of the potential N-glycosylation sites, contributes to the apparent moluclar mass of 60 kDa. This receptor binds IL-5 with intermediate (K_d ~ 500 pM 1 nM) affinity in man and upon association with the β_c-chain, a high affinity binding of approximately 150 pM is observed. The SOL IL-5Rα binds IL-5 with an affinity similar to membrane bound IL-5Rα, suggesting that the transmembrane and cytoplasmic domains of IL-5Rα contribute little energetically to IL-5 binding[31-34].

The β_c-chain

The β_c-chain is a 897 amino acids protein, including a 16 residue-long signal peptide, an extracellular part of 424 amino acids, a 27 residue-long transmembrane domain, and a cytoplasmic tail of 430 amino acids. The mature protein has a molecular mass of 120-130 kDa, and also is likely to be glycosylated. This receptor has no detectable affinity for one of the three cytokines, but plays an essential role in the formation of the high affinity complex and in signal transduction.

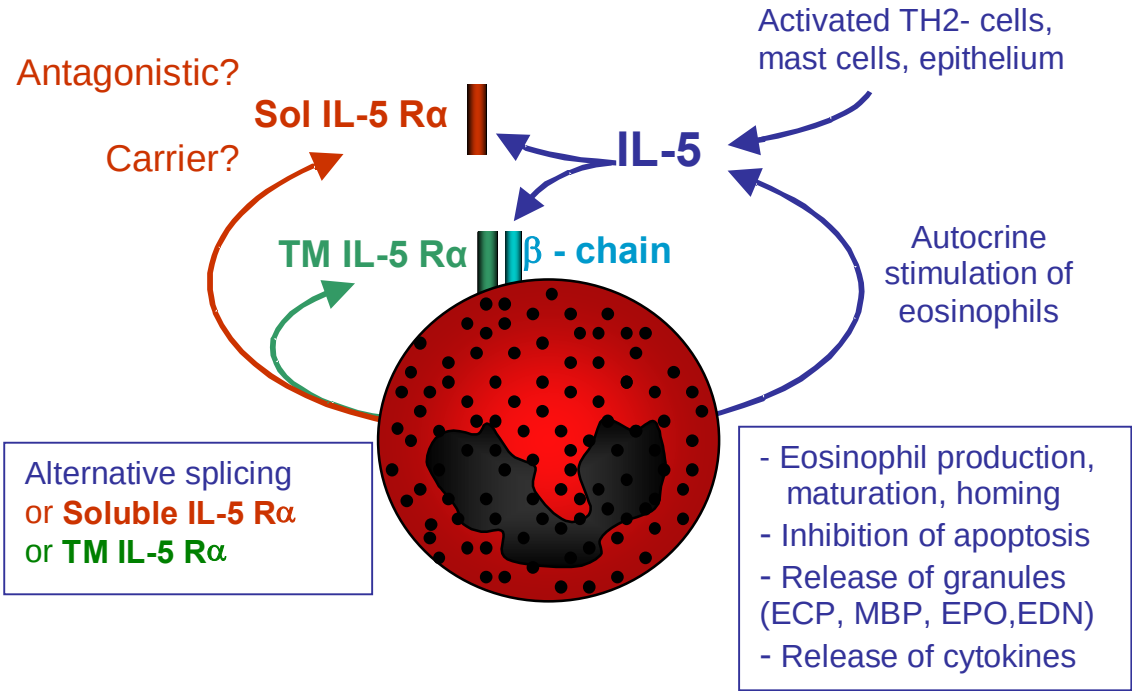

Figure 2: *Eosinophils, IL-5, SOL and TM IL-5Rα expression/interaction*

IL-5 and regulation of expression of its receptor subunits

The IL-5Rα-chain is expressed mainly on eosinophils and basophils, and on certain muscle cells [17]. Human eosinophils express, through alternative splicing three different transcripts from the same IL-5Rα gene [31-34], two soluble and one membrane-bound receptor. The expression of the latter depends on the utilization of a specific exon, leading to the loss of the membrane anchor. When expressed in heterologous cells, this SOL IL-5Rα protein shows receptor antagonist activity *in vitro* [31;32;35;36]. The expression level of SOL and TM IL-5Rα may control this agonist / antagonist balance [37]. Recent publications have demonstrated a complex pattern of regulation of hIL-5Rα expression. Clearly, IL-5 can affect the expression of its own receptor, and hence of eosinophil responsiveness, at the transcriptional, at splicing and at the protein level. Northern blot analysis of normal blood eosinophils illustrates that IL-3, IL-5 and GM-CSF down-regulate IL-5Rα expression at the mRNA level [38]. These authors showed that this regulation occurs very rapidly (reaching maximum inhibition within 2 hours), in a dose-dependent manner, which does not require protein synthesis. In a stable promyelocytic FDC-P1 cell-line containing a hIL-5Rα-chain minigene, in which cDNA and genomic DNA segments were combined [34], it was shown that IL-5 itself, but not IL-3 or GM-CSF, could stimulate a reversible switch from SOL toward TM hIL-5Rα isoform expression.

These results were confirmed using primary human cord blood-derived $CD34^+$ cells[34]. On peripheral blood eosinophils, Hellman et al. recently demonstrated that surface-anchored TM IL-5Rα was strongly down-modulated by recombinant IL-5, intermediately with both IL-5 and GM-CSF, and weakly with only GM-CSF, indicating that GM-CSF binding partially affects surface IL-5 receptor expression [39]. Furthermore, the proportion of CD69-positive eosinophils was significantly up-regulated to the same level by IL-5, GM-CSF as well as by the combination of both cytokines, indicating that activation of eosinophils, judged by CD69 up-regulation, is not mandatory linked to TM IL-5Rα down-regulation. Liu and co-workers could recently show a different control mechanism for expression of the membrane bound IL-5Rα on airway or circulating eosinophils [40;41]. After allergen challenge, IL-5Rα and β_c were markedly reduced on airway eosinophils, and this in contrast to circulating cells, soluble IL-5Rα concentrations were significantly elevated in bronchoalveolar lavages, and airway eosinophils exhibited a lack of responsiveness to IL-5[41]. These authors postulate that the reduced expression is due to an IL-5 driven proteolytic cleavage of the membrane bound isoform [40]. Finally, IL-5 induced activation of its receptor also results in a proteasomal degradation of the β_c cytoplasmic domain in the activated receptor complex [42]. As a consequence, signalling is terminated by deletion of the cytoplasmic phosphorylated residues. The remnant of the β_c is endocytosed and further degraded in the lysosomes.

IL-5R signal transduction

Signal transduction via the IL-5 receptor involves ligand binding and receptor dimerization,[43;44]. This involves the association of the IL-5Rα-chain with βc, which takes place by non-covalent as well as covalent means. The covalent linkage of IL-5Rα and βc is probably the most functionally relevant one as it is associated with tyrosine phosphorylation of the receptor [45]. IL-5 activates Lyn, Syk, and JAK2 and propagates signals through the Ras-MAPK and JAK-STAT pathways. Studies suggest that Lyn, Syk, and JAK2 tyrosine kinases and SHP-2 tyrosine phosphatase are important for eosinophil survival[1;46]. In contrast to their survival-promoting activity, Lyn and JAK2 appear to have no role in eosinophil degranulation or expression of surface adhesion molecules. Raf-1 kinase, on the other hand, is critical for eosinophil degranulation and adhesion molecule expression[1;46].

The precise role of the α subunit in IL-5 signalling is less clear. Apart from ligand binding, this subunit is essential for signal transduction, since deletion of its cytoplasmic part, which contains a critical JAK binding site, completely abolishes signalling [47]. There is a growing

body of evidence that, although IL-3, IL-5 and GM-CSF use the same β subunit, they also provoke cytokine-specific signals.

CLINICAL POTENTIAL FOR INTERLEUKIN-5 BLOCKAGE

IL- 5 is normally not found at high levels in healthy individuals. Next to nasal polyposis, a variety of diseases of the respiratory tract, skin, gut and of the haematopoietic system are associated with eosinophilia and with elevated levels of IL-5 mRNA and protein in bone marrow, in circulation and in tissue. Examples include allergic and non-allergic respiratory diseases, aspirin sensitivity, atopic dermatitis, hypereosinophilic syndrome, Churg-Strauss syndrome, food and drug allergies, and helminth infections (see also table 1). Indeed, in asthma as well as in nasal polyposis, elevated eosinophil numbers and IL-5 levels are described. Furthermore, segmental bronchial provocation of asthmatic patients results in eosinophil infiltration and accumulation in lung and also in the nose [48;49]. Similarly, nasal provocation resulted in eosinophil infiltration in the nasal and bronchial mucosa [50].

Table 1 : Eosinophil-Associated Diseases

- Parasitic infections
- Allergic diseases
  Asthma, allergic rhinitis, urticaria, allergic bronchopulmonary aspergillosis (ABPA), drug hypersensitivity reactions
- Respiratory tract
  Nasal polyposis, eosinophilic pneumonia, Loeffler's syndrome, hypersensitivity pneumonitis
- Toxic reactions
  Eosinophilia myalgia syndrome (L-tryptophan), toxic oil syndrome
- Neoplastic and myeloproliferative disorders
  Idiopathic hypereosinophilia syndrome (IHES), eosinophilic leukemia, lymphoma, angioimmunoplastic lymphadenopathy, immunotherapy with interleukin-2
- Connective tissue diseases
  Hypersensitivity vasculitis, Churg-Strauss syndrome, hypereosinophilic fasciitis
- Skin disorders
  Atopic dermatitis, scabies, eosinophilic cellulitis
- Immunodeficiency syndromes
  Wiskott-Aldrich syndrome, hyper IgE syndrome
- Gastrointestinal diseases
  Eosinophilic gastroenteritis, inflammatory bowel diseases

Interfering with IL-5 or IL-5R synthesis

The biological actions of IL-5 can be inhibited either by blocking the interaction IL-5/IL-5R using antibodies or IL-5 mutants, but also by preventing expression of IL-5 or components of its receptor. One way to achieve this is the use of antisense oligonucleotides. These are short, synthetic DNA sequences, which specifically hybridise to the mRNA of the target protein. This hybridisation results in the prevention of mRNA transport, splicing, transcription, but can also lead to degradation of the mRNA by endogenous ribonuclease H. Karras and co-workers showed that administration of an antisense oligonucleotide directed against IL-5 resulted in decreased IL-5 protein expression, and furthermore led to reduced lung eosinophilia and Ag-mediated late-phase airway hyper-responsiveness in a mouse model of asthma [51]. The fact that the abrogation of the late-phase airway response was not complete, suggested that, besides IL-5, additional pathways may contribute to airway hyper-reactivity.

The IL-5Rα-chain is another interesting target for antisense therapy. Antisense oligonucleotides were designed to reduce expression of the TM and SOL IL-5Rα isoforms *in vitro*, and to suppress eosinophilia *in vivo* upon IL-5 treatment or in a ragweed-induced allergic peritonitis model [51;52].

Interfering with ligand binding

A number of protein-based IL-5 antagonists have been reported, including single point mutants of IL-5 that occupy the receptor but fail to initiate receptor activation [53], neutralizing antibodies directed against IL-5, and the SOL IL-5Rα isoform that sequesters the ligand in solution [54].

Monoclonal antibodies

Humanized monoclonal antibodies (mAbs) have recently been used in several human clinical trials. mAbs offer clear advantages over traditional small molecular weight drugs. First, specific mAbs can be rapidly developed. Second, mAbs are highly selective for their targets and do not compete with the same drug metabolism, resulting in predictable biological effects and low risk of unfavorable drug interactions, respectively. Moreover, the pharmacokinetics of mAbs are predictable and the circulating half-life is often between days and weeks. Third, mABs can interfere with the binding of polypeptide hormones and cytokines with their receptors, which appears to be extremely difficult for small molecular weight compounds. Adversely, production costs are high, tissue penetration of mAbs may be restricted due to their size and oral delivery is problematic. Currently, therapeutic mAbs are given through

parenteral administration, although technologies allowing biological agents be delivered through the inhaled route are under development.

The profound inhibitory effect of anti-IL-5 mAbs on the development of lung eosinophilia and on airway hyper-responsiveness was demonstrated in several animal studies. Intraperitoneal as well as intranasal application of the anti-IL-5 mAb, TRFK-5, led to a long lasting inhibition of pulmonary eosinophilia and bronchial obstruction in mice [55]. In a monkey model of Ascaris-allergic asthma, Egan and coworkers could abolish inflammatory cell migration and airway hyperreactivity for up to three months by treating the monkeys with a single dose of a neutralizing humanized monoclonal antibody against hIL-5 [56]. The promising results of treatment with IL-5 mAbs observed in animal models made this approach a favourite candidate for targeting eosinophilic inflammation in humans. Similar anti-IL-5 treatment in humans, however, failed to demonstrate clinical efficacy in two independent studies. A single-dose phase I clinical trial was conducted with SCH55700 in patients with severe persistent asthma and demonstrated a significant decrease in peripheral blood eosinophils (lasting up to 90 days) and a trend towards improvement in lung function at the higher doses (30 days after dosing) [57]. SB240563 (Mepolizumab) was tested in mild asthmatics and caused a significant reduction in peripheral blood eosinophils and in post-challenge sputum eosinophils but did not alter FEV_1 values upon allergen challenge [58]. In another clinical trial, Flood-Page and coworkers observed a significant differential effect of IL-5 blockade on eosinophil counts in various body compartments.[59;60] After multiple dosing with mepolizumab, another anti-IL-5 mab, the authors found 100% reduction in blood eosinophils, but only 52% reduction in the bone marrow and a 55% decrease in the bronchial mucosa[60]. Completely blocking IL-5 activity *in vivo* therefore appears to be much more difficult than *in vitro*. Perhaps, the local tissue microenvironment, and/or the existence of IL-5-dependent autocrine activation mechanisms may limit *in vivo* antagonism [14;61]. Furthermore, only very limited amounts of IL-5 may be required to maintain eosinophil action and survival in the local tissue microenvironment. Hence, the *in vivo* levels of humanized anti-IL-5 mABs may be insufficient to block local effects. Therefore, complete detailed reports on the phase II trials in asthma, but also in other eosinophil-associated diseases such as nasal polyposis and atopic dermatitis are required to fully evaluate and understand the underlying mechanisms of anti-IL-5 treatment. Indeed, in recently published case reports in patients with hypereosinophilic syndrome, successful treatment with mepolizumab and reslizumab was demonstrated[62;63]. Anti-IL5 therapy effectively controlled the associated eosinophilic dermatitis with a drop of eosinophil counts, IL-5, eotaxin and ECP levels in serum[63].

Soluble IL-5Rα

The precise *in vivo* role of the SOL IL-5Rα protein requires more detailed study. Secreted receptor variants are a hallmark of the receptor family to which the IL-5Rα belongs. This mere evolutionary conservation underscores their functional importance, but the precise physiological role may differ from case to case. Models in which the antagonistic effects prevail include surface receptor down-modulation and ligand capture. On the other hand, secreted receptors may bind their ligands in circulation, protecting them from proteolytic breakdown and prolonging their serum half-life or facilitating ligand-mediated signaling. SOL IL-5Rα protein has antagonistic properties *in vitro* as demonstrated in eosinophil differentiation assays as well as in proliferation assays using IL-5 responsive cell lines [31;64]. It captures IL-5 in solution, and this IL-5/IL-5Rα complex is unable to associate and activate membrane bound βc chains [32;64]. Recently, the antagonistic effect of recombinant SOL IL-5Rα was demonstrated using nasal tissue explants. Culturing tissue with recombinant SOL IL-5Rα almost completely attenuated the ragweed-induced decrease in eosinophil precursors and increase in MBP-immunoreactive cell numbers [65]. This observation leads one to consider that endogenous production of soluble receptor may regulate IL-5 function *in vivo*. However, one of the potential problems of utilizing soluble receptors therapeutically is that they may actually prolong cytokine activity by reducing clearance rates[1].

Interfering with signal transduction

An intrinsic disadvantage of interfering with IL-5 function at the extracellular level is that all cellular responses are indiscriminately eliminated, resulting in possible unwanted side-effects. As an alternative, intervention at the level of intracellular signalling following receptor activation should be considered. Blockage of only a subset of (cell-specific) signals, or signal cascades, may be achieved by interfering with specific protein-protein interactions in a given pathway, or by blockage of specific modifying enzymes, such as kinases and phosphatases. An example of cascade specific inhibition of IL-5 signalling was recently provided by Alam and co-workers [66;67]. They characterised the binding site of Lyn kinase on the β_c receptor, and used this sequence information to design a cell-permeable β_c-derived peptide. This inhibitor furthermore blocked IL-5-dependent eosinophil differentiation and survival, but not eosinophilic degranulation *in vitro.* When applied *in vivo*, the Lyn-binding peptide significantly inhibited airway eosinophil influx in a mouse model of asthma[66].

ANTAGONISING THE IL-5 FUNCTION IN NASAL POLYPS

Interleukin 5 remains an interesting molecular target for pharmacological intervention in eosinophilic diseases. It's role as a central, non-redundant player in eosinophil function *in vivo* has been very well documented over the past years. Furthermore, within the haematopoietic system, it delivers specific signals to the eosinophilic and basophilic cells. Recent observations point to an additional function on airway smooth muscle cells, suggesting that IL-5 may directly contribute to bronchial hyper-responsiveness. Yet, initial studies using humanised anti-IL-5 monoclonal antibodies in asthma have been disappointing. Since than the eosinophil is in deep crisis and there is considerable confusion as to whether this cell is important in allergic diseases. If so, under what conditions?

In this context, similar studies in nasal polyposis may be of particular interest to understand these findings: Furthermore, nasal polyps have the advantage of being: 1) easily visualized by nasal endoscopy, 2) accessible for the local measurement of IL-5, IL-5Rα and other mediators, and 3) no attribution of airway smooth muscle cells is expected in NP.

The role of eosinophils in nasal polyposis is strongly suggested by observational studies and by in vitro data, however never firmly proven. Indeed, the increased concentrations of IL-5 and severe tissue eosinophilia were found in NP tissue and treatment of eosinophil-infiltrated polyp tissue with neutralizing anti-IL-5 monoclonal antibody (mAB), but not anti-IL-3 or anti-GM-CSF mAbs, resulted in eosinophil apoptosis and decreased tissue eosinophilia *in-vitro*[14]. However, as in asthma, only antagonizing the effect of IL-5 in nasal polyposis *in vivo* would be the ultimate test of the IL-5 / eosinophil hypothesis.

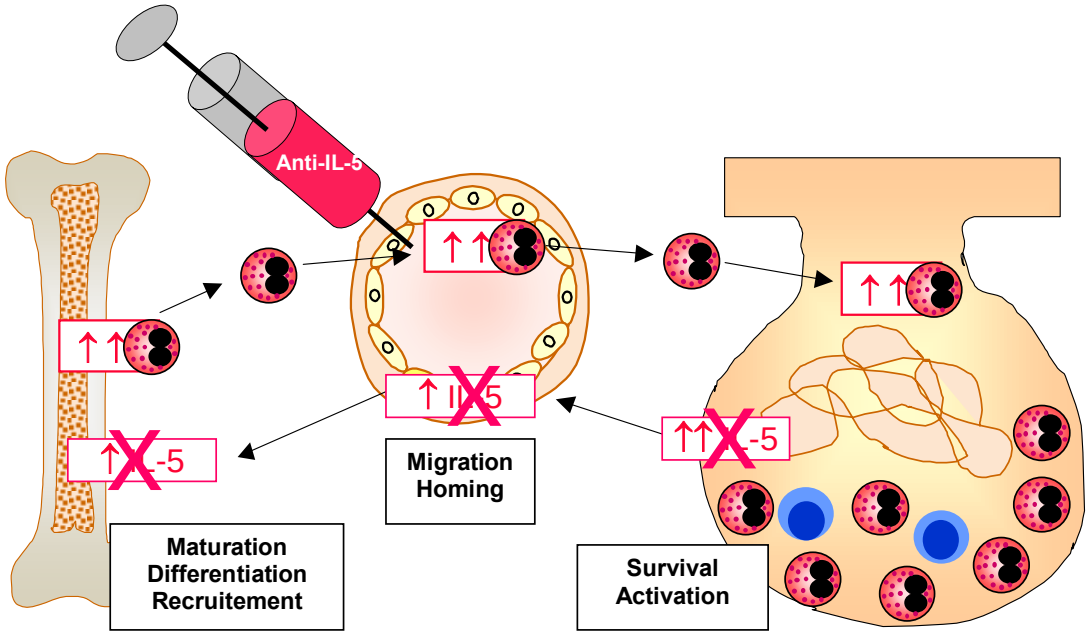

Figure 3: *Theoretical model of Anti-IL-5 treatment*

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CHAPTER 3

AIMS OF THE STUDY

AIMS OF THE STUDY

The aims of this thesis were to investigate the role of eosinophils in the pathogenesis of nasal polyposis. Especially the regulation of interleukin-5 and the interleukin-5 receptor α isoforms were studied with emphasis on future therapeutic strategies in nasal polyposis.

Chapter 4: To identify the most important factors in polyp growth, we did histologic studies on early stage manifestations of eosinophilic nasal polyps compared to their surrounding normal mucosa and mature polyps. Second we measured eosinophilic inflammatory mediators (IL-5, eotaxin, ECP, LTC4-D4-E4, TGF-β1) and extracellular matrix components (fibronectin, hyaluronic acid, albumin) in nasal polyp tissue, to investigate a possible relationship between eosinophilic inflammation and polyp constituents. Third we studied the effect of oral glucocorticoids on the eosinophilic inflammation in five nasal polyps that received oral corticoids prior to surgery.

Chapter 5: To investigate the possible impact of atopy on local IgE and eosinophilic inflammation in nasal polyposis, we measured total and specific IgE to common inhalant allergens in NP and non-polyp tissue homogenates.

Chapter 6: To determine the relationship between nasal carriage of Staphylococcus aureus, IgE formation to S. aureus enterotoxins and nasal polyposis. Furthermore, whether IgE is produced locally in nasal polyps or is a result of extravasation, we compared IgE specificities in tissue and serum. In this context B- and T-lymphocyte accumulations in NP tissue were histologically characterised.

Chapter 7: To develop specific ELISAs for studying the SOL IL-5Rα isoform at the protein level and to set up a PCR-based approach to monitor the expression of the SOL IL-5Rα mRNA, and to test the diagnostic potential of SOL IL-5Rα in eosinophil-associated disease such as nasal polyposis. In this context, SOL IL-5Rα was determined in various human samples including serum, nasal secretion and nasal tissue homogenates

Chapter 8: To study the expression of interleukin 5 receptor α isoforms in nasal polyp patients and controls, we established a real-time PCR, FACS and ELISA to investigate levels of SOL and TM-IL-5Rα transcripts and proteins in blood and in tissue. Furthermore, we studied whether in vitro stimulation of peripheral blood eosinophils with IL-5 could regulate the IL-5Rα isoform expression similar to the expression found in tissue eosinophils. Finally, we tested the biological activity of SOL-IL-5Rα in an hIL-5-driven proliferation assay.

Chapter 9: The primary objective of this study was to determine the safety and pharmacokinetics of Reslizumab (humanized anti-IL-5 monoclonal antibodies; SCH55700) given as a single intravenous dose of 3 mg/kg to subjects with severe nasal polyposis. In addition, the activity of Reslizumab on the clinical course of severe nasal polyps, on peripheral and nasal eosinophilic inflammation was evaluated. After all, this anti-IL-5 study gives us the ultimate opportunity to test role of IL-5 and eosinophils in nasal polyposis.

CHAPTER 4

NASAL POLYPOSIS: FROM CYTOKINES TO GROWTH

NASAL POLYPOSIS: FROM CYTOKINES TO GROWTH.

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Am J Rhinol 2000; 14(5): 279-90. * Equal contribution

ABSTRACT

Nasal polyposis (NP) is a chronic inflammatory condition, which is mostly characterised by an infiltration of eosinophils. How this eosinophilic inflammation leads to polyp formation remains largely unclear. In order to identify the most important factors in polyp growth, first we report the histological features of two early stage manifestations of eosinophilic nasal polyps compared to their surrounding normal mucosa and mature polyps. Histomorphologic analysis of these early stage manifestations of NP showed the presence of eosinophils, forming a subepithelial cap over a pseudocyst area, which was filled with albumin. In mature NP, a large pseudocyst area containing albumin was surrounded by subepithelial eosinophilia. Second, in an approach to quantify and to study possible relations between eosinophilic inflammation and changes in extracellular tissue components we measured interleukin-5 (IL-5), eotaxin, eosinophil cationic protein (ECP), leucotrienes (LTC4/D4/E4), transforming growth factor-beta 1 (TGF-β_1), fibronectin, hyaluronic acid and albumin in nasal tissue homogenates of 31 subjects. Nasal polyp samples (n=16) were obtained during routine endonasal sinus surgery, whereas control samples (n=15) were obtained from the inferior turbinate during septum surgery. In the group of polyp patients 11 received no treatment, whereas 5 were treated with oral glucocorticoids (GCS) within 4 weeks prior to surgery. IL-5 was measurable in 8 of 11 untreated NP, whereas IL-5 could not be detected in all 15 controls nor in 4 of 5 oral corticoid treated polyps. The comparison between the untreated polyp group and controls showed significantly higher concentrations of IL-5, eotaxin, ECP and albumin in polyp supernatants, whereas TGF-β_1 was significantly lower. In the oral GCS treated group, ECP and albumin were significantly reduced compared to untreated nasal polyps. The same tendency, but not reaching significance was seen for eotaxin and fibronectin, while no difference was found for LTC4/D4/E4 and hyaluronic acid between the groups. Our observations suggest a deposition of albumin (and possibly other plasma proteins) and extracellular matrix proteins, regulated by the subepithelial eosinophilic inflammation, as a possible pathogenic principle of polyp formation and growth. IL-5 and eotaxin are found to be key factors for eosinophilic accumulation and activation in NP. Oral corticoid treatment may lead to the shrinkage of NP by downregulation of the eosinophilic inflammation and reduction of the extravasation and deposition of albumin in NP.

INTRODUCTION

Nasal polyposis (NP) is a chronic inflammatory disease of the paranasal sinuses, with a prevalence of about 1 - 4 % in the general population. In subgroups of patients having specified airway diseases - allergic fungal disease, acetylsalicylic acid sensitivity, and asthma - much higher frequencies are found [1]. Most patients complain of nasal obstruction, secretion, loss of smell, headache and reduced general well-being. NP are benignant growths of the nasal and sinus mucosa which are mainly situated in the middle meatus. Their preferred site of origination was found to be the mucous membrane of the outlets from the sinuses [2]. Histopathologically NP consist of oedematous connective tissue covered with respiratory epithelium. The supepitehlial area is characterised by an eosinophilic inflammation in more than 80% of the cases, however, also non-eosinophilic NP exists and has to be careful differentiated, such as choanal polyps, or NP on the basis of chronic sinusitis or cystic fibrosis. Similarly, eosinophils are the major effector cells in allergic disease such as asthma and rhinitis. However, whereas atopy is a major risk factor for asthma, its contribution to polyp pathogenesis is questionable. Most studies have failed to show a higher occurrence of positive skin tests to inhaled allergens in patients with polyps than in the general population [2-4]. Extensive studies by Settipane showed that the prevalence of nasal polyps is higher in non-allergic rhinitis and asthma than in their allergic counterparts [5]. As polyp tissue is more readily accessible, it has been suggested to use nasal polyps as an ideal model for study of eosinophil-dominated immune inflammation in the airway mucous membrane.

The treatment of nasal polyps with systemic and topical corticoids is common clinical use. However, there is no medical or surgical treatment which guarantees cure and most clinicians treat polyp patients on an individual basis. Management of patients with severe nasal polyposis is still unsatisfactory and recurrences remain a problem with a general rate of recurrence of up to 40% [4]. Future progress in therapy seems to be closely linked to a better understanding of aetiology and pathogenesis of NP.

The pathogenesis of nasal polyps is far from clear. Woakes believed that formation of NP began as exudate in the nasal mucosa and that growth of nasal polyps was due to vascular stalk and vascular congestion [6]. Eggston et al. hypothesised that recurrent infections lead to chronic vascular changes in the nasal mucosa, blocking of intracellular fluid transport and oedema of the lamina propria[7]. Tos & Mogensen have suggested that early stage of polyp formation was characterised by infiltration and oedema of the nasal mucosa, rupture of the epithelium, and production of granulation tissue [8].

In order to identify the most important factors in polyp growth, first we report the histological features of two early stage manifestations of eosinophilic nasal polyps compared to their surrounding normal mucosa and mature polyps. Second we measured eosinophilic inflammatory mediators (interleukin(IL)-5, eotaxin, eosinophil cationic protein (ECP), leucotrienes (LT)C4-D4-E4, transforming growth factor (TGF)-β_1) and extracellular matrix components (fibronectin, hyaluronic acid, albumin) in nasal polyp tissue homogenates and compared those with normal nasal mucosa, in order to investigate a possible relationship between eosinophilic inflammation and polyp constituents. Third we studied the effect of oral glucocorticoids on the eosinophilic inflammation and the extracellular matrix components in five nasal polyps that were treated with oral corticoids prior to surgery.

MATERIAL AND METHODS

Patients

Nasal tissue was obtained from 16 polyp and 15 control patients at the department of Otorhinolaryngology of the University Hospital of Ghent, Belgium. Nasal polyp samples were obtained during routine endonasal sinus surgery, whereas control samples where obtained from the inferior turbinates during septal surgery. Bilateral nasal polyposis was diagnosed based on history, clinical examination, nose endoscopy and sinus CT-scan. In the group of polyp patients, 11 received no treatment, whereas 5 were treated with oral corticoids , more precisely a cumulative dose of minimum 150mg methyl-prednisolone within the last 4 weeks prior to surgery. The atopic status was evaluated by skin prick tests to common inhalant allergens, which were positive in 6 out of 15 controls and 9 out of 16 nasal polyp patients. A history of asthma was reported in 4 controls and 5 polyp patients. Informed consent was obtained from all the subjects, and the study was approved by the ethics committee of the Ghent University.

Immunohistochemistry

Within the group of nasal polyposis, two patients with an early stage of polyp formation localised on the middle turbinate were selected. Those patients had no allergy, no asthma, no aspirin sensitivity, nor previous treatment with systemic or topical corticoids prior to surgery. Parts of the middle turbinate with normal appearance, bearing a small polyp formation, were removed, further specimen were taken from the mature bilateral polyps. Samples were fixed in 4% formaldehyde (Phosphate buffered 6.8-7.2, Klinipath, Netherlands). The samples were embedded in paraffin and cut into 5µm sections and mounted on poly-L-lysin-coated slides.

After deparaffinization in parasolve, slides were hydrated through graded ethanol before use. For cellular staining, tissue sections were treated with trypsin for 20 minutes at 37°C to enhance immunohistohemical reactivity. Mouse anti-human eosinophil cationic protein (ECP) clone EG2 (1/400, Pharmacia, Sweden), mouse antihuman mast cell tryptase clone AA 1 (1/500, Dako, Denmark) and mouse antihuman α-smooth muscle actin monoclonal antibodies were used with the APAAP (alkaline phosphate-anti-alkaline phosphate) technique. For protein staining, sections were microwaved and incubated in blocking solution (TBS, 0.3% perhydrol, 0.1% Na-azide) for 20 minutes to block endogeneous peroxidase activity. Two polyclonal antibodies, rabbit anti-human albumin (1/40.000, Dako, Denmark), and rabbit antihuman fibronectin (1/4000, Dako, Denmark) were selected and stained using an LSAB-kit (Dako, Denmark). Sections were counterstained with hematoxylin and mounted.

Measurement of mediators and plasmatic proteins in controls and mature nasal polyps

All (n=31) freshly obtained specimen were weighed, and 0.5g tissue was transferred into 5 ml of 0.9% NaCl solution and homogenised at 1000 rpm for 5 minutes on ice. After homogenisation, suspensions were centrifuged at 3000 rpm for 10 minutes. Aliquots of the supernatants were prepared and stored at –80°C for analysis. Concentrations of IL-5, eotaxin, LTC4/D4/E4, TGF-β_1 and fibronectin were measured on tissue supernatants by using commercially available ELISA kits (R&D System, USA). ECP was measured by CAP system (Parmacia, Sweden), hyaluronic acid by RIA (Pharmacia, Sweden) and albumin by nephelometry.

Statistical analysis

Between group comparisons were made by using the non parametric Mann Whitney test. Spearman rank correlation coefficient was used to asses the relationships between the parameters. $P < 0.05$ was considered statistically significant.

RESULTS

Histologic study of early stage polyps

In the following, we differentiate between ‘early stage’ and ‘mature’ nasal polyps. The analysis was focused on the description of the inflammatory cells, i.e. mast cells, eosinophils and myofibroblasts, and extracellular proteins, fibronectin and albumin, in nasal polyps.

The main characteristic of the early stage polyp formation is the presence of pseudocysts in the core of the polyp. The extracellular matrix in this region contains a loose connective tissue, fibroblasts and only a few inflammatory cells. In contrast, there is an accumulation of

inflammatory cells at the top of the early stage polyp (Fig. 1). In mature polyps, oedema is major, many pseudocysts are visible, but the cellular component is less expressed.

The cellular component

Numerous EG2-stained eosinophils were present at the top of the early stage polyps, forming a subepithelial cap over the pseudocyst area. In contrast, the basis of the polyp and the adjacent mucosa has a normal appearance and showed no eosinophilia. In mature polyps, the location of eosinophils seemed less organised, some regions are fully infiltrated by them, others less. Most eosinophils are localised subepithelially and some infiltrate into the epithelium.

In contrast, mast cells were found in the pedicle and the adjacent mucosa, but not in the top and core of the early stage polyps. In mature polyps, mast cells were diffusely distributed over the polyp, without any accumulation.

The distribution of myofibroblasts was limited to the stalk and the centre of the polyps, near to the pseudocysts, whereas they were scattered within mature polyps. No myofibroblasts were seen in the adjacent mucosa

The protein component

The overall staining of albumin in the normal mucosa and the subepithelial regions of polyps is dense and compact. In the core of the early stage polyp and of mature polyps, staining is less compact. However, albumin staining is pronounced and resembles a network-like structure within the pseudocysts (Fig. 2). Fibronectin deposition was noticed together with eosinophils at the top of small polyps, and formed a network-like structure in the polyp centre (Fig. 3). In the adjacent mucosa, fibronectin staining was much less intense.

Concentrations of mediators and plasmatic proteins

In an approach to quantify the eosinophilic inflammation and the extracellular matrix components, we measured the concentrations of IL-5, eotaxin, ECP, LTC4/D4/E4, TGF-β_1 , fibronectin, hyaluronic acid and albumin in inferior turbinate and nasal polyps tissue homogenates (table 1 and Fig. 4). IL-5 was measurable in 8 of 11 untreated nasal polyps, whereas IL-5 could not be detected in all 15 controls nor in 4 out of 5 oral corticoid treated polyp patients. Comparison between controls and untreated nasal polyps showed significant higher concentrations of eotaxin, ECP and albumin in polyp supernatants, whereas TGF-β_1 was significantly lower. Fibronectin, hyaluronic acid and LTC4/D4/E4 levels were not

different between controls and untreated nasal polyps. When we compared controls and oral corticoid treated polyps, there were no significant differences except for fibronectin, which was lower in the oral treated NP. Concentrations of ECP and albumin were significantly reduced in oral corticoid treated polyps compared to their untreated counterparts. The same tendency, but not reaching significance, was seen for eotaxin and fibronectin. TGF-β_1 tissue concentration was higher in the oral corticoid treated polyps compared to untreated polyps without reaching statistical significance, while no difference was found for LTC4/D4/E4 and hyaluronic acid. Within the group of untreated NP, IL-5 and eotaxin were correlated with ECP, whereas albumin correlated with fibronectin. Comparing the atopic with non-atopic patients within the control and within the nasal polyp group didn't reveal any significant differences for the above mentioned parameters.

DISCUSSION

The aim of this study was to identify cells and mediators possibly involved in polyp formation and growth, their interrelationship as well as changes following oral steroid treatment.

In a first part we first report the histological features of two early stage manifestations of human eosinophilic nasal polyps compared to their surrounding normal mucosa and mature polyps. Histomorphologic analysis of these early stage manifestations of NP showed the presence of eosinophils, forming a subepithelial cap over a pseudocyst area, which was filled with albumin. In mature NP, a large pseudocyst area containing albumin was surrounded by subepithelial eosinophilia. The retention of albumin, most likely driven by the eosinophilic inflammation, therefore seems to be a hallmark of the polyp formation.

Second, in an approach to quantify and to study possible relations between eosinophilic inflammation and changes in the concentrations of extracellular tissue components, we measured IL-5, eotaxin, ECP, LTC4/D4/E4, TGF-β_1, fibronectin, hyaluronic acid and albumin in nasal tissue homogenates of control and NP patients. IL-5, eotaxin, ECP and surprisingly albumin were significantly upregulated in untreated polyps compared to controls, supporting the histologic findings and linking eosinophilic inflammation to albumin deposition in NP.

Third, oral corticoid treatment could reduce IL-5, eotaxin, ECP, albumin and fibronectin concentrations in NP, reaching significance for ECP and albumin. This clearly demonstrates that the eosinophil activation together with albumin deposition can be reduced by oral GCS treatment, leading to the shrinkage of nasal polyps.

Table 1: Influence of GCS Treatment on Polyp tissue

	Controls (n=15)	versus	Untreated NP (n=11)	versus	Oral GCS NP (n=5)	versus controls
IL-5 (pg/ml)	not detectable		159,1		1 of 5 detectable	
Eotaxin (pg/ml)	78,0	p = 0,008	965,9	ns	403,2	ns
ECP (µg/l)	666,5	p = 0,003	5134,8	p = 0,036	1035,4	ns
TGF-β1 (pg/ml)	9952	P < 0,001	2585	ns	5203	ns
Albumin (g/l)	8,576	p < 0,001	19,646	p = 0,020	12,477	ns
Fibronectin (µg/ml)	22,65	ns	20,16	ns	14,04	p = 0,032
Hyaluronan (pg/ml)	8781	ns	9471	ns	7008	ns
LTC4/E4/D4 (pg/ml)	2932	ns	4267	ns	2688	ns

Median concentrations of IL-5, eotaxin, ECP, TGF-β1, albumin, fibronectin, hyaluronic acid and LTC4/D4/E4 in inferior turbinates (controls), untreated, and orally corticoid treated nasal polyp tissue homogenates. Statistical analyses were performed by the Mann Whitney test for unpaired comparisons. $p < 0,05$ was considered statistically significant (ns = not significant).

Nasal polyps have been described in numerous publications and several theories on their formation have been postulated. They have been regarded as a neoplastic disorder, a result of glandular hyperplasia [9]or altered ion transport mechanisms[10]. Tos described polyp formation in the middle ear of rats and proposed that polyp formation is initiated by the rupture of the epithelium, followed by a prolapse of granulation tissue[8;11;12]. Norlander manipulated the respiratory mucosa of white rabbits to induce polyp formation[13]. However, to the best of our knowledge, histological documentation of the first crucial stages of nasal polyp formation in human has not been described up until now.

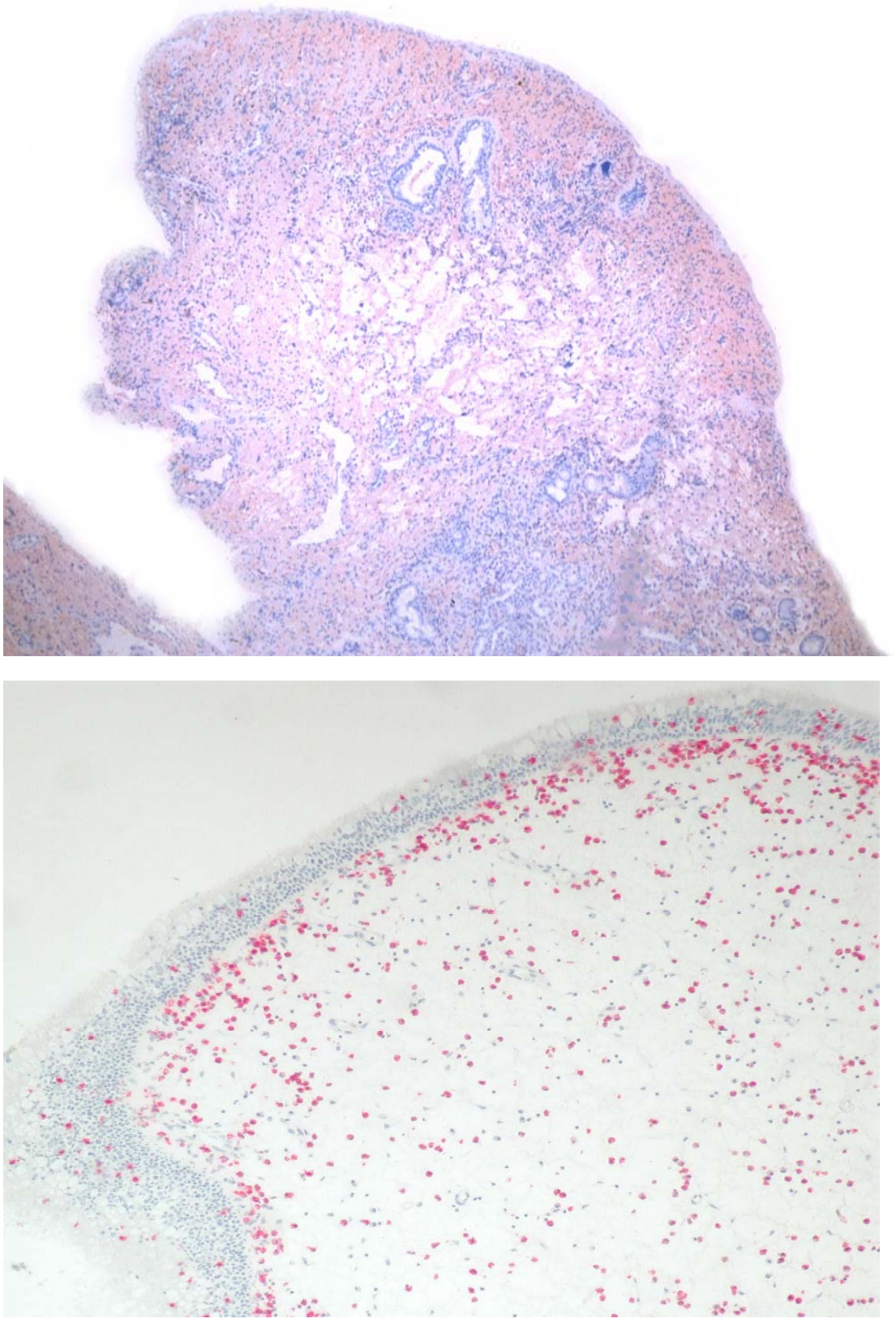

Figure 1: *(A) An early stage nasal polyp on a middle turbinate, Albumin stained, overview (original magnification: 40X). (B) Subepithelial eosinophilic inflammation in the mature polyp, EG2 stained (original magnification: 200X).*

Nasal polyps are characterised by a marginal, subepithelial eosinophilic inflammation and a central oedema with pseudocyst formation. Even in the early stage polyps, this key principal could be found regularly. Numerous EG2-stained eosinophils were present at the top of the early stage polyp, forming a subepithelial cap over the pseudocyst area. Activated, EG2-positive eosinophils have been described to predominate in polyp stroma and to participate in the development and maintenance of mature polyp disease[14;15]. According to our findings, the same principal seems to be true for the early stage polyps.

In contrast to eosinophils, mast cells were not found in the top and core of the early stage polyps, but in the pedicle and the adjacent mucosa. In mature polyps, mast cells were more diffusely distributed without any accumulation. Drake-Lee showed that mast cells are more abundant in the submucosa and were not more frequent in the epithelium of nasal polyps [16]. Ultrastructural studies of nasal polyps in human have described degranulation of mast cells[17]. Our results indicate that mast cells are absent in the early stage nasal polyp, which at least suggests that mast cells are not directly involved in the initiation of polyp formation.

A rather small number of myofibroblasts were found mainly in the stalk and the centre of the early stage polyps, near to the pseudocysts, whereas no myofibroblasts were seen in the adjacent mucosa. Wang and Escudier have shown that myofibroblasts were more abundant in nasal polyps compared to normal nasal mucosa and that their local development could be controlled by TGF-β [18]. They suggested the involvement of myofibroblasts in NP formation and growth by inducing extracellular matrix accumulation.

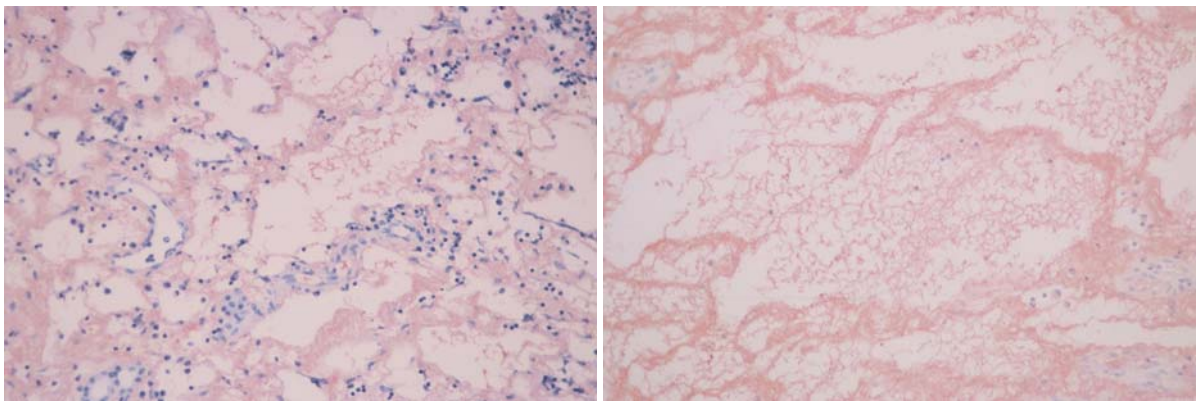

Figure 2: *Albumi-stained early stage polyp (A) and mature polyp (B), showing network-like albumin deposition in the pseudocysts (original magnification: 200X).*

Fibronectin deposition was noticed together with eosinophils at the top of small polyps, and it formed an network-like structure in the polyp centre and within pseudocysts. It has been shown, in vitro, that human eosinophils preferentially survive on tissue fibronectin compared with plasma fibronectin[19]. Recently a correlation among oedematous morphology, eosinophil infiltration, and fibronectin expression was described in NP[20]. According to Greiff, NP could consist of oedematous tissue, which is very rich in plasma proteins[21]. To our knowledge, however, there are no previous studies on albumin staining in nasal polyps. In the present study we demonstrate that albumin is retained in the pseudocyst area of mature and early stage NP, surrounded by an eosinophilic inflammation.

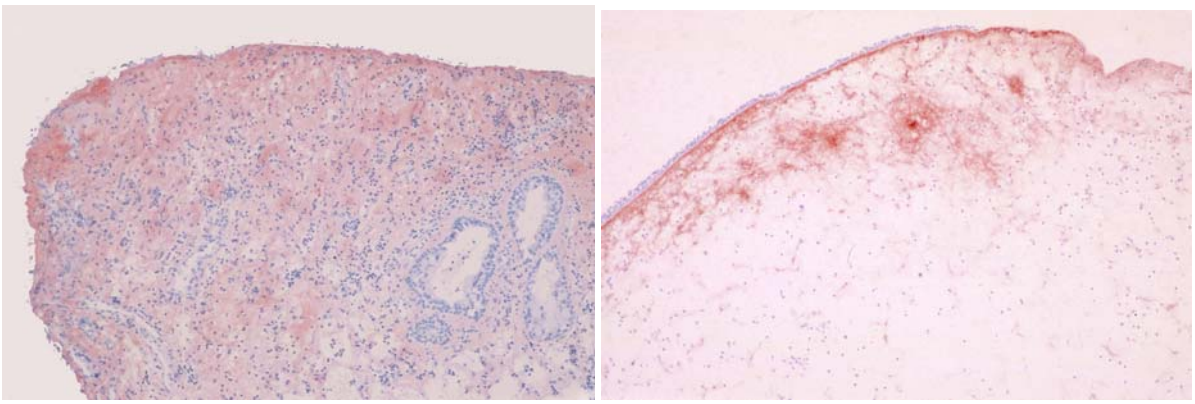

Figure 3: *Fibronectin stained early stage polyp (A) and mature polyp (B). Fibronectin deposition is noted espescially subepithelially in the area with eosinophilic inflammation and is denser in the early stage polyps than in mature NP (Original magnification: 100X).*

Eosinophils contain an armoury of distinctive cytoplasmatic granules necessary for killing parasites, which also contribute to tissue damage and inflammation. Four principal granule proteins have been identified including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). In addition, eosinophils contain preformed cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-4, IL-5, TGF-β and tumor necrosis factor-α (TNF-α) [22].

Eosinophil granule proteins such as ECP have been shown to increase vascular permeability in a hamster cheek pouch model[23]. Here we show high ECP concentrations together with high albumin concentrations in nasal polyps versus controls, supporting the hypothesis that eosinophils could induce plasma exudation. Biewenga found higher albumin concentrations in nasal secretions of polyp patients compared to healthy subjects[24]. According to Perrson et al, in non-polyp airways - in health and in disease - plasma exudation may not readily produce oedema, but may rather result in a quantitative lumenal entry of plasma[25]. In polyps, however,

the extravasated plasma - for reasons of distance, binding force, or by some barrier or extracellular matrix abnormality – may not as easily find its way to the airway surface. Our observations suggest a central deposition of albumin (and possibly other plasma proteins) and extracellular matrix proteins such as fibronectin, regulated by the subepithelial eosinophilic inflammation, as a possible pathogenic principle for polyp formation and growth.

A sustained eosinophilic inflammation is the hallmark of nasal polyps, which is also true in the early stage of polyp formation. This can be explained by an increase of eosinophilic migration into the tissue, by prolonged survival of these cells, or by a combination of both [14]. The recruitment of granulocytes to sites of inflammation is a complex process that potentially may be regulated by cytokines and chemokines. Induction of eosinophil proliferation in the bone marrow, promotion of the release of eosinophils from the bone marrow, and inhibition of eosinophil apoptosis can be promoted by IL-3, IL-5 and GM-CSF (Th2 cytokines), while IL-1β, IL-4, IL-5, and TNF-α may regulate trafficking by activating adhesion molecules in the vascular endothelium [26].

Several chemokines of the CC-family, including eotaxin and RANTES, have been reported to attract and activate eosinophils via high affinity binding to the CC-chemokine receptor CCR3[27;28]. RANTES immunoreactivity was reported in nasal polyps [29]. However, in an earlier study we did not find a difference in RANTES protein concentrations between nasal polyps and control tissue [14].

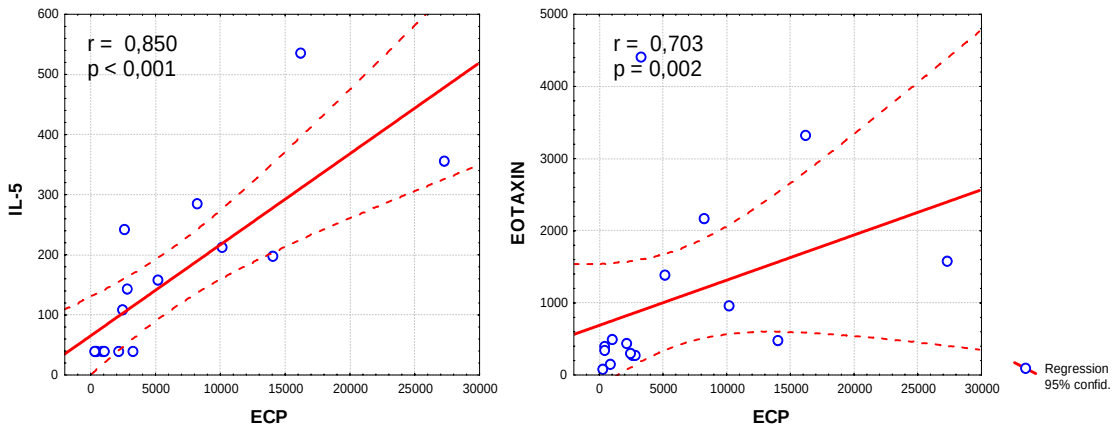

Figure 5: *Spearman rank correlations between IL-5 and ECP, and eotaxin and ECP in all nasal polyps (n=16). The concentrations of IL-5, eotaxin and ECP are expressed in pg/ml.*

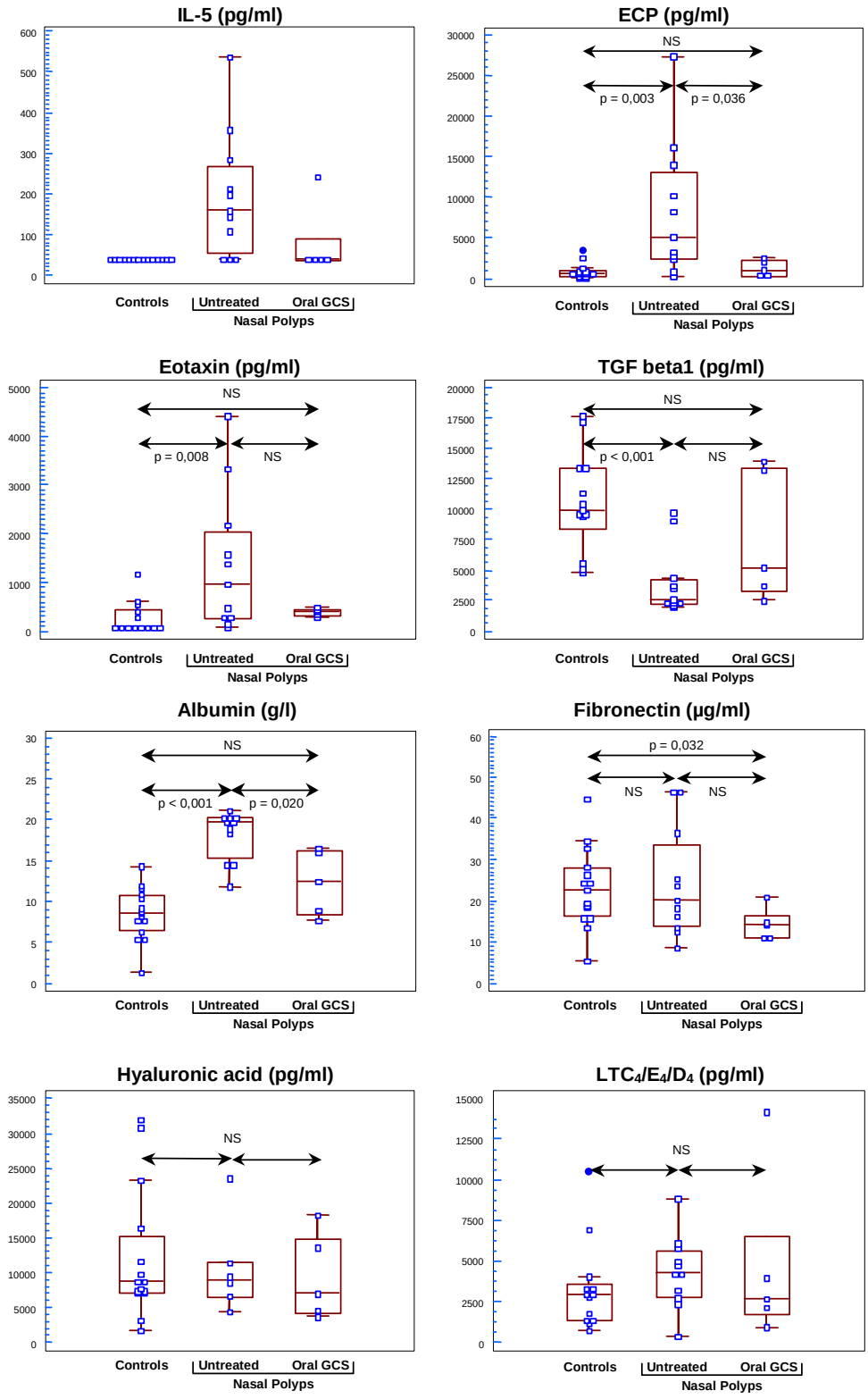

Figure 4: *Measurement of IL-5, ECP, eotaxin, TGF-β1, albumin, fibronectin, hyaluronic acid and LTC4/D4/E4 in inferior turbinates (controls), untreated and orally corticoid treated nasal polyp tissue homogenates. In the basic Box-and-whisker plot, the central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. The horizontal line extends from the minimum to the maximum value, excluding "outside" and "far out" values, which are displayed as separate points. Statistical analyses performed by the Mann Whitney two-tailed test for unpaired comparisons (ns = not significant).*

Recently, Jahnsen et al. showed significantly increased mRNA expression for eotaxin and eotaxin-2 in NP compared to turbinate mucosa[27]. It was also shown that eotaxin secreted from inflamed tissue may play an important role in initiating both blood and tissue eosinophilia [26]. In an in vivo model system, eotaxin has been reported to co-operate with IL-5 in inducing eosinophil infiltration. Furthermore, IL-5 is not only essential for mobilising eosinophils from the bone marrow, but also plays a critical role in regulating eosinophil homing and migration into tissues in response to eotaxin[26]. Corresponding to these findings, we here demonstrate IL-5 and eotaxin both being upregulated in NP, furthermore showing a significant correlation to eosinophil activation as evaluated by ECP concentrations in tissue.

Eosinophilic infiltration into tissues is usually followed by elimination of these cells by programmed cell death. Cytokine-mediated inhibition of apoptosis clearly contributes to the accumulation of eosinophils in tissues[30;31]. Cytokines such as IL-3, IL-5, GM- CSF or γ-IFN dramatically increase the life-span of eosinophils by inhibition of the programmed cell death in vitro [14]. Comparing protein levels of different cytokines and chemokines in NP versus control mucosal tissue, we were able to demonstrate an impressive upregulation of IL-5 synthesis in NP in several studies[14;32;33]. Furthermore, NP but not control nasal tissue, expressed IL-5 mRNA. Simon et al. demonstrated that anti-IL-5 mAb, but not anti-IL-3 or anti-GM-CSF mAb decreased tissue eosinophil numbers by inducing apoptosis in an elegant polyp model [31].

IL-5 is essential for the maturation and differentiation of eosinophils, and is involved in the homing, activation, and degranulation of these cells. It is well established that IL-5 can act both on eosinophil production from bone marrow progenitors and on circulating mature eosinophils via a specific receptor[34]. Activated TH2 cells, mast cells, basophils, eosinophils and recently airway epithelial cells have been shown to be potential cellular sources of IL-5 [35]. In nasal polyps, we previously demonstrated that eosinophils may represent a major source of IL-5, thus creating a possible autocrine loop for activation and survival[14].

Eosinophils were also suggested to be an important source of TGF-β in nasal polyps[36-39]. TGF-β is a potent fibrogenic cytokine that stimulates extracellular matrix formation, acts as a chemoattractant for fibroblasts, but largely inhibits the growth and activity of invading inflammatory cells[40]. There are three principal isoforms of TGF-β, namely, TGF-β_1, β_2 and β_3. TGF-β_1 is described to be the main isoform in fibrosis and in nasal polyps[36]. Corresponding to our data, Eisma showed a decreased expression of TGF-β_1 and enhanced expression of TGF-β_2 in nasal polyps versus normal control tissue[37]. Alam et al demonstrated

that TGF-β inhibits the synthesis of IL-5, abrogates the survival-prolonging effect of hematopoietins (IL-5 and GM-CSF) on eosinophils, and induces apoptosis[40]. They suggested that there is a fine balance between the production of TGF-β and IL-5 by eosinophils and that TGF-β may act as a homeostatic regulatory mechanism that counteracts the action of IL-5 and programs cell death. Other data showed that TGF-ß in low concentrations can induce eosinophil chemotaxis, whereas higher concentrations reduce eosinophil survival mediated by IL-5, IL-3 and GMCSF[41]. This corresponds to our findings in NP, were we found high concentrations of IL-5 and low concentrations of TGF-β1 compared to normal nasal mucosa.

Nasal polyps may be treated by long-term local glucocorticosteroid (GCS) therapy, short-term systemic corticoid therapy, or endoscopic sinus surgery[2]. The clinical efficacy of topical corticoids in nasal polyps has been confirmed by several placebo controlled studies, showing a reduction of symptoms and polyp scores [42-48]. The effect of systemic corticoids on nasal polyps is generally recognised but less well studied. Van Camp and Clement [49] reported 25 NP patients who were treated with oral GCS and reported a positive effect on all symptoms. However, within five months after successful oral GCS therapy, a strong tendency for recurrence was found. Nevertheless, GCS have a prominent anti-inflammatory effect and are found to be effective in the treatment of eosinophil related inflammatory disease such as asthma. Several studies already showed that GCS may reduce the numbers of eosinophils in polyp tissue[27;50-53]. This could be due to a reduction of eosinophil influx, a shortening of eosinophil survival or a combination of both. GCS have been shown to reduce the endothelial expression of adhesion molecules[54;55] and suppress eotaxin levels in NP, both important for a reduction of eosinophil recruitment [56].

In this study, we found that IL-5 was measurable in 8 of 11 untreated NP, whereas IL-5 could not be detected in 4 of 5 oral GCS treated polyps. This suppression of IL-5 could well explain the reduction in eosinophil activation, demonstrated by a significantly suppressed ECP level in our study. As discussed before, eosinophil granule proteins such as ECP are known to affect vascular permeability [23]. Microvascular effects of GCS in NP have not been explored specifically. Biewenga showed decreased albumin and immunoglobulin levels in nasal secretions of patients with GCS-treated NP [24]. Interestingly, in our study, GCS treatment also showed a significant reduction of albumin retention in polyp tissue. These data support the hypothesis that GCS lead to the shrinkage of NP by down-regulation of the eosinophilic inflammation and reduction of the extravasation and deposition of albumin in NP extracellular matrix.

SUMMARY

Our findings support the hypothesis that eosinophils are the major effector cells in the pathogenesis of nasal polyps. Even in the early stage of polyp formation, the inflammatory reaction is mainly based on eosinophils. Their accumulation may be explained by an increased migration into the tissue, a prolonged survival, or a combination of both, based on the regulation by cytokines and chemokines. IL-5 and eotaxin are found to be essential for the accumulation and activation of eosinophils. Here we demonstrate that IL-5 and eotaxin are both up-regulated in nasal polyp supernatants, whereas TGF-ß1 is down-regulated. The correlation of these factors with eosinophil activation, evaluated by ECP-measurement, indicates their crucial role in eosinophil inflammation.

Our observations furthermore suggest the deposition of plasmatic proteins such as albumin and extracellular matrix proteins, regulated by the subepithelial eosinophilic inflammation, as a possible pathogenic principle of polyp formation and growth. Oral GCS treatment significantly reduced ECP and albumin content in NP, suggesting that GCS could lead to the shrinkage of NP by down-regulation of the eosinophilic inflammation and reduction of the deposition of albumin in NP extracellular matrix.

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CHAPTER 5

TOTAL AND SPECIFIC IGE IN NASAL POLYPS IS RELATED TO LOCAL EOSINOPHILIC INFLAMMATION

TOTAL AND SPECIFIC IGE IN NASAL POLYPS IS RELATED TO LOCAL EOSINOPHILIC INFLAMMATION

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J Allergy Clin Immunol 2001; 107(4): 607-14. * Equal contribution

ABSTRACT

Background: Nasal polyps (NP) are characterized by eosinophilic inflammation and often coexist with asthma. However, the role of atopy and IgE in NP pathogenesis is unclear.

Objective: To determine whether there is an association between total and specific IgE to a variety of allergens in polyp and non-polyp tissue and markers of eosinophilic inflammation or skin test results.

Methods: Homogenates were prepared from nasal tissue of 20 NP and 20 non-polyp subjects and analyzed for concentrations of IL-5, IL-4, eotaxin, LTC4/D4/E4, sCD23 and histamine (ELISA). ECP, tryptase, total and specific IgE for inhalant allergens and Staphylococcus aureus enterotoxins were measured (ImmunoCAP).

Results: The concentrations of total IgE, IL-5, eotaxin, ECP, LTC4/D4/E4, and sCD23 were significantly higher in NP tissue, compared to non-polyp tissue. Total IgE was significantly correlated to IL-5, ECP, LTC4/D4/E4, sCD23 and to the number of eosinophils in NP. Based on the presence of specific IgE antibodies in tissue, three NP groups were defined: Group NP I demonstrated no measurable specific IgE, Group NP II selected specific IgE. The third group demonstrated a multiclonal specific IgE, including IgE to Staphylococcus enterotoxins, a high total IgE and a high prevalence of asthma.

Conclusions: These studies suggest that there is an association between increased levels of total IgE, specific IgE and eosinophilic inflammation in nasal polyps, which may be of relevance in the pathophysiology of nasal polyposis. Similarly, the presence of specific IgE to SEA and SEB also points to a possible role of bacterial superantigens.

INTRODUCTION

Clinically, nasal polyps (NP) are characterized by edematous masses in the nasal and paranasal cavities, leading to nasal obstruction, secretion, loss of smell, headache and reduced general well being. Nasal polyposis is believed to be a multifactorial disease that is frequently associated with asthma and aspirin sensitivity, but the mechanisms underlying the etiology of this disease are so far ill defined[1;2].

Although an allergic etiology of NP had been presumed since the early 1930s[3], this suggestion was challenged in the 1970s, when a retrospective study by Settipane et al[4] demonstrated that more NP were found in the non-atopic group compared to atopics, and subsequent studies demonstrated that multiple positive skin tests were less common in NP patients, compared to the general population[5]. However, tissue IgE concentrations have been found to be increased, irrespective of skin test results, suggesting a possible local IgE production[5;6]. Remarkably, studies of seasonal allergic patients with NP have demonstrated that symptoms and markers of eosinophilic inflammation are not influenced by the season[5;7]. Thus, the relationship between NP, IgE and allergy is not clearly understood so far.

A drawback in elucidating the etiology and pathologic mechanisms of this disease is the lack of a widely accepted classification, which includes clinical history and histology, to differentiate between the various forms of NP. Stammberger[8] has recently proposed a classification for NP and suggested that bilateral polyps are frequently linked to asthma and aspirin sensitivity. Indeed, bilateral polyps are the most predominant type of NP, covering 80-90% of polyp disease in Europe and USA, and are associated with tissue eosinophilia in most cases[9].

A key to understanding polyp pathophysiology is the regulation of recruitment, activation and survival of eosinophils, as well as their effect on polyp formation and growth[10]. We have demonstrated that synthesis of IL-5 and survival of eosinophils is significantly increased in NP tissue compared with non-polyp control tissue[11;12]. Additionally, ex-vivo studies demonstrated that eosinophil survival was decreased by incubation with anti-IL-5 neutralizing monoclonal antibodies, suggesting a functional role for IL-5 in the regulation of eosinophil survival[12].

In the current study, we aimed to investigate the possible impact of atopy (skin prick test positivity) and local IgE on eosinophilic inflammation. Therefore, total and specific IgE to common inhalant allergens were measured in NP and non-polyp tissue homogenates. Since Staphylococcus aureus enterotoxins have been shown to modify atopic dermatitis[13;14] and

unstable asthma[15;16], we also investigated the nasal tissues for the presence of specific IgE to these enterotoxins, to elucidate whether or not these may also play a role in the pathophysiology of NP.

METHODS

Patients

Nasal tissue was obtained from 20 bilateral NP and 20 non-polyp patients, outside the pollen season, at the Department of Otorhinolaryngology, Ghent University Hospital. Approximately 0.5 cm^3 tissue strips were collected from the surface of each inferior turbinate (turbinotomy, 0.2 to 0.5g of tissue) or bilateral NP (0.2 to 2.0g of tissue), during routine septal and sinus surgery, respectively.

Bilateral polyposis was diagnosed based on history, clinical examination, nasal endoscopy and sinus CT-scan, according to the classification of Stammberger[8]. A history of asthma was reported in 4 non-polyp and 11 polyp patients, with two subjects in the polyp group also demonstrating a history of aspirin sensitivity. The atopic status of all patients was evaluated by skin prick tests using a standard panel of allergens, according to the guidelines of the European Academy of Allergology and Clinical Immunology[17]. The reaction to a skin prick test was considered positive, if the wheal area caused by allergen was greater than 7mm² (diameter greater than 3 mm). The panel of allergens comprised: grass pollen mix *(Dactylis glomerata, Festuca elatior, Lolium perenne, Phleum pratense, Poa pratensis);* mites *(Dermatophagoides Pteronyssinus, Dermatophagoides farinae, Tyrophagus putrescentiae, Acarus siro, Lepidoglyphus destructor);* moulds *(Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum)* and *Curvularia spicifera* (ALK-Abelló, Denmark)**.** Negative and positive controls (10 mg/ml histamine solution) were also included with each skin prick test. Patients treated with oral corticoids within the last 4 weeks prior to surgery were excluded. The ethical committee of the Ghent University Hospital approved the study, and informed consent was obtained from all subjects.

Measurement of cytokines, mediators and IgE in tissue homogenates

All (n=40) freshly obtained tissue specimens were weighed, and 1 ml of 0.9% NaCl solution was added per every 0.1g tissue. The tissue was then homogenized with a mechanical homogenizer (B. Braun Melsungen, Germany) at 1000 rpm for 5 minutes on ice as described previously[18]. After homogenization, the suspensions were centrifuged at 3000 rpm for 10 minutes at 4°C, and the supernatants separated and stored at – 80°C until analysis. All

supernatants were assayed for IL-5, IL-4, eotaxin, sCD23, histamine (R&D System, Minneapolis, USA) and LTC4/D4/E4 (Amersham Pharmacia Biotech, Buckinghamshire, UK) by ELISA using commercially available kits. ECP, tryptase, total and specific IgE were measured by the Uni-CAP system (Pharmacia & Upjohn, Upsala, Sweden)[19;20]. Specific IgE was determined for grass pollen mix (*Dactylis glomerata, Festuca elatior, Lolium perenne, Phleum pratense, Poa pratensis*); house dust mix (Hollister-Stier Labs, *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blatella germanica*); moulds mix (*Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum, Candida albicans, Helminthosporium halodes*); Staphylococcus aureus enterotoxins SEA and SEB; and *Curvularia spicifera Bipolaris*. ImmunoCap coated with human serum albumin or glycine were used to evaluate any non-specific binding of IgE.

Immunohistochemistry

Samples were fixed in 4% formaldehyde (phosphate buffered 6.8-7.2, Klinipath, Duiven, Netherlands) and embedded in paraffin wax prior to cutting into 5µm sections and mounting on poly-L-lysin-coated slides. After deparaffinization in parasolve (Prosan, Merelbeke, Belgium), the sections were hydrated through graded ethanol and stained (Pappenheim) for standard histomorphologic analysis. For mast cell staining, tissue sections were treated with trypsin (0,1%; Sigma-Aldrich, Bornem, Belgium) for 20 minutes at 37°C (to enhance immunohistohemical reactivity) and then incubated with mouse anti-human mast cell tryptase clone AA 1 (1/500, Dako, Glostrup, Denmark) for 1h, prior to staining by the standard APAAP (alkaline phosphate-anti-alkaline phosphate) technique[21;22]. Sections were counterstained with hematoxylin.

All tissue sections were examined with an Olympus light microscope by two independent observers, blinded to the experimental conditions. The numbers of eosinophils and mast cells were counted in the epithelium and in the adjacent lamina propria in 10 randomly selected fields (final magnification X400) and the results were expressed as the mean number of positive cells per field.

Statistical analysis

Data are expressed as median and interquartile range (IQR). When comparisons were made between groups, significance between-group variability was first established using Kruskal-Wallis test. The Mann Whitney U-test was then used for between-group comparison. Spearman rank correlation coefficient (r) was used to assess the relationships between the parameters. P-values less than 0.05 were considered statistically significant.

RESULTS

Protein concentrations and immmunohistochemistry in non-polyp vs. polyp tissue

Analysis of total IgE, IL-5, ECP, LTC4/D4/E4, eotaxin and sCD23 in supernatants prepared from tissue homogenates demonstrated that the concentrations of these mediators were significantly higher in NP tissue, compared to non-polyp tissue (Table I). In contrast, no significant differences were found in the levels of IL-4, histamine and tryptase.

Similarly, immunohistochemical analysis of whole NP and non-polyp tissue samples demonstrated that the numbers of eosinophils in both the epithelium and in the lamina propria were also significantly higher in NP tissue, compared to non-polyp tissue (Table I). Contrary to the findings for eosinophils, however, significantly higher numbers of mast cells were found in the lamina propria of non-polyp tissue, compared with polyp tissue, and there were no differences in epithelial mast cells.

Table I. Statistical comparisons between non-polyp and nasal polyp tissue.

ELISA	***Inferior turbinates (n=20, Median and IQR)***	***Mann Whitney test***	***Nasal polyps (n=20, Median and IQ range)***
Total IgE (kU/l)	22.3 (6.6 – 142.4)	***p < 0.001***	479.6 (199.7 – 1479.0)
sCD23 (U/ml)	All BDL	***p < 0.001***	5 BDL; 17.5 (5.5 – 32.0)
IL-5 (pg/ml)	19 BDL	***p < 0.001***	5 BDL; 205.2 (47.5 – 353.3)
IL-4 (pg/ml)	17.1 (12.7 – 33.9)	p = 0.578	18.3 (11.1 – 29.6)
LTC4/D4/E4 (pg/ml)	2932.5 (1387.2 – 3713.4)	***p = 0.025***	4594.7 (3405.4 – 6011.6)
ECP (µg/ml)	539.8 (118.4 – 899.6)	***p < 0.001***	10077.5 (3518.7 – 22740.7)
Eotaxin (pg/ml)	16 BDL	***p < 0.001***	1 BDL; 1516.7 (461.6 – 3534.1)
Histamine (mM)	20.4 (9.0 – 43.2)	p = 0.055	31.2 (20.7 – 67.8)
Tryptase (mg/ml)	11.1 (5.4 – 15.7)	p = 0.197	6.7 (3.5 – 11.2)
Immunohistochemistry			
Eosinophils in epithelium	0.1 (0 – 0.2)	***p = 0.017***	0.8 (0.1 – 2.9)
Eosinophils in lamina propria	4.2 (1.8 – 15.4)	***p = 0.003***	52.5 (9.6 – 76.0)
Mast Cells epithelium	0.6 (0.2 – 1.9)	p = 0.853	0.7 (0.2 – 1.4)
Mast Cells lamina propria	7.8 (5.7 – 18.8)	***p = 0.003***	3.9 (1.9 – 6.2)

The concentrations (Median, IQR) of total IgE***,*** sCD23, IL-5, IL-4, LTC4/D4/E4, ECP, eotaxin, histamine and tryptase were measured in homogenates of non-polyp (inferior turbinate) and nasal polyp tissue. The number of eosinophils and mast cells were counted on Pappenheim and tryptase stained paraffin slides. Statistical analyses were performed by the Mann Whitney U-test. $P < 0.05$ was considered statistically significant. (BDL= below detection level)

Total IgE in atopic vs. non-atopic subjects

In 10 out of 20 non-polyp and in 13 out of 20 NP patients, the skin prick test was positive for at least one allergen. In the non-polyp group, 5 patients with positive skin prick tests and symptoms to grass pollens or to house dust mite showed elevated total IgE and corresponding elevated specific IgE concentrations for grass pollens or house dust mite in tissue homogenates. The median (IQR) IgE concentrations in non-polyp tissue were 11.9 (4.7 – 38.4) kU/l in non-atopic subjects and 45.9 (16.0 – 389.4) kU/l in atopic subjects ($p < 0.05$).

Comparison between atopic and non-atopic patients within the NP group did not show any significant differences for any of the above-mentioned parameters. In NP tissue of non-atopic and atopic patients, the total IgE concentrations were found to be 836.0 (49.5 – 5011.1) kU/l and 467.0 (203.9 – 1354.5) kU/l, respectively (Figure 1). Additionally, there was no correlation between the levels of IgE and skin prick test positivity in these individuals.

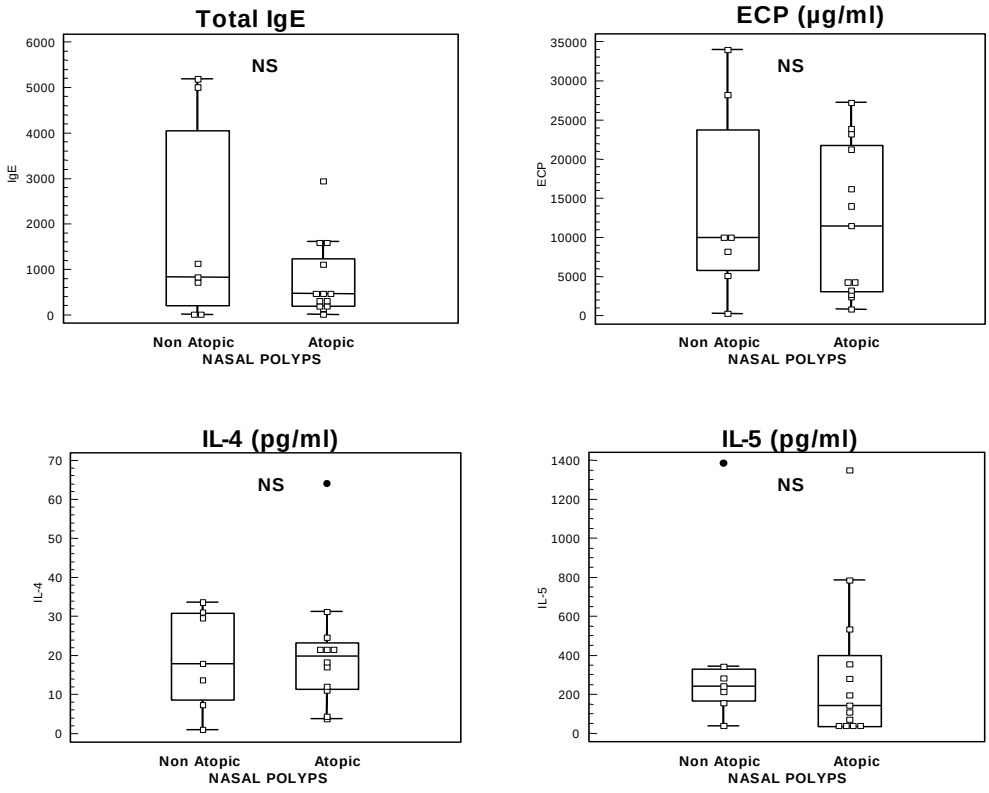


Figure 1. *Comparison of IgE, IL-4, IL-5 and ECP concentrations between non-atopic and atopic nasal polyps showing no significant differences between the groups. The Box-and-whisker plot represents the median, the lower to upper quartile, the minimum to the maximum value, excluding "outside" and "far out" values, which are displayed as separate points. Statistical analyses performed by the Mann Whitney U-test (NS = not significant).*

However, assessment for any correlations between IgE and other indices of inflammation demonstrated that in NP tissue the concentration of total IgE was significantly correlated with concentrations of sCD23 ($r = 0.737$; $p < 0.001$), ECP ($r = 0.457$; $p < 0.05$), LTC4/D4/E4 ($r = 0.588$; $p < 0.01$), and IL-5 ($r = 0.676$; $p < 0.001$), and numbers of eosinophils in both the epithelium ($r = 0.731$; $p < 0.001$) and the lamina propria ($r = 0.497$; $p < 0.05$). In contrast, total IgE in non-polyp tissue was correlated with histamine ($r = 0,654$; $p < 0.05$) and tryptase ($r = 0.538$; $p < 0.05$).

Nasal polyp groups based on specific IgE in nasal tissue

A positive ImmunoCap to inhalant allergens was detectable in tissue homogenates of 15 out of 20 nasal polyp patients. In 2 of 9 samples tested against ImmunoCap with either non-allergenic protein (HSA) or no protein (glycine), positive reactions were obtained. The lack of correlation with total IgE supports the hypothesis of a specific response. The reaction with HSA of the atopic patient with a skin test positive to Guinea pig could be due to allergenic similarities. IgE antibody to carbohydrate matrix of the ImmunoCap could explain the other reaction.

Based on the presence of specific IgE antibodies in tissue homogenates, we were able to arbitrarily distinguish three nasal polyp groups (Table II): group NP I was characterized by no measurable specific IgE and a low total IgE. In group NP II, selected specific IgE directed to house dust mite mix, grass pollen or mould mix were measurable and total IgE was increased. The third group (NP III) demonstrated a multiclonal IgE and high total IgE tissue concentrations (> 450 kU/l). sIgE to Staphylococcal enterotoxins SEA and SEB was also measured consistently in this group of patients, whereas these specific antibodies were not detectable in any of the other specimens.

Whereas in NP I, no patient suffered from asthma, in NP III, 8 out of 10 patients had a history of asthma and 2 patients reported a clinical history of aspirin sensitivity. Furthermore, the numbers of eosinophils in the tissue and the concentrations of total IgE, sCD23, ECP, LTC4/D4/E4, eotaxin and IL-5, but not histamine, tryptase or IL-4, were significantly higher in NP III, compared to non-polyp controls and NP I subjects (Figure 2).

Table II. Nasal polyp groups based on the presence of specific IgE in nasal polyp tissue.

Nasal polyp Groups	*Skin prick test*		Asthma	*Total IgE*	Specific *IgE to* (kU_A/l)					Dummy Caps	
				(kU/l)	*gx1*	*hx2*	*mx2*	SEA,SEB	*C. Bip*	*Glycine*	*HSA*
NP Group I	Non atopic	negative	-	8.2	BDL	BDL	BDL	BDL	BDL	/	/
		negative	-	49.5	BDL	BDL	BDL	BDL	BDL	/	/
	Atopic	hdm, mx,	-	212.3	BDL	BDL	BDL	BDL	BDL	/	/
		gx,	-	9.7	BDL	BDL	BDL	BDL	BDL	/	/
		hdm	-	140.6	BDL	BDL	BDL	BDL	BDL	/	/
NP Group II	Non atopic	negative	-	1131.8	BDL	4.7	BDL	BDL	BDL	/	/
	Atopic	hdm, gx	asthma	195.5	31.0	BDL	BDL	BDL	BDL	/	/
		hdm	asthma	343.2	BDL	BDL	12.4	BDL	BDL	/	/
		hdm	asthma	317.6	BDL	16.3	BDL	BDL	BDL	/	/
		hdm	-	1114.2	BDL	7.6	BDL	BDL	BDL	BDL	BDL
NP Group III	Non atopic	negative	-	5011.1	11.0	9.7	7.9	9.8	6.1	BDL	BDL
		negative	asthma	710.7	6.4	6.9	5.6	6.5	4.4	BDL	BDL
		negative	-	836.0	3.8	4.7	5.2	5.7	3.0	BDL	BDL
		negative	APA	5189.9	5.1	5.8	6.8	4.8	4.0	BDL	BDL
	Atopic	gx	asthma	488.6	11.1	19.0	10.0	15.4	9.8	BDL	BDL
		gx	asthma	2948.4	4.4	5.2	4.9	5.7	2.6	2.3	9.8
		hdm	asthma	467.0	17.5	9.4	6.5	36.1	BDL	/	/
		hdm, gx	asthma	1594.7	75.9	40.1	76.6	4.1	7.4	BDL	BDL
		hdm, gx	APA	470.7	9.6	10.9	8.2	7.0	4.4	/	/
		hdm	asthma	1612.0	4.2	4.3	4.1	3.7	2.6	1.4	2.3

Nasal polyp patients are grouped based on the presence of specific IgE in nasal polyp tissue: NP I (undetectable specific IgE), NP II (selected specific IgE), NP III (multiclonal IgE). The atopic status was evaluated by skin prick tests to common inhalant allergens: grass pollen mixture (gx), mites (hdm), moulds (mx) and Bipolaris. Skin prick tests for Staphylococcus aureus were not available and therefore not performed. APA: Patients suffering from the triad of aspirin sensitivity, polyposis and asthma. Polyp total IgE concentrations (kU/l) and specific IgE (kU_A/l) were determined for grass pollen mix (gx1), house dust mix (hx2), mould mix (mx2), S aureus enterotoxins (SEA and SEB) and Curvularia spicifera Bipolaris (C. Bip). Specific IgE to HSA and glycine was determined to exclude unspecific binding. Data from each individual are given separately (/ = not tested; BDL = below detection level).

DISCUSSION

This is the first report to describe a relationship between concentrations of total and specific IgE and the severity of eosinophilic inflammation in human nasal polyp tissue. Remarkably, these findings are unrelated to skin prick test results and symptoms, differing from a typical atopic disease such as allergic rhinitis. We could demonstrate a multiclonal specific IgE in 50% of NP samples. The presence of specific IgE antibodies to Staphylococcal enterotoxins SEA and SEB in these samples points to a possible role of bacterial superantigens as disease modifiers.

In line with previous studies[11;12;18;23-25], we were able to demonstrate a significant upregulation of IL-5 protein in NP, showing a firm correlation to ECP protein. It is widely accepted that IL-5 is an important cytokine for mature eosinophil activation[26]. The important functional role of IL-5 in NP has been demonstrated by experiments showing the induction of programmed cell death by neutralizing monoclonal antibodies (anti-IL-5 mAb)[12]. In addition, our study has also demonstrated that the increased concentration of eotaxin was correlated to ECP, suggesting a key role of this C-C chemokine for eosinophil recruitment in collaboration with IL-5.

Upregulated total IgE concentrations were paralleled by an increase of the soluble low affinity IgE receptor sCD23. Membrane-bound CD23 on B-cells was described to downregulate IgE formation upon binding to IgE[27], and the soluble receptor could interfere by capturing IgE and prevention of binding to the receptor. Thus, high concentration of sCD23 would be consistent with elevated total IgE.

We further analyzed specific IgE to common inhalant allergens and selected moulds in comparison to skin prick test results in non-polyp mucosa from the inferior turbinate of atopic and non-atopic patients. As expected, increased total IgE and specific IgE in the inferior turbinates of subjects allergic to inhalant allergens was found in correspondence with the skin prick test results. In marked contrast, atopy based on positive skin prick tests to inhalant allergens was not related to total or specific IgE in NP homogenates. Contrary to other reports[28], no impact of atopy could be found on any of the mediators or cytokines in the polyp group, questioning the role of common allergens in nasal polyp pathophysiology.

Based on the finding of specific IgE, we were able to differentiate three groups of NP patients:

- Group NP I with undetectable specific IgE and low total IgE (5-220 kU/l)
- Group NP II with selected specific IgE antibodies and increased total IgE (190-1150 kU/l)
- Group NP III with multiclonal specific IgE and high total IgE (450-5200 kU/l)

In the last group, representing 50% of the polyp patients in this study, all polyps demonstrated specific IgE responses to SEA and SEB, which were not found in any other subject. Following the classification into these three groups of polyps, we could demonstrate that total IgE, sCD23, IL-5 and ECP all follow a common pattern, with significantly increased concentrations in NP III, compared to either non-polyp or polyp group I samples. This is the first study to show that total IgE is related to IL-5 and ECP protein concentrations, and thus possibly to eosinophilic inflammation. Interestingly, 8 out of 10 patients in this group also

suffered from asthma, indicating not only a more severe local, but also systemic disease in these patients.

In contrast to other publications[5;29;30], we could not find increased histamine or tryptase levels within polyps compared to non-polyp tissue, questioning the role of mast cells in the pathogenesis of mature NP. In line with our finding, however, significantly lower numbers of mast cells were found in the lamina propria of NP compared to non-polyp tissue. Whilst it could be speculated that mast cells in NP were undetectable by immunohistochemistry due to degranulation, the lack of increased histamine and tryptase concentrations in NP tissue does not support this view.

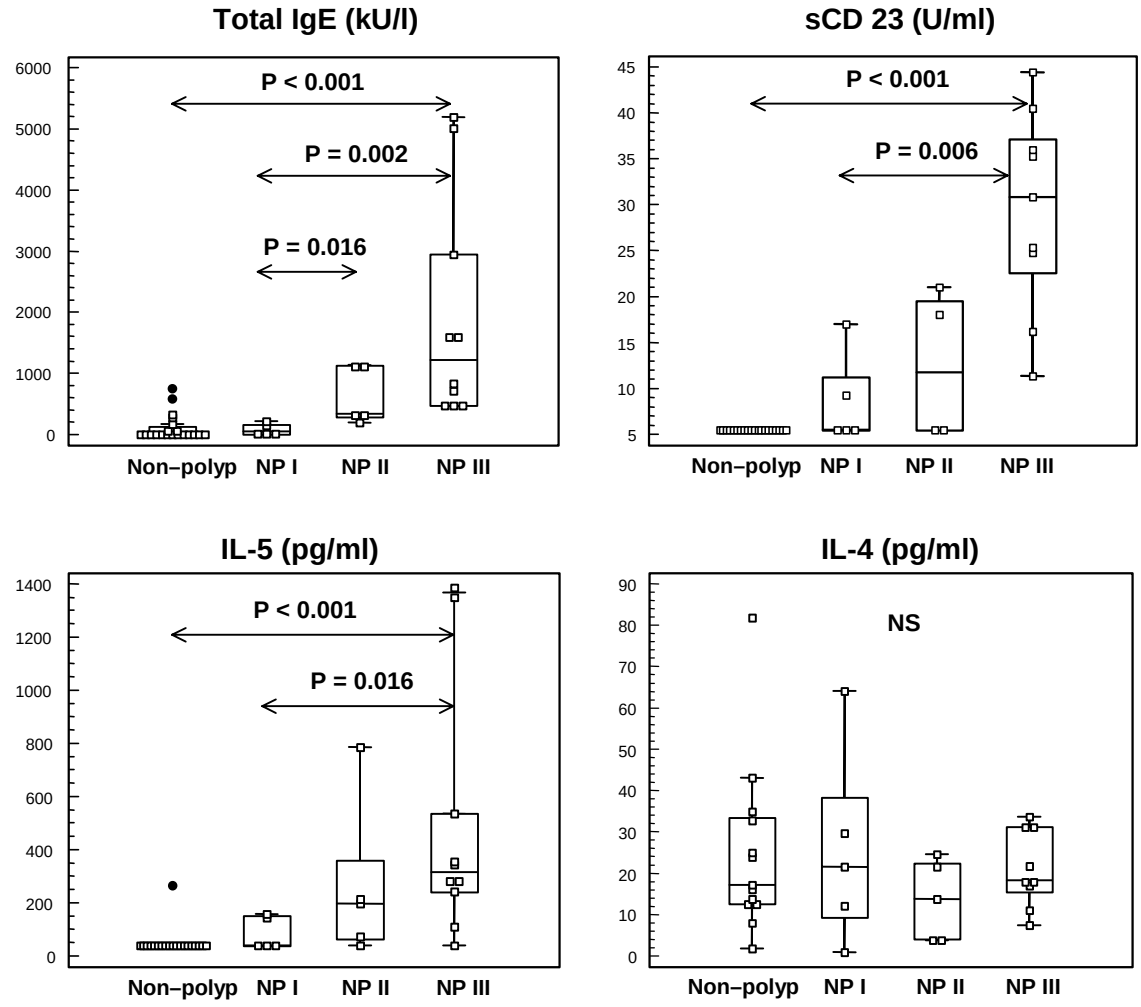


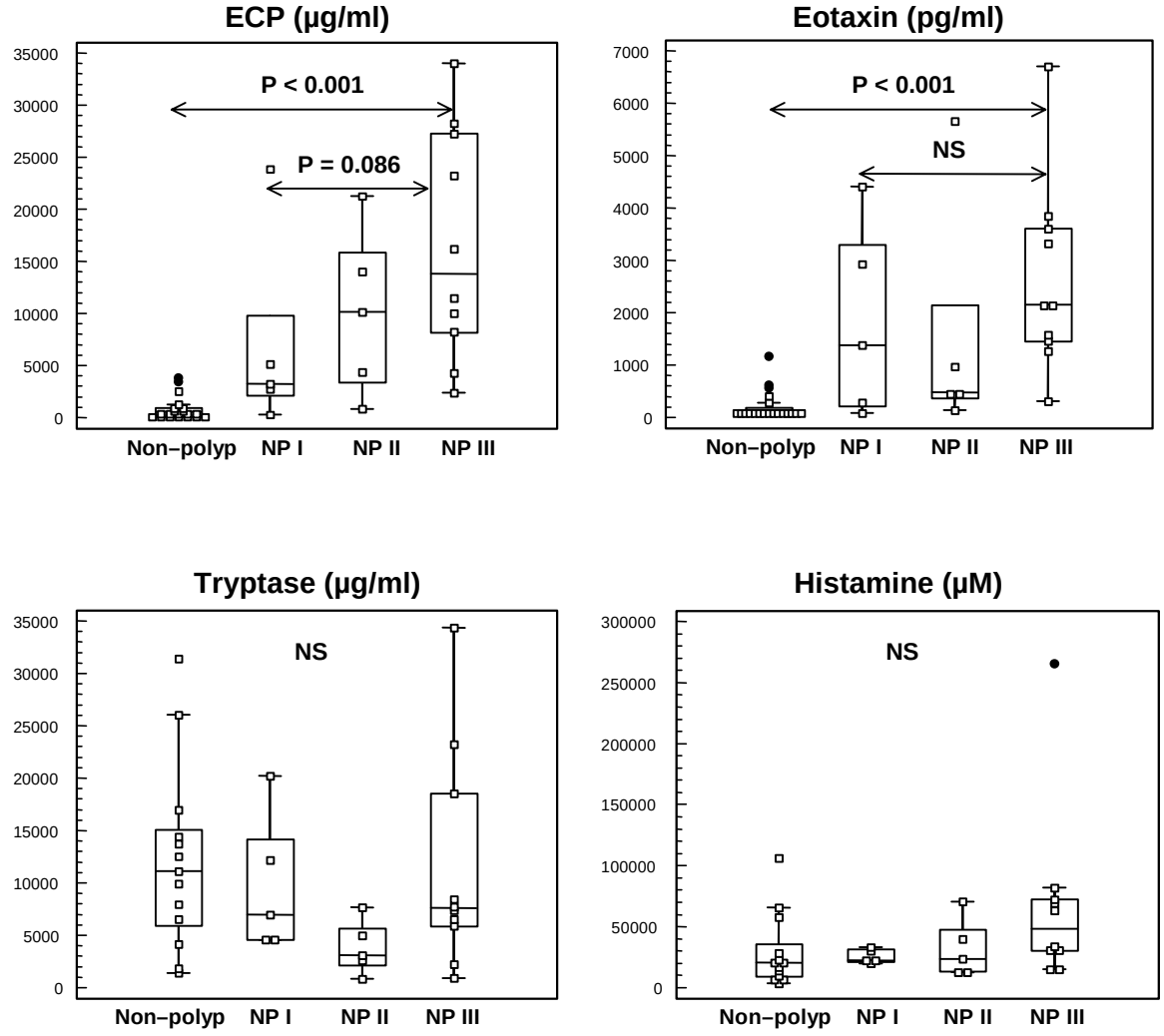
Figure 2. *Measurement of IgE, sCD-23, IL-5, and IL-4 tissue concentrations in inferior turbinates (non-polyp) and nasal polyp groups: NP I (undetectable specific IgE), NP II (selected specific IgE), NP III (multiclonal IgE). The Box-and-whisker plot represents the median, the lower to upper quartile, the minimum to the maximum value, excluding "outside" and "far out" values, which are displayed as separate points. Statistical analyses performed by the Mann Whitney U-test (NS = not significant).*

The high IgE protein concentrations could be preferentially derived from the serum, as an extravasation and deposition of serum proteins in polyp tissue has been shown[31]. However, allergen-induced heavy-chain switching to IgE occurs locally within the nasal mucosa of patients with seasonal allergic rhinitis[31] and increased local IL-4 mRNA, IgE heavy chain (Cε) and IgE heavy chain promoter (Iε) RNA[32] has been demonstrated after allergen exposure. Thus, a local IgE production also seems possible in NP. Interestingly, although IL-4 has been shown to be necessary for IgE synthesis, we could not demonstrate increased IL-4 protein concentrations in NP with high total IgE compared to non-polyp tissue. This, however, confirms our previous findings[12], where IL-4 mRNA expression in nasal polyp samples was also found to be low and not significantly different from controls. On the other hand, it has been demonstrated that only a small quantity of IL-4 is necessary to induce IgE synthesis in the presence of high IL-5 concentrations[33;34], and that IL-13 may replace IL-4[35]. Furthermore, Leung et al[36;37] have demonstrated an increased IgE production, induced by SSAg, in lymphocytes from patients with SAR in the absence of exogeneous IL-4.

Although the demonstration of specific IgE to SSAg within polyp tissue in the present study proves former contact to enterotoxins, the exact role of specific IgE needs further clarification. IgE to SSAg has been demonstrated to mediate basophil degranulation[36;37] and SSAg are thought to act as superantigens through unconventional interaction with the T-cell receptor[16;38]. There is recent evidence that SSAg may also play an important role in atopic dermatitis[13]. Anti-SEB IgE titers have been shown to correlate with the severity of disease in patients with mild to severe atopic dermatitis[14]. The majority of T cells found in lesional skin expressed a T cell receptor Vß repertoire that corresponded to the SSAg produced by the colonizing S aureus strain[39]. However, enterotoxins may also function as classic antigens through antigen-presenting cells resulting in specific sensitization[36], since SSAg also induce the synthesis of chemokines in a human epithelial cells[40] and may thus recruit T-cells and eosinophils to the site of infection. Furthermore, SEB has also been shown to induce a lymphocyte-dependent airway inflammation, including recruitment of eosinophils, in mice[16].

It is unclear whether the different groups of NP defined in the present study represent different disease entities or rather an evolution from group NP I to III. So far, we were unable to relate specific IgE to the age of the patient or duration of disease. Furthermore, it has to be elucidated whether SSAg´s represent causative or only modulating factors, being dependent upon a former damage of the mucosa by unrelated pathomechanisms.

In summary, our studies have demonstrated that in marked contrast to allergic rhinitis, specific IgE in nasal polyps is unrelated to skin prick test positivity, and total IgE correlates to markers of eosinophilic inflammation, but not to mast cell activation markers. In 50% of bilateral eosinophilic NP, a multiclonal IgE, including specific IgE to Staphylococcal enterotoxins A and B, has been demonstrated, suggesting a possible role of superantigens as disease modifiers. We suggest that nasal polyps may have pathophysiologic principles in common with atopic dermatitis and asthma with respect to the role of SSAg. Furthermore, we have developed a concept to differentiate bilateral nasal polyps into further three sub-groups, based on our findings for the presence of specific IgE antibodies within polyp tissue.



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CHAPTER 6

ORGANISATION OF SECONDARY LYMPHOID TISSUE AND LOCAL IGE FORMATION TO STAPHYLOCOCCUS AUREUS ENTEROTOXINS IN NASAL POLYP TISSUE

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Allergy 2004 (In press)

ABSTRACT

Background: Bilateral nasal polyposis (NP) is characterized by high concentrations of IgE in NP-tissue, which show no relation to the atopic status. We aimed to study the relationship between systemic and local IgE formation, nasal carriage of *Staphylococcus aureus* and nasal polyposis.

Methods: In serum and nasal tissue homogenates from 24 NP patients and 12 controls, we determined concentrations of total IgE and IgE antibodies to inhalant allergens and S. aureus enterotoxins (SAEs; A,B,C,D,E,TSST) by ImmunoCAP. Tissue cryosections were stained for CD3, CD20, CD38, CD23, FcεRI, IgE and SEA/SEB.

Results: We demonstrated a higher incidence of *S. aureus* colonisation (17/24) and IgE antibodies to SAEs in NP tissue (12/24) compared to controls (resp. 3/12 and 0/12). Total IgE and IgE antibodies in serum and NP-tissue were dissociated due to local polyclonal IgE formation in NP-tissue. Staining of NP tissue revealed follicular structures characterised by B- and T- cells, and lymphoid accumulations with diffuse plasma cell infiltration.

Conclusions: We demonstrated the organisation of secondary lymphoid tissue in polyp tissue and a polyclonal hyper-immunoglobulinemia E associated with the presence of IgE antibodies to SAEs, colonization with *S. aureus,* and tissue eosinophilia in a relevant subgroup of polyp patients.

INTRODUCTION

A hallmark of bilateral nasal polyposis in western countries is the pronounced tissue eosinophilia that can be found in about 70 to 90 % of cases as well as elevated local IgE levels in polyp fluid [1-5]. Therefore, an allergic aetiology of nasal polyps has been presumed, but never firmly demonstrated [6]. In a retrospective study by Settipane and Chafee, polyps were present in 2.8% of atopic patients, but in 5.2% of non-atopic subjects [7]. Furthermore, nasal polyps occur more frequently in subgroups of patients with asthma and aspirin sensitivity [8]. About 40 to 80% of patients with aspirin sensitivity suffer from polyposis, and about 15% of polyp patients are hypersensitive to aspirin [9]. In studies involving large series of patients with nasal polyposis, asthma was found in 20% to 70%, and non-allergic asthma is significantly more frequently linked to polyps compared to allergic asthma [8;9]. In a recent study we could demonstrate that atopy based on positive skin prick tests to inhalant allergens was not related to total IgE or IgE antibodies in NP tissue and that there was no impact of atopy on the levels of IL-5, IL-4, eotaxin, LTC4/D4/E4, sCD23, ECP and number of tissue eosinophils in NP tissue [2]. Whether the high concentrations of IgE antibodies in polyp tissue are the result of a local production or are preferentially derived from the serum remains to be elucidated. In about half of the polyp specimen we demonstrated polyclonal IgE formation specific IgE to *Staphylococcus aureus* enterotoxins (SAE), high levels of local IgE antibodies, and a high prevalence of asthma [2]. Similarly, we demonstrated that IgE antibodies to SAE were more often found in serum from patients with severe asthma than in those with mild asthma [10]. Recent evidence suggests that SAE could modify airway disease by acting as superantigens [11;12]. Superantigens are microbial or viral protein toxins with potent immunostimulatory properties. These properties are attributable to their unique ability to crosslink major histocompatibility complex (MHC) class II molecules with T cell receptors forming a trimolecular complex that activates a much larger number of resting T cells (as many as one in five T cells) than does a conventional antigen (one in 10^5-10^6 T cells) [13]. Several bacterial superantigens have been described, of which the family of classical *Staphylococcal aureus* enterotoxins (SEA, SEB, SEC, SED, SEE and TSST) are the most widely studied. In addition, *Staphylococcal* protein A (SPA)[14] as well as the SAE TSST-1 have also been demonstrated to possess B-cell superantigen moieties, inducing polyclonal IgE synthesis [15;16]. Because of the widespread carriage of *S. aureus* and the ubiquitous expression of their toxins it is clear that the nasal immune system is under constant challenge by these extremely powerful agents, which are therefore likely to influence the response to other challenges.

It was therefore tempting to study the relationship between nasal carriage of Staphylococcus aureus, IgE formation to Staphylococcus aureus enterotoxins and nasal polyposis. Furthermore, we aimed to determine whether IgE is produced locally in nasal polyps or is a result of extravasation, by comparison of IgE specificities in tissue and serum and by analysis of B- and T-lymphocyte accumulations in tissue.

METHODS

Patients

Nasal tissue and serum were obtained from 12 control and 24 bilateral NP patients at the Department of Otorhinolaryngology, Ghent University Hospital. All control subjects (median age 33.6 years, range 18-71 years; 5 F/ 7 M) were skin prick test negative, in generally good health, and none had a history of nasal or sinus disease, allergic disease (asthma, rhinitis, dermatitis), upper respiratory tract infection in the previous month, use of any intranasal medications, decongestants, antihistamines or oral steroids during the last month. Bilateral polyposis was diagnosed based on history, clinical examination, nasal endoscopy and sinus CT-scan. The median age of NP patients was 46 years (range 22-78 years; 7 F/ 17 M). A history of asthma was reported in 8 nasal polyp patients. Twelve patients were atopic, as indicated by positive cutaneous skin prick test responses to common aeroallergens [17]. The panel of allergens comprised: grass pollen mix *(Dactylis glomerata, Festuca elatior, Lolium perenne, Phleum pratense, Poa pratensis);* mites *(Dermatophagoides Pteronyssinus, Dermatophagoides farinae, Tyrophagus putrescentiae, Acarus siro, Lepidoglyphus destructor);* moulds *(Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum)* and *Curvularia spicifera* (ALK-Abelló, Denmark)**.** Negative and positive controls (10 mg/ml histamine solution) were also included with each skin prick test. Patients treated with oral corticosteroids within the last 4 weeks prior to surgery were excluded. The ethical committee of the Ghent University Hospital approved the study, and informed consent was obtained from all subjects.

Measurement of total IgE, IgE antibodies and albumin in serum and tissue homogenates

All samples were immediately processed, separated and stored in aliquots at – 80°C until analysis. Approximately 0.5 cm^3 tissue strips were collected from the surface of each inferior turbinate (turbinotomy, 0.2 to 0.5g of tissue) or bilateral NP (0.2 to 2.0g of tissue), during routine septal and sinus surgery, respectively. All (n=24) freshly obtained tissue specimens were weighed, and 1 ml of 0.9% NaCl solution was added per every 0.1g tissue. The tissue

was then homogenized with a mechanical homogenizer (B. Braun Melsungen, Germany) at 1000 rpm for 5 minutes on ice as described previously[4]. After homogenization, the suspensions were centrifuged at 1500 *g* for 10 minutes at 4°C, and the supernatants separated. Blood samples were allowed to clot at room temperature for 20-30 minutes, centrifuged at 1500 *g* for 10 minutes at 4°C, and serum was separated.

All supernatants and sera were assayed for total IgE and IgE antibodies by the UniCAP system (Pharmacia Diagnostics, Upsala, Sweden)[18] and for albumin by nephelometry. IgE antibodies were determined for Staphylococcus aureus enterotoxins (SEA, SEB, SEC, SED, SEE and TSST-1); grass pollen mix (*Dactylis glomerata, Festuca elatior, Lolium perenne, Phleum pratense, Poa pratensis*); trees mix *(Alnus incana, Betula verrucosa, Corylus avellana, Quercus alba, Salix caprea)*; house dust mix (Hollister-Stier Labs, *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blatella germanica*) and moulds mix (*Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum, Candida albicans, Helminthosporium halodes*). ImmunoCAP coated with human serum albumin or glycine were used to evaluate any non-specific binding of IgE.

Immunohistochemistry

Specimens were snap frozen in liquid nitrogen cooled methyl butane and stored at –80°C. Cryostat sections were prepared (6μm) and mounted on SuperFrost Plus glass slides, packed in aluminium paper and stored at –30°C until staining.

Sections were stained for CD3, CD20, CD38, CD23, FcεRI and IgE. Specimens were fixed in acetone. Endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxide in TBS containing 0.1% sodium azide for 20 minutes. Primary antibodies or negative controls, consisting of the corresponding isotype control, were incubated for 1 hour and detected using the LSAB technique conjugated with peroxidase according to the manufacture's instructions (labelled streptavidin-biotin, Dako, Copenhagen, Denmark). The peroxidase activity was detected using AEC Substrate chromogen (Dako), which results in a red-stained precipitate. Finally sections were counterstained with Hematoxyline and mounted.

Biotinylated SAE staining and blocking experiments

Specimens were fixed in acetone and endogenous peroxidase was blocked. Biotinylated SEA (40 μg/ml) and a mixture of non-biotinylated SEA/ biotinylated SEA (1000μg/ml / 40 μg/ml), to determine the specificity of the biotinylated SAE staining, (Toxin Technology ,Sarasota, US) were applied and incubated overnight at 4°C. The sections were rinsed with TBS and incubated with streptavidine-conjugated peroxidase (Dako) for 30 minutes. After rinsing with

TBS, sections were incubated with AEC chromogen (Dako) for 10 minutes rinsed, counterstained with hematoxyline and mounted.

Statistical analysis

Data are expressed as median and interquartile range (IQR). When comparisons were made between groups, significance of between-group variability was first established using the Kruskal-Wallis test. The Mann Whitney U-test was then used for between-group comparison. Spearman rank correlation coefficient (r) was used to assess the relationship between the parameters. The Chi-square test was used for comparison of frequencies. P-values less than 0.05 were considered statistically significant.

RESULTS

Nasal culture from the middle meatus demonstrated staphylococcal colonisation in 17/24 NP patients and in 3/12 controls (p=0.02). Table I demonstrates a significant increase in concentrations of IgE, albumin, and eosinophil counts in nasal polyp tissue compared to control tissue. Total IgE and IgE antibody concentrations were in all cases higher in tissue compared to serum. The IgE/albumin ratios in polyp tissue and in serum were dissociated, indicating that tissue IgE is rather the result of a local IgE production than of extravasation. Furthermore, IgE antibodies in polyp tissue only showed a partial relation to IgE antibody in serum and to skin prick tests (Table II). In a subgroup of sixteen patients a particular pattern of IgE expression was found in NP tissue. This polyclonal type of IgE expression is characterised by IgE antibodies to all measured common aeroallergens and is associated with the highest levels of IgE antibodies in NP tissue. IgE specific to SAE could be demonstrated in 12 out of 24 NP tissue homogenates and in 3 out of 24 serum samples of NP subjects, but in none of the controls. Eosinophil counts and IgE concentrations were significantly higher in those nasal polyps positive for IgE antibodies to SAEs than in SAE negative samples ($p<0.001$) (Figure 1).

Follicular structures were found in 6 out of 24 NP samples, whereas diffuse lymphoid accumulations were found in all NP samples (Figure 2). Follicular structures stained positive for IgE, CD3, CD20, and CD23, whereas FcεRI was found only outside the follicle (Figure 3). Plasma cells expressing CD38 were absent from the inner area of the follicular structures, but prominent in the diffuse lymphoid accumulations. The latter stained positive for IgE, CD3, CD20 and, FcεRI, but not for CD23 (Figure 3). SAE binding was positive in follicular structures and in some areas with lymphoid accumulations. The specificity of the SAE

binding was confirmed by staining with an excess of non-biotinylated SEA to biotinylated SEA, which completely blocked the signal. Follicular structures or SAE staining were not found in control tissue.

Table I: IgE concentrations and eosinophil counts in controls versus nasal polyps

NASAL TISSUE	*Controls (n=12) Median (± IQR)*	*versus*	*Nasal polyps (n=24) Median (± IQR)*
IgE (kU/l)	17 (7-25)	p < 0.001	654 (156-1263)
Albumin (g/l)	15.6 (12.7-17.3)	p = 0.002	19.7 (17.7-23.2)
IgE/Albumin	1.28 (0.90-2.14)	p < 0.001	27.08 (10.2-59.8)
SAE IgE ab + (%)	All negative (0%)	p = 0.009	12/24 (50 %)
Eosinophils (%)	0.3 (0.1-0.7)	p < 0.001	11.5 (2.9-24.3)
SERUM			
IgE (kU/l)	31 (15-64)	p = 0.004	160 (50-291)
Albumin (g/l)	48.1 (46.2-49.7)	NS	44.5 (40.9-54.3)
IgE/Albumin	0.32 (0.67-1.19)	p = 0.04	3.5(1.12-6.93)
SAE IgE ab + (%)	0/8 (0%)	NS	3/24 (12.5%)
Eosinophils (%)	0.9 (0.4-1.8)	p < 0.001	4.8 (2.6-7.1)

DISCUSSION

We demonstrated the organisation of secondary lymphoid tissue in nasal polyp tissue and a polyclonal hyper-immunoglobulinemia E associated with the presence of IgE specific to SAEs, colonization with *S. aureus,* and increased eosinophilic inflammation in a relevant subgroup of nasal polyp patients. Furthermore, the dissociation in IgE levels and IgE antibodies between nasal polyp tissue and serum suggests that IgE in NP are mainly due to a local production. We demonstrated binding of biotinylated SAE to follicular structures and in lymphoid accumulations in NP tissue. The follicular structures were mainly characterized by an accumulation of B-cells and T cells, whereas lymphoid accumulations showed diffuse plasma cell infiltration. These data suggest an organization of secondary lymphoid tissue with polyclonal B-cell activation in nasal polyps due to chronic microbial colonization and release of enterotoxins, which is likely to be the cause of IgE switch and formation.

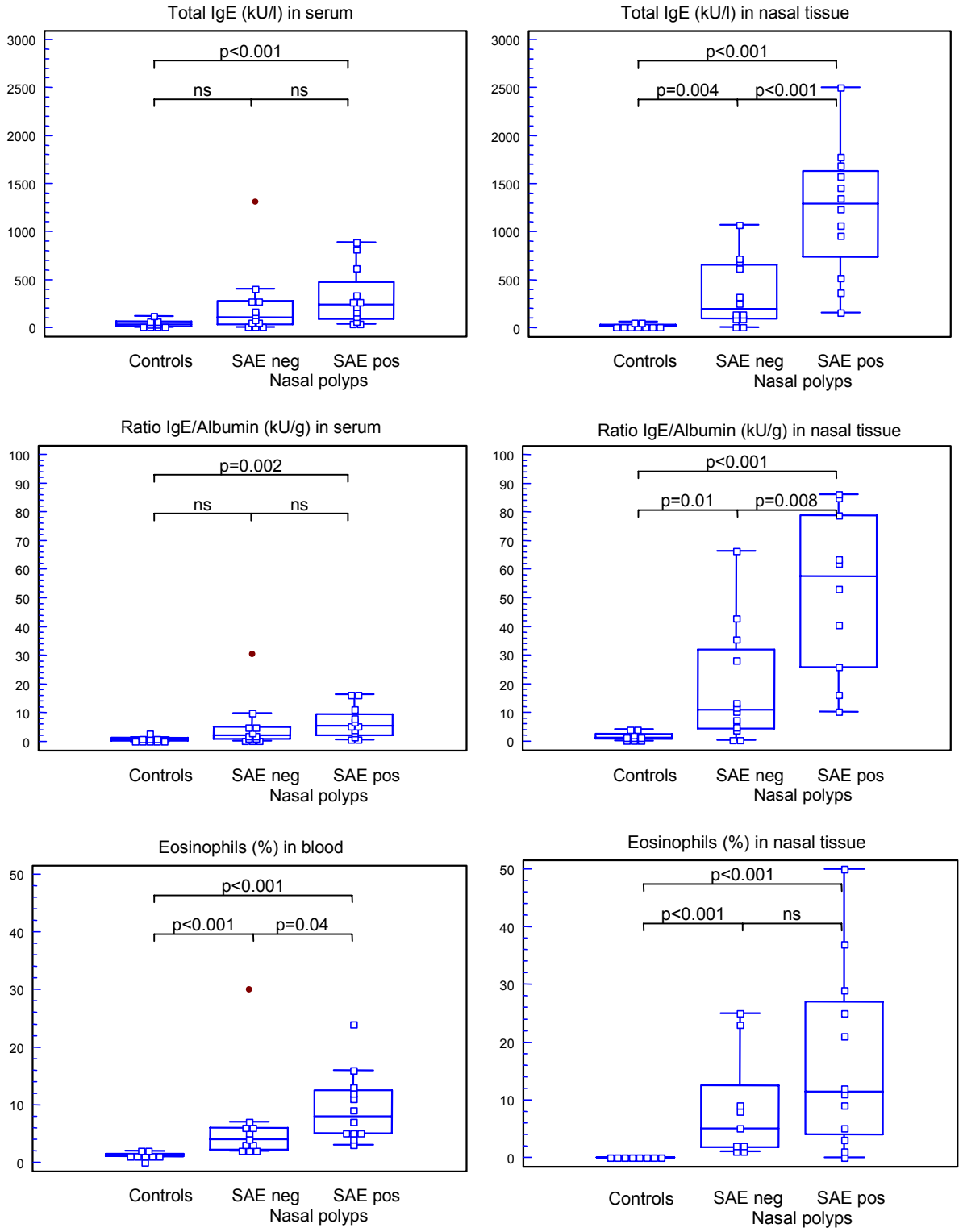

***Figure 1**: Comparison of total IgE concentrations, IgE/albumin ratio's and eosinophil percentages in serum and nasal tissue of controls, NP patients without or with IgE antibodies to SAE (NP+SE). The Box-and-whisker plot represents the median and the 10th, 25th, 75th, 90th percentile. Statistical analyses performed by the Mann Whitney U-test (NS = not significant).*

The nasal vestibule is the ecologic reservoir for *S. aureus, with* about 25% of the population being permanent carriers[19]. A large proportion of the population (approximately 60%) harbours *S. aureus* intermittently, and the strains change with varying frequency [19]. Notably, only a minority of people (approximately 20%) almost never carries *S. aureus* [19]. The differences could be due to host factors and/or to competition between members of the nasal flora (e.g. *Corynebacterium spp.*)[20]. Indeed, a higher incidence of *S. aureus* colonisation of skin or mucous membranes may be due to injuries, abnormal leukocyte function and inflammation (e.g. atopic dermatitis), viral infections (e.g. influenza), metabolic abnormalities (e.g. diabetes mellitus and uraemia) and miscellaneous other conditions (e.g. malnutrition, old age, malignancies, etc). Whereas nearly all strains of *S. aureus* produce enzymes and haemolysins that contribute to their pathogenicity, it has been generally accepted that only some strains produce superantigens. However, recent data using multiplex PCR-DNA enzyme immunoassays and considering both, the newly described SAE genes (SEG, SEH, SEI, and SEJ) and the classical toxins (SEA, SEB, SEC, SED, SEE and TSST), demonstrated that the overall rate of toxin gene-positive isolates reached 73.0%. More than half of the isolates (55.0%) harboured the combination of SEG and SEI, whereas other toxin genes amplified were TSST (20.3%), SEA (15.9%), and SEC (11.2%)[20]. In the present study we demonstrate a significantly higher incidence of nasal colonisation with *S. aureus* in NP patients (71%) than in controls (25%). However, only in 50% of polyp tissues, IgE antibodies to classical SAE´s were detectable. These data probably underestimate the impact of enterotoxins, as contact to only the classical, but not to the newly described enterotoxins was studied due to the current lack of validated tests for IgE antibodies to the latter.

Detailed analysis of Table II reveals two patterns of IgE expression: “the allergic type” and “the polyclonal type” that can be found either isolated or combined. The “allergic” type of IgE expression is characterized by increased concentrations of total IgE and IgE antibody specificities in nasal tissue that correspond those in serum and to the skin prick test results. In clear contrast, the “polyclonal” IgE expression is a local process and IgE antibodies found in polyp tissue are only partially reflected in serum of the same patients and are independent of the skin prick results. Notably, this polyclonal expression pattern is associated with a hyper-immunoglobulinemia and only a small fraction of the total can be explained by IgE antibodies. Polyclonal expression was described in 16/24 NP tissues and was associated with IgE antibodies to SAEs in 12 cases, indicating that other than the classic enterotoxins might have acted as superantigens (most probably from *S. aureus*, but possibly also from other

Table II. IgE antibody expression in serum and nasal polyp tissue

		SERUM											*NASAL TISSUE*										
Diagnose	**Skin Prick Test**	**Total IgE (kU/l)**	**IgE (kU_A/l)**				**IgE ab to s aureus enterotoxins (kU_A/l)**						**Total IgE (kU/l)**	**IgE (kU_A/l)**				**IgE ab to s aureus enterotoxins (kU_A/l)**					
			gx1	**hx2**	**mx2**	**tx9**	**SEA**	**SEB**	**SEC**	**SED**	**SEE**	**TSST1**		**gx1**	**hx2**	**mx2**	**tx9**	**SEA**	**SEB**	**SEC**	**SED**	**SEE**	**TSST1**
NP	negative	13,3	/	/	/	/	/	/	/	/	/	/	6,1	/	/	/	/	/	/	/	/	/	/
NP	negative	8,8	/	/	/	/	/	/	/	/	/	/	10,5	/	/	/	/	/	/	/	/	/	/
NP	negative	78,5	/	/	/	/	/	/	/	/	/	/	97,1	/	/	/	/	/	/	/	/	/	/
NP+Asth	negative	20,2	/	/	/	/	/	/	/	/	/	/	141,3	/	/	/	/	/	/	/	/	/	/
NP	negative	277,0	/	/	/	/	/	/	/	/	/	/	145,3	/	/	/	/	/	/	/	/	/	/
NP	negative	48,5	/	/	/	/	/	/	/	/	/	/	97,4	/	/	4,17	/	/	/	/	/	/	/
NP	negative	50,2	/	/	/	/	/	/	/	/	/	/	250,7	/	/	5,78	5,01	/	/	/	/	/	/
NP	negative	157,0	/	/	/	/	/	/	/	/	/	/	160,1	/	/	5,88	4,57	/	/	/	/	/	4,03
NP+Asth	negative	278,0	/	/	/	/	/	/	/	/	/	/	1776,5	4,62	4,29	12,21	9,13	/	/	/	/	/	5,94
NP	negative	114,0	/	/	/	/	/	/	/	/	/	/	957,4	6,66	6,22	11,68	9,39	4,59	/	/	/	/	4,91
NP	negative	887,0	/	/	/	/	/	0,44	0,54	/	0,49	/	1455,0	3,91	4,02	6,95	5,21	4,85	/	/	/	/	/
NP	negative	265,0	/	/	/	/	/	2,36	2,54	/	1,05	/	1233,7	7,13	5,92	13,71	9,65	4,61	11,19	12,17	6,03	18,97	7,46
NP	hdm	163,5	/	3,11	/	/	/	/	/	/	/	/	322,9	nd	18,85	4,30	4,30	/	/	/	/	/	/
NP	hdm	130,0	/	1,72	/	/	/	/	/	/	/	/	620,4	4,50	6,92	7,03	5,93	/	/	/	/	/	/
NP	gx, tx	273,0	1,51	/	0,92	3,39	/	/	/	/	/	/	718,1	5,25	nd	9,51	10,71	/	/	/	/	/	/
NP	hdm, gx	1310,0	0,59	34,70	/	/	/	/	/	/	/	/	688,3	4,56	111,81	4,34	3,91	/	/	/	/	/	/
APA	hdm	402,5	/	0,38	/	/	/	/	/	/	/	/	1073,7	4,69	4,14	6,43	4,25	/	/	/	/	/	/
NP	Gx	64,8	4,71	/	/	/	/	/	/	/	/	/	514,7	37,10	6,59	14,71	11,19	5,38	/	/	/	/	6,80
NP	hdm, tx	815,0	/	1,17	/	26,60	0,39	0,40	/	/	0,44	0,84	1686,8	6,16	7,92	10,46	23,99	4,51	/	/	/	/	4,18
NP+Asth	Gx	37,5	0,54	/	/	/	/	/	/	/	/	/	1348,2	9,84	6,34	nd	nd	4,05	/	/	3,94	/	/
NP+Asth	hdm	38,5	/	0,38	/	/	/	/	/	/	/	/	365,2	43,01	/	5,72	/	/	8,14	7,70	4,40	/	/
NP+Asth	hdm, gx	331,0	0,46	18,10	/	/	/	/	/	/	/	/	1066,5	5,78	45,91	12,54	10,47	7,96	4,69	/	/	6,22	14,18
APA	hdm, gx	615,0	5,20	3,06	/	/	/	/	/	/	/	/	2500,0	66,81	11,21	12,40	10,23	/	/	4,24	/	4,46	/
APA	hdm, gx	217,0	1,38	6,35	/	0,97	/	/	/	/	/	/	1577,7	7,65	15,75	nd	nd	4,77	/	/	/	8,43	6,65

Legends: NP: nasal polyps; NP+Asth: nasal polyps with concomitant asthma; APA: aspirin hypersensitivity, polyps and asthma syndrome; hdm: house dust mite; gx: grass mix; hx: house dust mite mix; mx: moulds mix; tx: trees mix; nd: not done; /: below detection level

germs). Furthermore, 12 of the 16 polyp patients showed a combined expression pattern, with the polyclonal type of IgE expression in addition to an existing allergy.

The earliest quantitative studies on humoral responses to antigenic stimuli showed that the amount of measurable specific antibody produced often only represented a small fraction of the newly synthesized total [16]. Parasites, viruses, fungi and bacteria can obliterate specific immune responses by triggering the machinery of polyclonal lymphocyte responses, thus resulting in a lack of specificity of antibodies or T-cell responses to the microbial agents [21]. There is convincing evidence that cytokines released form activated T-cells may substitute non-specifically for TH cells in stimulating B cells[22]. Two types of polyclonal stimuli exist that can trigger B lymphocyte proliferation and differentiation in the absence of antigen: (i) those derived from microbial products, such as lipopolysaccharide or unmethylated single-stranded DNA motifs (CpG oligonucleotides), which stimulate B cells via TLR4 (Toll-like receptor 4) and TLR9, respectively; and (ii) T cells activated by a third-party antigen, which stimulate B cells in a noncognate fashion via CD40 ligand [23] and cytokine production, referred to as bystander help. Recent data indicated that memory B cells have two response modes. In the antigen-dependent mode, they undergo a massive expansion and differentiation towards short-lived plasma cells [24;24;25]. In contrast, in the polyclonal mode, all memory B cells respond to microbial stimuli by undergoing continuous proliferation and differentiation. In this way, a constant level of plasma cells and immunoglobulin in serum could theoretically be maintained throughout a human life-span [26]. Because this mechanism is non-specific, it would act indiscriminately to maintain the broad spectrum of antibody specificities generated during the antigen-driven immune response as well as non-antibody active immunoglobulin molecules. The protection is relative to a particular pathogen or toxin and may be mediated by pre-formed antibodies, by rapid secondary responses or by a combination of both [24;25]. However, in the case of powerful bacterial toxins such as superantigens, protection relies entirely on pre-existing neutralizing antibodies since there is no time to mount a secondary response to achieve protection.

The high IgE concentrations in polyp tissue could be preferentially derived from the serum, as an extravasation and deposition of serum proteins in polyp tissue has been shown [1]. However, IgE has been found in NP tissue enriched over serum suggesting that IgE synthesis occurs locally in the mucosa. In mucosal tissues of allergic rhinitis and asthma patients, mRNA for the ε-chain of IgE was associated with a significant proportion of B-cells by using *in situ* hybridization [27-30]. This supports the hypothesis of local IgE synthesis since mRNA expression would be required for synthesis and secretion of IgE. Using immunohistochemical

methods, different research groups have reported varying success in visualizing IgE protein associated with B-cells in nasal mucosa. Recently, however, KleinJan et al. visualized IgE protein in association with both B-cells and plasma-cells in the nasal mucosa of patients with allergic rhinitis [31]. In our present study we describe two prominent IgE positive areas: first, the follicular structures, which are characterised by an organisation of T-cells (CD3) and B-cells (CD20), found in 6 out of 24 NP samples; second, in all polyps we found diffuse lymphoid accumulations that contain T-cells (CD3) and plasma-cells (CD38). B-cell foci have been described before in nasal polyp tissue [32], associated with a reduced expression of activin A in those areas, which points to a reduced suppression of plasma cells development [32;33]. There is increasing evidence that SAEs can directly affect the frequency and activation of the B-cell repertoire. Functional studies in B-cells have shown that *S. aureus* protein A induces proliferation of these cells, following signal transduction involving protein kinase C (PKC), mitogen-activated protein kinase (MAP kinase), serum responsive factor (SRF) and Bcl-2 gene expression [34]. Studies with TSST-1 have shown that this staphylococcal superantigen may play an important role in the modulation of allergic disease, since it can augment isotype switching and synthesis of IgE, both *in vitro* [23] and *in vivo,* in a SCID mouse model [35]. Although TSST-1-induced activation of B-cells *in vitro* is indirect and dependent on increased expression of CD40 ligand on T-cells, a more recent study has also provided evidence for a direct effect by demonstrating TSST-1-induced expression on B-cells of B7.2 [15], a molecule that has been shown to enhance Th2 responses and to be involved in IgE regulation.

An augmented local synthesis of IgE may, under appropriate circumstances, increase allergic reactivity, but when it is excessive, it has been suggested to actually suppress specific reactivity by saturation of Fcε receptors on mast cells through polyclonal IgE and/or an inhibition of specific antibody synthesis to environmental allergens by the polyclonal response [36]. These mechanisms could explain why nasal symptoms and markers of nasal mucosal inflammation did not increase in relation to natural seasonal allergen exposure in highly ragweed-allergic patients with polyps [37] and why nasal provocation in nasal polyps is largely unsuccessful although elevated IgE levels are present [38;39]. However, the backside of this local polyclonal IgE production may be the permanent triggering of the IgE-mast cell-FcεRI cascade that maintains polyp growth[40].

Conclusion

We demonstrate the organization of secondary lymphoid tissue in nasal polyposis with polyclonal B-cell activation and hyper-immunoglobulinemia E associated with the presence

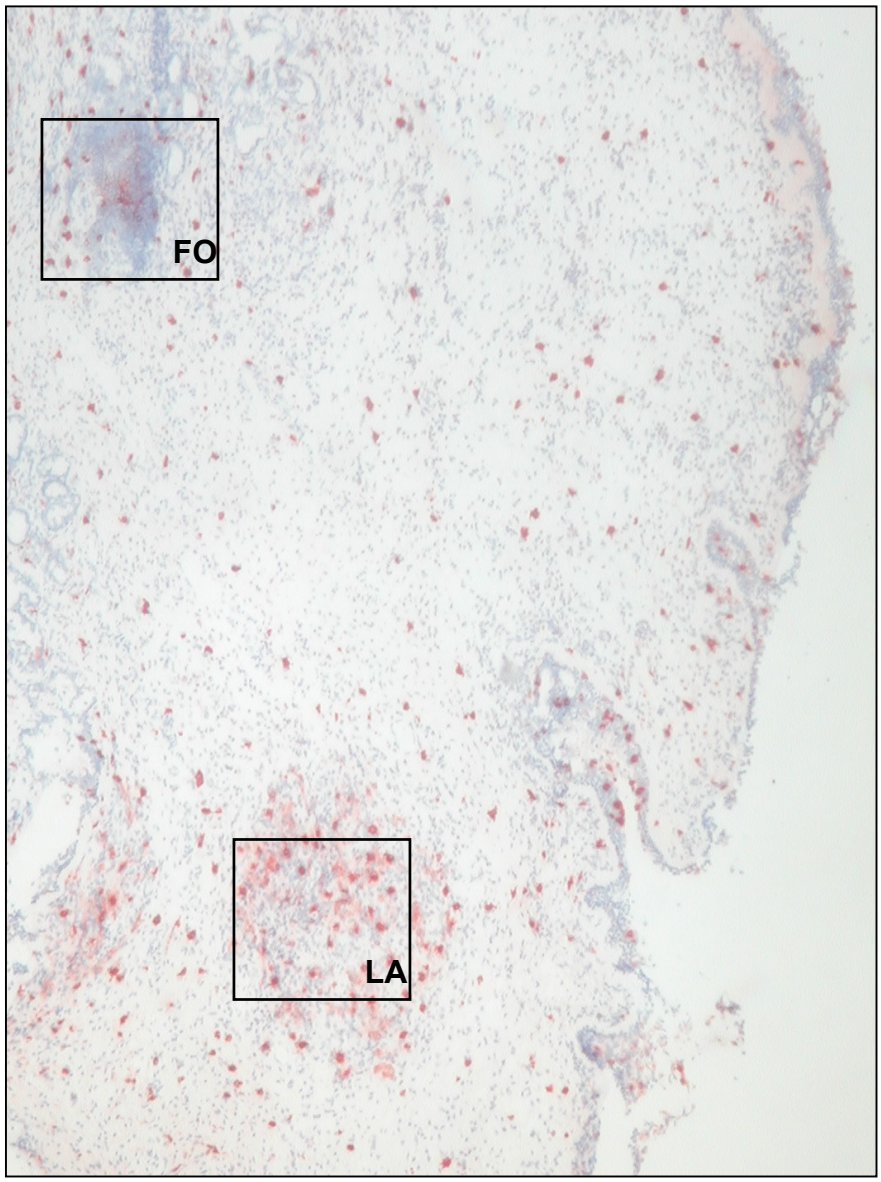

Figure 2 *Nasal polyp tissue stained for IgE demonstrating a follicular structure (FO) and lymphoid accumulations (LA) (x10)*

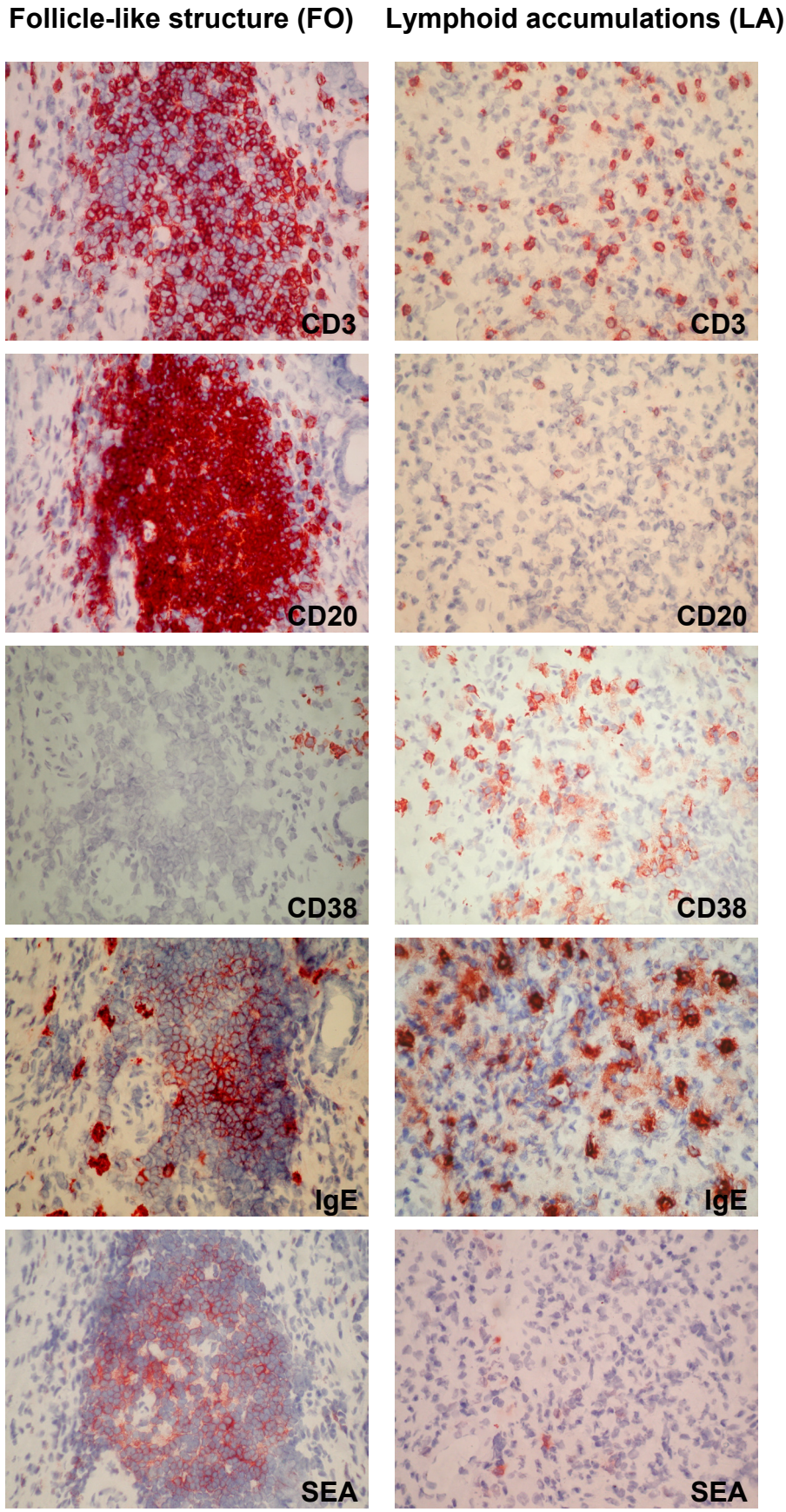

Figure 3: Follicular structures and lymphoid accumulations in nasal polyp tissue are stained for CD3, CD20, CD38, IgE, and SAE binding (x40).

of locally formed IgE antibodies to staphylococcal enterotoxins, and increased eosinophilic inflammation in a relevant subgroup of nasal polyp patients. The increased prevalence of colonization with *Staphylococcus aureus* and subsequent chronic microbial stimulation in NP may be a causative or disease modulating factor, being dependent upon a former damage of the mucosa by unrelated pathomechanisms.

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CHAPTER 7

ENHANCED SOLUBLE INTERLEUKIN-5 RECEPTOR ALPHA EXPRESSION IN NASAL POLYPOSIS.

ENHANCED SOLUBLE INTERLEUKIN-5 RECEPTOR ALPHA EXPRESSION IN NASAL POLYPOSIS.

Gevaert P, Bachert C, Holtappels G, Novo CP, Van Der HJ, Fransen L et al.

Allergy 2003; 58(5):371-379.

ABSTRACT

BACKGROUND: Alternative splicing of the IL-5Rα-subunit leads to the generation of a signaling, membrane-anchored (TM) isoform, or a secreted (SOL), antagonistic variant. Given the key role of IL-5 in eosinophil function, we investigated SOL IL-5Rα expression pattern in an eosinophil-associated disease such as nasal polyposis (NP).

METHODS: SOL IL-5Rα ELISA and quantitative real-time PCR were established and applied in serum, nasal secretion and nasal tissue of controls (n=12) and NP patients (n=42) with or without asthma.

RESULTS: Analysis of serum, nasal secretion and nasal tissue samples revealed that SOL IL-5Rα protein concentrations were significantly increased in NP versus control tissue. Within the NP group, there was a significant up-regulation of SOL IL-5Rα in patients with systemic airway disease. These findings were confirmed at the mRNA level, using an optimised real-time RT-PCR procedure.

CONCLUSIONS: This report demonstrates SOL IL-5Rα transcript and protein up-regulation in nasal polyposis. SOL IL-5Rα differentiates nasal polyps with or without concomitant asthma. Since SOL IL-5Rα is strongly up-regulated for disease and has antagonistic properties *in vitro*, our studies shed new light onto the mechanisms of specific immuno-modulatory therapies, such as anti-IL-5.

INTRODUCTION

Interleukin-5 is a haematopoietic growth factor essential for eosinophil development, activation and survival. The eosinophil is regarded as an important effector cell in various chronic pathologies including asthma, allergic rhinitis and nasal polyposis. However, the recent failure to demonstrate clinical efficacy of humanized antibodies directed against IL-5 in human asthma trials, has led to a re-evaluation of the role of eosinophils [1]. A better understanding of the biology of IL-5 and the regulation of its receptors seems to be mandatory to understand the effect of anti-IL-5 treatment in eosinophil-associated diseases.

Nasal polyposis is an excellent model for eosinophil-associated diseases, as 80 to 90% of bilateral nasal polyps are characterized by abundant eosinophilic infiltration. Nasal polyps are swellings of the lamina propria mucosa, a pathology that is frequently associated with asthma and often treated by surgical removal of polyps that block airways, facilitating the use of this tissue for investigation. High amounts of IL-5 can be detected at the mRNA as well as at the protein level in NP [2;3]. The highest levels of IL-5 protein were observed in polyp homogenates of patients with generalized eosinophilic diseases such as asthma and aspirin sensitivity [2;4;5].

The biological signal from IL-5 is mediated through a receptor consisting of a specific IL-5-binding α-chain, and a signal-transducing common β-chain, which is shared with the receptors for IL-3 and GM-CSF [6;7]. When the β-chain associates with the specific IL-5-binding α-chain, a high affinity complex is formed [8]. In contrast to the pan-haemopoietic βc-chain, the IL-5Rα receptor seems to be expressed on a restricted range of cell types in humans, principally eosinophils, basophils, and their precursors. However, recent publications have demonstrated eosinophil-independent airway hyperresponsiveness and IL-5Rα gene expression in human bronchus smooth muscle [9;10].

The IL-5 receptor α-subunit (IL-5Rα) can appear in either a membrane-anchored (TM) active form or a soluble (SOL) variant with antagonistic properties *in vitro* [6;11]. The various expression patterns of these forms are regulated at the transcriptional level by alternative splicing [12]. The antagonistic properties of SOL IL-5Rα have been demonstrated in binding assays, eosinophil differentiation assays [11] and in nasal tissue explants [13]. It is suggested that eosinophils are able to control their responsiveness to IL-5 by regulated expression of the IL-5Rα isoforms. Surprisingly, mature, circulating eosinophils predominantly express the SOL IL-5Rα transcript (own data) [11], which raises the question whether this reflects a role in vivo. However, until now the human SOL IL-5Rα protein could not be demonstrated in clinical samples or in supernatants of cultured eosinophils.

The aims of this study were to develop specific ELISAs for studying the SOL IL-5Rα isoform at the protein level and to set up a PCR-based approach to monitor the expression of the SOL IL-5Rα mRNA, and to test the diagnostic potential of SOL IL-5Rα in eosinophil-associated disease such as nasal polyposis.

METHODS

Patients

We collected serum, nasal secretions and nasal polyp tissue of 42 nasal polyp patients (13 F/ 29 M; mean age 42.6 years, range 22-78 years) during routine sinus surgery at the Department of Otorhinolaryngology at the Ghent University Hospital, Belgium. Bilateral polyposis was diagnosed based on history, clinical examination, nasal endoscopy, and sinus CT-scan. A history of asthma was reported in 18 nasal polyp patients, with nine subjects additionally demonstrating a history of aspirin sensitivity. Twenty-one patients were atopic, as indicated by positive coetaneous skin prick test responses to common aeroallergens. Subjects were excluded if they had received any steroids or antibiotics during the month before the surgery.
Control serum, nasal secretions and nasal tissue (inferior turbinates) were obtained from twelve subjects during routine septal surgery. All control subjects (5 F/ 7 M; mean age 33.6 years, range 18-71 years) were skin prick test negative, in generally good health, and none had a history of nasal or sinus disease, allergic disease (asthma, rhinitis or dermatitis), upper respiratory tract infection in the previous month, use of any intranasal medications, decongestants, antihistamines or oral steroids. The ethics committee of the Ghent University Hospital approved this study and a written informed consent was obtained from all subjects.

IL-5Rα ELISA

Production of monoclonal antibodies

For the production of monoclonal antibodies (mAbs) against SOL IL-5Rα, Balb/C mice were immunized with 30μg Baculovirus-secreted human IL-5Rα in CFA/IFA. Monoclonal antibodies were generated by fusing spleen cells of these immunized mice with SP2/0 myeloma cells. Stable hybridomas were obtained by screening on SOL IL-5Rα coated plates. Eleven hybridomas that produced specific antibodies against SOL IL-5Rα were selected and were classified into 4 complementation groups. All these hybridomas were grown under serum-free conditions. The supernatants were harvested and purified on Protein A Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden).

Characterization of the monoclonal antibodies

A chequer board experiment was set up whereby each mAb was cross-paired with the other mAbs in an ELISA format. Briefly, microtiter plates were coated with SOL IL-5Rα. For this, sub-saturating concentrations for each biotinylated antibody were determined first. Then competition between biotinylated and unlabeled antibodies for binding to coated SOL IL-5Rα was performed.

FDC-P1-Cl7 originated by transfection of the FDC-P1 cell line to stably express the TM IL-5Rα at high levels. Cells were incubated with mAbs at 2µg/ml, stained with Alexa488 conjugated anti-mouse Ig (Molecular Probes, Leiden, The Netherlands) and analysed by FACS. Non-transfected FDC-P1 cells were used as a negative control.

Development of an ELISA for soluble IL-5Rα

Very careful assessment of antibody selection resulted in the development of several different ELISA's. Based on background, sensitivity and reproducibility in human samples, a sandwich ELISA for SOL IL-5Rα detection was selected with combinations of mAbs from group 2 (15B8) and 4 (1G3) (Innogenetics, Ghent, Belgium).

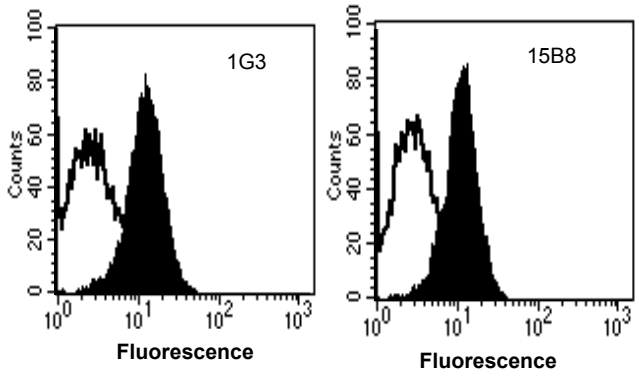
Microtiter plates (Maxisorb, Nunc Denmark) were coated overnight with 1G3 at 2 µg/ml. The plates were washed once with washing buffer (PBS + 0.02% Tween20) and blocked for 30 minutes with blocking buffer (PBS + 0.1% casein). The plates were incubated for 2h at room temperature on a shaker with the samples or standards diluted in blocking buffer. A cellular extract of COS cells transfected with IL-5Rα was used as standard. The concentration of the extract was determined with purified Baculo IL-5Rα as standard. After washing, the plates were incubated for 1h with the biotinylated mAb 15B8 at 0.5µg/ml in blocking buffer. The plates were washed and incubated for 30 minutes with HRP-conjugated streptavidin (Jackson, West Grove, Pennsylvania, US) at 100 ng/ml. After washing, the plates were incubated with TMB (Roche, Brussels, Belgium) in phosphate-citrate buffer at pH 4.3 for 30 minutes. The reaction was stopped by acidification and the plates were read at 450-620 nm.

In vitro bioassay for hIL-5

Ba/F3-hIL-5R-Luc originated by transfection of the Ba/F3 cell line to stably express the IL-5Rα. Additionally, luciferase cDNA, driven by a constitutive CMV promoter, was co-transfected and stably integrated into the cell line. Chemiluminescent luciferase detection correlated with proliferation that was dependent on the hIL-5 concentration.

To evaluate the neutralizing capacity of the mAbs the cells were incubated with a low concentration of hIL-5 alone or together with a varying concentration of the mAbs. After 48

hours of incubation, the cells were assayed for luciferase by chemiluminescence measurement.



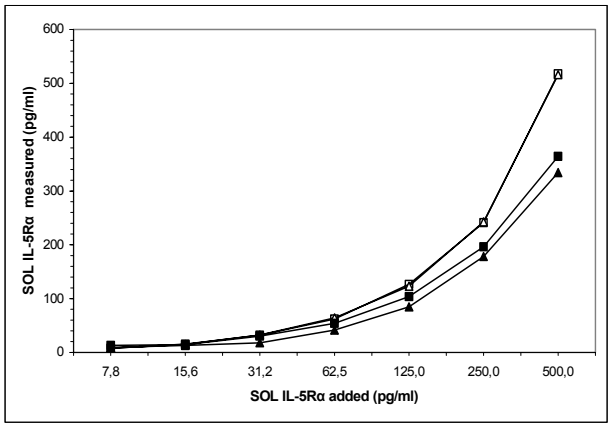


Figure 1: Characterization of monoclonal antibodies and SOL IL-5Rα ELISA

a) FACS analysis using mAb 1G3 in the upper – and 15B8 in the lower histogram. Open and filled curves represent control FDC-P1 and hIL-5Rα-expressing FDC-P1-Cl7 cells, respectively.

b) To exclude interference of IL-5 with the SOL IL-5Rα ELISA, standard curves (7.8 to 500 pg/ml SOL IL-5Rα) were run in blocking buffer (PBS + 0.1% casein) in the absence (□) or presence of 250 pg/ml rhIL-5 (△). A similar experiment was carried out in serum that was diluted 1/5 in blocking buffer in the absence (■) or presence of 250 pg/ml rhIL-5 (▲).

Characterization of the IL-5Rα ELISA

The reproducibility of the SOL IL-5Rα ELISA was assessed in five different serum samples by serial testing (20 times). The recovery in the ELISA was determined by spiking 400 pg/ml of recombinant IL-5Rα in seven different serum samples. To exclude possible interference of IL-5 in ELISA for SOL IL-5Rα the standard curve (in blocking buffer; PBS + 0.1% casein) was run both with and without the addition of 250 pg/ml rhIL-5 (R&D Systems, Minneapolis, USA). A similar experiment was carried out on 5 different serum samples. All serum samples were diluted 1/5 in blocking buffer (PBS + 0.1% casein) and a 2-fold dilution series ranging from 500 pg/ml to 7.8 pg/ml SOL IL-5Rα was added. Further, blocking experiments were carried out on three serum and three tissue samples by pre-incubation with 2 µg/ml capture antibody (1G3) for two hours at 4°C.

Measurement of IL-5 Rα protein concentrations

All freshly obtained samples were immediately processed, separated and stored in aliquots at – 80°C until analysis. Blood samples were allowed to clot at room temperature for 20-30 minutes, centrifuged at 1500 *g* for 10 minutes at 4°C. Nasal secretions were obtained by placing sinus packs (IVALON® 4000 plus) in both nasal cavities for exactly 5 minutes. The quantity of secretions collected was determined by comparing the weight of the sinus pack before and after insertion. In order to mobilize the nasal secretions out of the sinus pack, three millilitres of 0.9% NaCl solution were added to the tube, which was stored at 4°C for two hours. The sinus pack was then placed into the shaft of a syringe (placed into another tube) and centrifuged at 1500 g for 10 minutes at 4°C to recover all fluid. Nasal tissue specimens were weighed, and 1 ml of 0.9% NaCl solution was added for every 0.1 g of tissue. The tissue was then homogenized with a mechanical homogeniser (B. Braun Melsungen, Germany) at 1000 rpm for 5 minutes on ice as described previously [4;5]. After homogenisation, the suspensions were centrifuged at 1500 *g* for 10 minutes at 4°C, and the supernatants separated and stored at – 80°C until analysis.

All samples were assayed by a research ELISA for SOL IL-5Rα (described above, Innogenetics, Gent, Belgium) and IL-5 (R&D Systems, Minneapolis, USA). For comparison to an established marker, eosinophil cationic protein (ECP) (3) was measured by the Uni-CAP system (Pharmacia & Upjohn, Uppsala, Sweden).

IL-5Rα Real Time PCR

As a standard, we used PCR products generated from plasmids containing the cDNA sequences for SOL hIL-5Rα. The cDNA sequence of the SOL isoform was amplified using a forward primer complementary to a common sequence of hIL-5Rα (nts 1033 to 1056) and a SOL hIL-5Rα specific reverse primer (nts 1279 to 1298). All primers were designed using the Primer Express 2.0 Software (Applied Biosystems, Foster City, US) and purchased from Invitrogen. The PCR resulted in a unique band that was purified by using the PCR Gel extraction QIAquick (Qiagen, Hilden, Germany) spin columns. The purified fragments were quantified using PicoGreen reagent (Molecular Probes, Leiden, The Netherlands). Equimolar 10-fold dilutions of the PCR products were used to generate standard curves.

Measurement of SOL IL-5 Rα mRNA in blood and nasal tissue

RNA was isolated from snap frozen blood and nasal tissue of 7 controls and 20 NP patients using the Rneasy Kit (Qiagen, Hilden, Germany). Total RNA was quantified using RiboGreen

Kit (Molecular Probes) and 0.5 μg was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen) and random hexamers (Amersham Pharmacia Biotech, Buckinghamshire, UK). cDNA equivalent to 25 ng was used to perform the Real Time PCR. The Real Time amplifications were performed using the 1X SYBR Green I Mastermix (Qiagen, Hilden, Germany) and a set of primers including a common and a specific primer for SOL hIL-5 Rα. All the PCRs were performed in a 5700 SDS thermal cycler (Applied Biosystems, Foster City, US). Each sample was tested in duplicate. The quantity of each amplicon was calculated from the values of each standard curve and normalized by the quantities obtained for β-actin transcripts [14].

Statistical analysis

Data are expressed as median and interquartile range (IQR). When comparisons were made between groups, significant between-group variability was first established using Kruskal-Wallis test. The Mann Whitney U-test was then used for between-group (unpaired) comparison. Spearman rank correlation coefficient (r) was used to assess the relationships between the parameters. The intra- and inter-assay precision was expressed as coefficient of variation (%CV). The ability of a test to discriminate diseased cases from normal cases was evaluated using Receiver Operating Characteristic (ROC) curve analysis. The cut-off values corresponding with the highest accuracy (minimal false negative and false positive results) were selected and the subsequent sensitivity and specificity were used. Further, comparison of ROC curves was performed to test the statistical significance difference between the areas under two ROC curves. P-values less than 0.05 were considered statistically significant.

RESULTS

IL-5Rα ELISA

Careful assessment of antibody selection from different complementation groups resulted in several useful ELISA's. Based on background, sensitivity and reproducibility in human samples, one sandwich ELISA consisting of two monoclonal antibodies (1G3/15B8biot) for detection of SOL IL-5Rα was selected.

These antibodies were further characterized by FACS analysis and using an IL-5-specific bioassay. As shown in Figure 1a, strong staining of cells expressing the IL-5Rα is observed for both antibodies. In an *in vitro* bioassay on Ba/F3-hIL-5R-Luc cells, 15B8 was shown to neutralize IL-5 activity, whereas all other antibodies (including 1G3) were non-neutralizing.

Because 15B8 is a neutralizing antibody, the potential non-capture of an IL-5 occupied soluble receptor could bias the results of the ELISA. However, competition experiments between IL-5 and 15B8, analysed by FACS, revealed that at equimolar concentrations 15B8 was able to chase IL-5 from its receptor, even when IL-5 was added 1 hour before 15B8. This indicates that the possibility to underestimate the level of receptor, in samples with IL-5 present, is low or negligible, particularly when 15B8 is used as secondary antibody. Furthermore, standard curves in buffer and in human serum were not affected by addition of IL-5, excluding cross interference by endogenous IL-5 (Figure 1b).

The established ELISA reached a sensitivity of 8 pg/ml for detection of SOL IL-5Rα. The intra-assay precision was assessed in five different serum samples by serial testing (20 times) in one run. The coefficient of variation (%CV) ranged from 3.1% to 7.0%. The inter-assay precision was determined in five different serum samples by determinations in 20 separate runs, resulting in a %CV of less than 10% and therefore acceptable. The average recovery - after addition of 400 pg/ml of recombinant SOL IL-5Rα in human serum - was 95% with a range between 83% and 103%. Addition of 2 μg/ml capture antibody (1G3) significantly blocked (86 to 97%) SOL IL-5Rα detection in human serum samples.

SOL IL-5Rα protein expression is increased in nasal polyposis

Analysis of SOL IL-5Rα, IL-5, and ECP in supernatants prepared from tissue homogenates demonstrated that the concentrations of these mediators were significantly higher in NP tissue, compared to normal control tissue (Table I). Within the NP group, there was a significant increase of SOL IL-5Rα in patients with concomitant asthma compared to patients without asthma. SOL IL-5Rα/IL-5 ratio's were calculated in all NP samples and 3 to 1200 times higher SOL IL-5Rα levels were present compared to IL-5 concentrations. Furthermore, within the NP group SOL IL-5Rα/IL-5 ratios were significantly higher in NP tissue with concomitant asthma (Figure 2a). Increased eosinophil percentages were found in NP tissue with and without concomitant asthma compared to control tissue (Table I). Assessment for any correlations between SOL IL-5Rα and eosinophilic markers demonstrated that in NP tissue, SOL IL-5Rα significantly correlated with concentrations of IL-5 ($r = 0.405$; $p = 0.006$), ECP ($r = 0.843$; $p < 0.001$), and percentage of eosinophils ($r = 0.536$; $p < 0.001$).

With nasal secretions we aimed to have a less invasive and easy to use technique in comparison with nasal biopsy to monitor SOL IL-5Rα concentrations. Within the nasal polyp group SOL IL-5Rα and ECP concentrations were higher in secretions of NP patients with asthma than those without asthma (Table I). Using 2341 pg/ml SOL IL-5Rα as best cut-off

value, the sensitivity was 83% and specificity was 100% for the differentiation of NP patients with or without asthma, whereas with a best cut-off value for ECP of 1546 µg/l, a sensitivity of 44% and specificity of 95% could be reached (Figure 3a). Comparison of areas under the ROC curves for SOL IL5Rα and ECP were significantly different in favour of SOL IL-5Rα ($p = 0.023$) (Figure 3b). A significant correlation between SOL IL-5Rα concentrations in nasal secretions and nasal tissue confirms that sampling of nasal secretions might be a valuable alternative for biopsies for SOL IL-5Rα monitoring (Figure 3c).

In serum we found increased SOL IL-5Rα concentrations in NP patients with concomitant asthma compared to controls and NP patients without asthma (Table I). Whereas SOL IL-5Rα could be measured in all serum samples, IL-5 protein concentrations were only measurable in four subjects (all with concomitant asthma). SOL IL-5Rα concentrations correlated significantly ($r = 0.595$; $p = 0.005$) between serum and nasal tissue samples.

Table I: SOL IL-5Rα expression in nasal tissue and serum

Nasal Tissue	*Controls (n=12) Median (± IQR)*	*versus*	**Nasal polyps** *(n=24) Median (± IQR)*	*versus*	*Nasal polyps & Asthma (n=18) Median (± IQR)*	*versus controls*
Sol IL-5 Rα (pg/ml)	885 (43-301)	p = 0.004	2496 (1244-7287)	p < 0.001	26096 (17640-34287)	p < 0.001
IL-5 (pg/ml)	All BDL	p = 0.002	100 (43-301)	p = 0.04	230 (112-420)	p < 0.001
Ratio Sol IL-5 Rα / IL-5	/	/	21 (11-41)	p = 0.002	95 (32-284)	/
ECP (µg/l)	220 (94-306)	p < 0.001	3268 (856-7036)	p < 0.001	15737 (11331-28544)	p < 0.001
Eosinophils (%)	0.3 (0-0.7)	p = 0.001	5.3 (1.2-21.72)	NS	20.7 (8.7-24.6)	p < 0.001
Nasal Secretion						
Sol IL-5 Rα (pg/ml)	519 (301-595)	p = 0.07	667 (430-1135)	p < 0.001	7568 (3389-12869)	p < 0.001
IL-5 (pg/ml)	All BDL	/	15/24 BDL	NS	118,72 (30-172)	p = 0.006
ECP (µg/l)	139 (45-300)	p = 0.03	516 (200-853)	p = 0.02	1064 (389-2243)	p < 0.001
Serum						
Sol IL-5 Rα (pg/ml)	286 (237-426)	NS	369 (282-562)	p = 0.01	759 (475-1030)	p = 0.005
ECP (µg/l)	12 (11-16)	NS	25 (18-29)	NS	43 (23-65)	p = 0.008
Eosinophils (%)	0.9 (0.4-1.8)	p = 0.002	4.8 (2.6-7.1)	p = 0.007	9.4 (6.8-16.9)	p < 0.001

Median and interquartile ranges (IQR) of SOL IL-5Rα, IL-5, ECP concentrations and eosinophil percentages are compared in nasal tissue, nasal secretions and serum of controls and nasal polyp patients with or without asthma. BDL= Below detection level. NS=Not significant.

SOL IL-5Rα mRNA Real-Time PCR

Analysis of peripheral blood and nasal tissue samples revealed that relative amounts of SOL IL-5Rα isoform (copies of mRNA/µl), were significantly higher in samples of patients with NP compared to controls. Within the NP group, there was a significant increase of SOL IL-5Rα mRNA in NP tissue from patients with concomitant asthma compared to patients without asthma. A similar tendency was observed for SOL IL-5Rα in peripheral blood (Figure 4). There was a significant correlation between SOL IL-5Rα in peripheral blood and nasal tissue ($r = 0.668$; $p = 0.006$). Assessment for any correlations between SOL IL-5Rα mRNA isoform expression and eosinophilic markers demonstrated that relative amounts of SOL IL-5Rα mRNA were significantly correlated with protein concentrations of IL-5 ($r = 0.619$; $p = 0.002$), SOL IL-5Rα ($r = 0.659$; $p = 0.001$), ECP ($r = 0.617$; $p = 0.003$), and the percentage of eosinophils ($r = 0.779$; $p < 0.001$) in nasal tissue. In peripheral blood samples, relative amounts of SOL IL-5Rα mRNA were correlated to ECP ($r = 0.604$; $p = 0.02$) and percentage of eosinophils ($r = 0.761$; $p=0.001$), but not to SOL IL-5Rα serum concentrations.

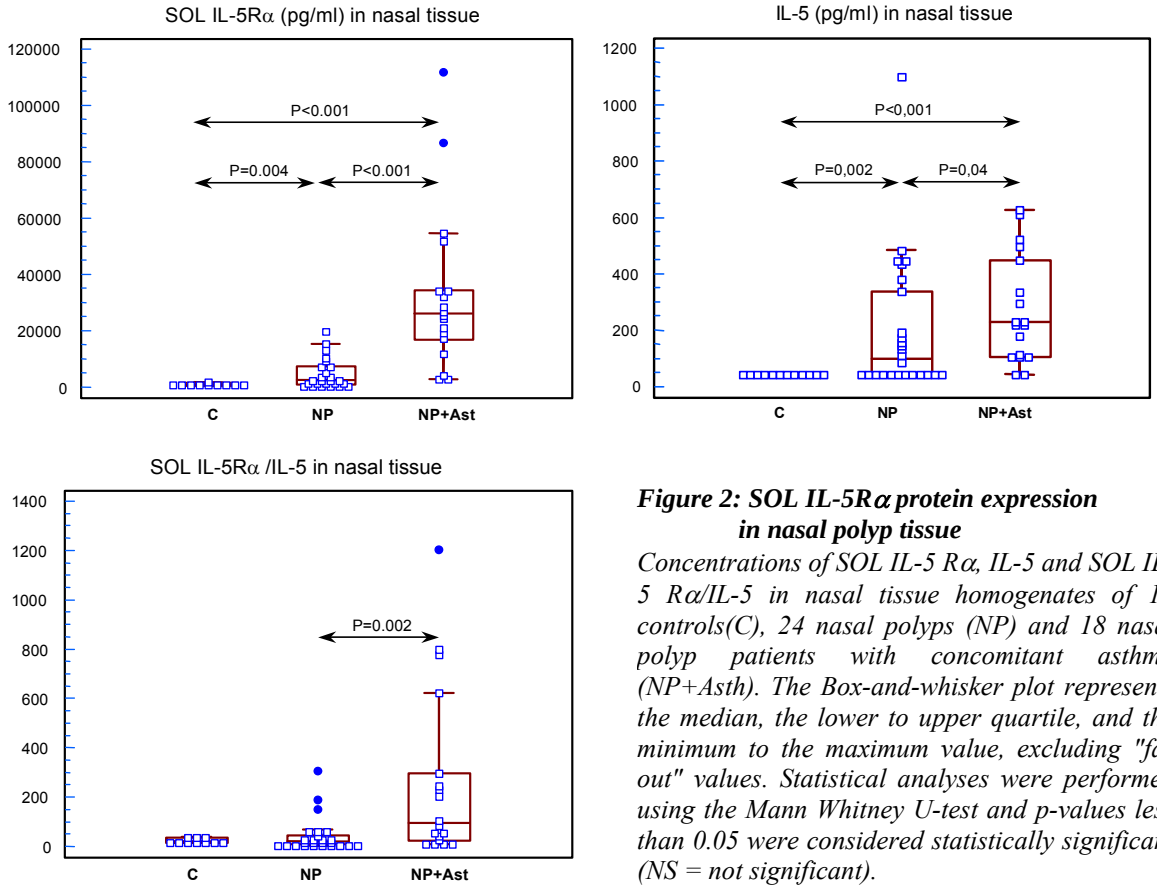


Figure 2: SOL IL-5Rα protein expression in nasal polyp tissue

Concentrations of SOL IL-5 Rα, IL-5 and SOL IL-5 Rα/IL-5 in nasal tissue homogenates of 15 controls(C), 24 nasal polyps (NP) and 18 nasal polyp patients with concomitant asthma (NP+Asth). The Box-and-whisker plot represents the median, the lower to upper quartile, and the minimum to the maximum value, excluding "far out" values. Statistical analyses were performed using the Mann Whitney U-test and p-values less than 0.05 were considered statistically significant (NS = not significant).

DISCUSSION:

This paper demonstrates human SOL IL-5Rα protein in clinical samples such as serum, nasal secretion and tissue homogenates. So far, only expression of the murine SOL IL-5Rα protein could be demonstrated in sera and ascitic fluids of mice bearing chronic B-cell leukaemia [15]. We have established a sensitive, specific and reliable ELISA that enables determination of SOL IL-5Rα concentrations in various human body fluids such as serum, nasal secretions, urine, and peritoneal fluid (not all data shown). In human serum, the SOL IL-5Rα protein is detectable in all samples with a median concentration of 210 pg/ml in healthy controls. Notably, we found that the SOL IL-5Rα was up-regulated in samples from patients with nasal polyposis. These findings were confirmed at the mRNA level, using an optimised real-time RT-PCR procedure.

Nasal polyposis is an excellent model for eosinophil-associated diseases and is often associated with asthma and/or aspirin sensitivity. IL-5 can be detected at the mRNA as well as at the protein level in high amounts by immunohistochemistry and by in situ hybridisation, and could be co-localized to eosinophils [3]. Highest levels of IL-5 protein were observed in polyp homogenates of patients with generalized eosinophilic diseases such as asthma and aspirin sensitivity [2;4;5]. Our data now reveal that SOL IL-5Rα mRNA and protein are up-regulated in nasal polyposis and are correlated with eosinophil counts, ECP and IL-5 concentrations. In addition, SOL IL-5Rα levels in nasal tissue or blood of NP patients are up-regulated and related to the extension of disease, more specifically with the involvement of local (NP) or systemic airway disease (NP with concomitant asthma). With the collection of nasal secretion we aimed to have a less invasive and easy to use technique in comparison to the nasal biopsy to determine SOL IL-5 Rα concentrations. In nasal secretion, SOL IL-5 Rα concentrations were well detectable and there was a strong correlation to the SOL IL-5 Rα concentrations in nasal tissue confirming that collection of nasal secretion is a valuable alternative for biopsies. Furthermore, ROC curve analysis demonstrates that SOL IL-5 Rα in nasal secretion seems to be a superior marker than an established marker such as ECP for the differentiation of NP patients with or without asthma. Taken together, our results indicate that the measurement of SOL IL-5Rα in serum and nasal secretion can be used for objectively monitoring severity of nasal polyposis, and may offer an attractive alternative for currently used clinical scoring parameters.

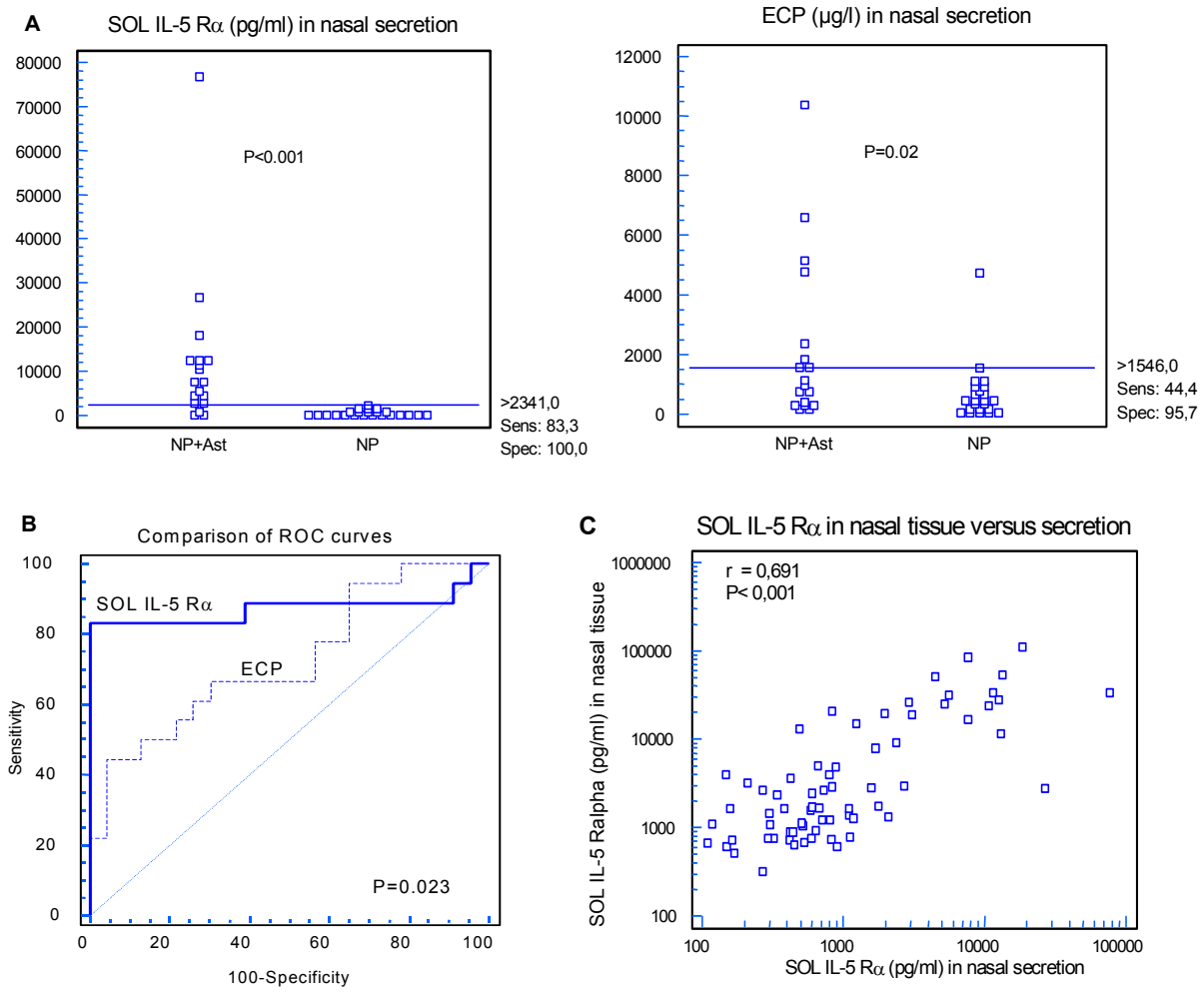


Figure 3: SOL IL-5Rα protein expression in nasal secretions
A) *Concentrations of SOL IL-5 Rα and ECP in nasal secretions of 24 nasal polyps (NP) and 18 nasal polyp patients with concomitant asthma (NP+Asth). The mentioned cut-off values are selected corresponding with the highest accuracy (minimal false negative and false positive results).* ***B)*** *Comparison of ROC curves of SOL IL-5Rα and ECP in the differentiation between nasal polyps with or without concomitant asthma. The statistical significance difference between the areas under the ROC curves was tested and p-values less than 0.05 were considered statistically significant.* ***C)*** *Correlation between SOL IL-5Rα concentrations in nasal secretions and nasal tissue confirms that sampling of nasal secretions might be a valuable alternative for biopsies SOL IL-5Rα monitoring.*

We demonstrate abundant SOL IL-5Rα expression in NP with concomitant asthma often dramatically exceeding the IL-5 concentrations. Indeed, in nasal tissue SOL IL-5Rα levels up to 1200 times higher than IL-5 concentrations can be found. It therefore appears that in the most severe disease manifestations, a regulatory system may operate to antagonize the ongoing eosinophilic inflammation by the synthesis of SOL IL-5Rα. It has been shown that differential splicing of the IL-5Rα gene can generate different mRNA isoforms: a signaling competent membrane-anchored TM IL-5Rα isoform, and at least one secreted variant (SOL IL-5Rα). The expression of the latter depends on the utilization of a specific exon, leading to the loss of the membrane anchor. When expressed in heterologous cells, this SOL IL-5Rα

protein shows receptor antagonist activity *in vitro* [6;11;16;17]. However, the precise *in vivo* role of the SOL IL-5Rα protein requires more detailed study. Secreted receptor variants are a hallmark of the receptor family to which the IL-5Rα belongs. This mere evolutionary conservation underscores their functional importance, but the precise physiological role may differ from case to case. Models in which the antagonistic effects prevail include surface receptor down-modulation and ligand capture. On the other hand, secreted receptors may bind their ligands in circulation, protecting them from proteolytic inactivation and prolonging their serum half-life or facilitating ligand-mediated signaling. The SOL IL-5Rα protein has antagonistic properties *in vitro* as demonstrated in eosinophil differentiation assays as well as in proliferation assays using IL-5 responsive cell lines [11;18]. It captures IL-5 in solution, but this IL-5/IL-5Rα complex is unable to associate and activate membrane bound βc chains [6]. Recently, the antagonistic effect of recombinant SOL IL-5Rα has been demonstrated on nasal tissue explants. Culturing tissue with recombinant SOL IL-5Rα almost completely attenuated the ragweed-induced decrease in eosinophil precursors and increase in MBP-immunoreactive cell numbers [13]. This observation suggests that endogenous production of soluble receptor may regulate IL-5 function *in vivo*. However, the observed correlation between SOL IL-5Rα levels and disease severity questions the antagonistic role for the SOL IL-5Rα *in vivo*.

A number of protein-based IL-5 antagonists have been reported, including SOL IL-5Rα that sequesters the ligand in solution [18], single point mutants of IL-5 that occupy the receptor but fail to initiate receptor activation [7;19], and neutralizing antibodies directed against IL-5 [20]. Treatment of eosinophil-infiltrated polyp tissue with neutralizing anti-IL-5 monoclonal antibody (mAB) resulted in eosinophil apoptosis and decreased tissue eosinophilia *in vitro* [21]. In a monkey model of Ascaris-allergic asthma, Egan and coworkers could abolish inflammatory cell migration and airway hyperreactivity for up to three months by treating the monkeys with a single dose of the SCH55700 (Reslizumab) neutralizing humanized monoclonal antibody against hIL-5 [22]. Similar anti-IL-5 treatment in humans, however, failed to demonstrate clinical efficacy in two independent studies. A single-dose phase I clinical trial was conducted with SCH55700 in patients with severe persistent asthma and demonstrated a significant decrease in peripheral blood eosinophils (lasting up to 90 days) and a trend towards improvement in lung function at the higher doses (30 days after dosing) [23]. SB240563 (Mepolizumab) was tested in mild asthmatics and caused a significant reduction in peripheral blood eosinophils and in postchallenge sputum eosinophils but did not alter FEV_1 values upon allergen challenge [1].

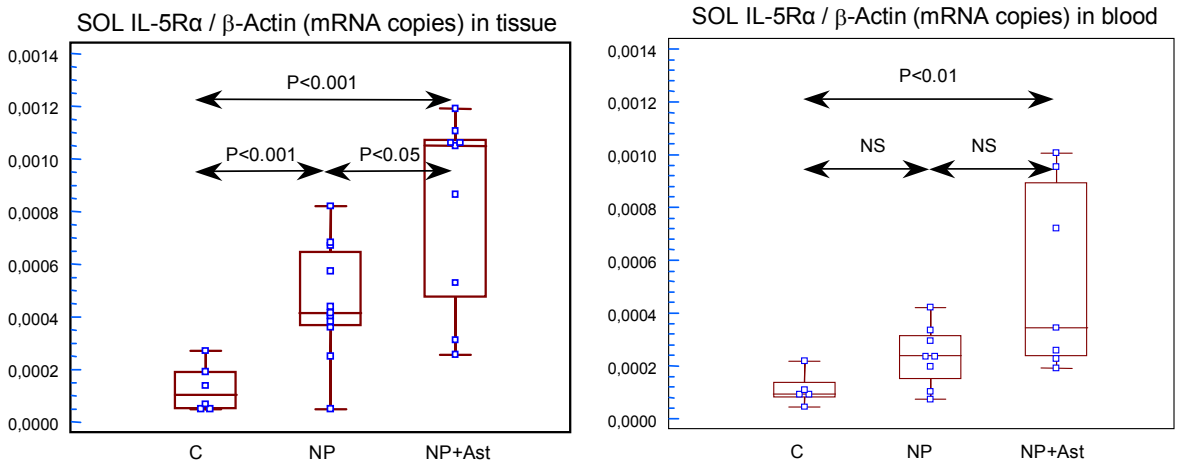


Figure 4: SOL IL-5Rα mRNA expression (real-time PCR)
Relative amounts of SOL hIL-5Rα / β-actin (copies mRNA/μL) in nasal tissue (a) and in peripheral blood (b) of 7 controls(C), 10 nasal polyps (NP) and 9 nasal polyp patients with concomitant asthma (NP+Asth). Statistical analyses performed using the Mann Whitney U-test (NS = not significant).

Our findings suggest that high amounts of SOL IL-5Rα may not be capable of controlling eosinophilia *in vivo*. In line with this, antagonizing IL-5 activity in patients with humanized anti-IL-5 mABs was largely unsuccessful. Completely blocking IL-5 activity *in vivo* therefore appears to be much more difficult than *in vitro*. Perhaps, the local tissue microenvironment, and/or the existence of IL-5-dependent autocrine activation mechanisms [3,21] may limit *in vivo* antagonism [2]. A reassessment of the *in vivo* role of the SOL IL-5Rα may therefore help to understand recent findings in anti-IL5 treatment. Accurate measurement of SOL IL-5Rα levels may distinguish responders from non-responders, or help in the titration of anti-IL-5 doses. Assessment of endogeneous SOL IL-5Rα levels might then be considered as a tool for management of anti-IL-5 therapy. With reliable methods now in place to monitor SOL IL-5Rα protein and mRNA expression, detailed studies of the mechanisms controlling IL-5 activity under normal conditions and in eosinophil-associated diseases can now be performed.

CONCLUSION:

This report demonstrates the presence of the SOL IL-5Rα transcript and protein in various human samples including serum, nasal secretion and nasal tissue homogenates. SOL IL-5Rα is up-regulated in nasal polyposis and differentiates nasal polyps with or without concomitant asthma. Since SOL IL-5Rα is strongly up-regulated for disease and has antagonistic properties *in vitro*, our studies shed new light into the mechanisms of specific immuno-modulatory therapies, such as anti-IL-5.

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Ref Type: Abstract

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CHAPTER 8

REGULATED INTERLEUKIN 5 RECEPTOR α ISOFORM EXPRESSION IN BLOOD AND TISSUE EOSINOPHILS.

REGULATED INTERLEUKIN 5 RECEPTOR α ISOFORM EXPRESSION IN BLOOD AND TISSUE EOSINOPHILS.

Gevaert P, Hellman C, Lundblad L, Lundahl L, Holtappels G, Perez Novo C, Fransen L, van Cauwenberge P, Tavernier J, Bachert C; Submitted 2003.

SUMMARY

Regulated alternative splicing of the IL-5Rα-subunit results in a signaling, transmembrane (TM) isoform, or a soluble (SOL). We examined SOL and TM-IL-5Rα expression and regulation at both protein and transcript level *in vitro* and *in vivo* by FACS, ELISA and real-time PCR. SOL and TM-IL-5Rα protein and mRNA expression were significantly increased in peripheral blood and nasal tissue from nasal polyp (NP) patients versus control subjects. In polyp tissue, SOL-IL-5Rα expression correlated to disease severity and eosinophil percentages, whereas TM-IL-5Rα levels were inversely correlated to eosinophils and SOL-IL-5Rα expression. Tissue eosinophils are activated (increased CD69) and show a decreased TM-IL-5Rα expression compared to blood eosinophils. Human blood eosinophils were incubated with IL-5 for 2 and 24 hours, which caused a significant down-regulation of TM-IL-5Rα mRNA and protein, and up-regulation of SOL-IL-5Rα protein. In a hIL-5-driven proliferation assay, SOL-IL-5Rα proved to be antagonistic only in very high quantities and therefore the endogenous concentrations might be insufficient to block IL-5 activity. This report demonstrates that expression of SOL and TM-IL-5Rα differs according to the eosinophil activation state and localization in the body and may therefore be involved in the fine-tuning of the eosinophil homeostasis.

INTRODUCTION

Interleukin 5 (IL-5) plays a central role in an eosinophil's life span: it supports eosinophilopoiesis and eosinophil differentiation, contributes to eosinophil migration, tissue localization and function, and prevents eosinophil apoptosis[1;2]. Given the likely role of eosinophils in chronic inflammatory diseases, recent research focused at antagonizing the IL-5 function. It appears from recent studies that, although this can easily be achieved *in vitro*[3;4], blocking IL-5 function *in vivo* is much more difficult than originally anticipated[5;6]. Flood-Page and coworkers observed a significant differential effect of IL-5 blockade on eosinophil counts in various body compartments[7]. After multiple dosing with mepolizumab, there was a 100% median reduction in eosinophils in blood, 79% in bronchoalveolar lavage, but only a 52% in the bone marrow and a 55% decrease in the bronchial mucosa[7]. The exact reason for a different effect of anti-IL-5 in different body compartments is unclear, but recent data indicated varying IL-5 sensitivity and IL-5 receptor expression at different stages of the cell's maturation and localization[8].

The interleukin 5 receptor (IL-5R) is a complex consisting of two chains [9]. The α-chain is ligand-specific and binds IL-5 with intermediate affinity (*K*d of 500 pM). Association with the βc-chain leads to a high affinity receptor complex (*K*d of approximately 150 pM). The βc-chain does not bind IL-5 by itself but provides the major determinants for signaling and is shared with the receptors for IL-3 and GM-CSF, which also have their own ligand-specific α-subunits. In contrast to the pan-haemopoietic βc-chain, the IL-5Rα receptor seems to be expressed on a restricted range of cell types in humans, principally eosinophils, basophils, and their precursors. However, recent publications have demonstrated eosinophil-independent airway hyperresponsiveness and IL-5Rα gene expression in human bronchus smooth muscle [10;11].

Regulated alternative splicing of the IL-5Rα-subunit leads to the generation of either a signaling, membrane-anchored (TM) isoform, or a soluble (SOL) variant with antagonistic properties *in vitro* [9;12]. It is suggested that eosinophils are able to control their responsiveness to IL-5 by regulated expression of the IL-5Rα isoforms. However, this regulated expression may change during eosinophil differentiation, maturation and localization. On CD34+ progenitor cells, it has been demonstrated that IL-5 induces a switch from predominantly SOL isoform to TM-IL-5Rα mRNA expression at the splicing level [13]. In mature blood eosinophils, IL-5, IL-3 and GM-CSF down-regulate IL-5Rα mRNA in a dose dependent manner at the promoter level [14]. Liu and coworkers showed that IL-5 receptor expression on

airway eosinophils is down-modulated *in vivo* after inhaled allergen challenge and that this may be due to cleavage by membrane-associated metalloproteinases[15;16]. In addition, Gregory and coworkers have demonstrated that exposure of blood eosinophils to IL-3, IL-5, or granulocyte-macrophage colony-stimulating factor *in vitro* leads to sustained down regulation of surface IL-5Rα expression and reduced responsiveness to IL-5, but without sustained changes in CCR3 expression[17]. We have previously demonstrated that the TM-IL-5Rα is down-regulated on eosinophils in peritoneal dialysis fluid, as compared to blood eosinophils, from patients with peritoneal fluid eosinophilia[18], whereas SOL-IL-5Rα concentrations were up-regulated (unpublished data). This indicates that IL-5Rα isoforms on mature eosinophils can be dynamically regulated at the extra-vascular site. However, all data are based on tissue-dwelt eosinophils (BAL eosinophils and peritoneal fluid eosinophils) which may have different properties compared to resident tissue eosinophils. Recently, we demonstrated an up-regulation of SOL-IL-5Rα protein in serum and nasal polyp tissue[19]. We hypothesize that the expression of the IL-5Rα isoforms may be different depending on eosinophil activation state, maturation and localization.

Taken these observations into account, we sought to study SOL and TM-IL-5Rα proteins and transcripts in blood and in tissue eosinophilia. Therefore a real-time PCR, FACS and ELISA were established to determine IL-5Rα isoform expression in peripheral blood and nasal tissue from control subjects and nasal polyp (NP) patients. Furthermore, we aimed to investigate whether *in vitro* stimulation of peripheral blood eosinophils with IL-5 could regulate the IL-5Rα isoform expression similar to the expression found in tissue eosinophils and its relation to membrane type-1 matrix metalloproteinases (MT1-MMP or MMP-14). Finally, the biological activity of SOL-IL-5Rα in an hIL-5-driven proliferation assay was to be tested.

RESULTS

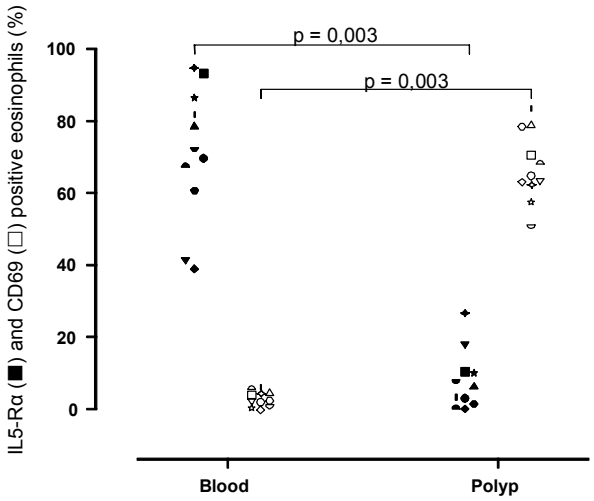
SOL and TM-IL-5Rα protein expression in blood and tissue

We found increased median (IQR) concentrations of SOL-IL-5Rα in serum from 17 NP patients with concomitant asthma (778 pg/ml; 501-1191) compared to 16 controls (285 pg/ml; 226-465; $p = 0.009$) and 17 NP patients without asthma (328 pg/ml; 272-448; $p < 0.001$). Whereas SOL-IL-5Rα could be measured in all serum samples, IL-5 protein concentrations were only measurable in four subjects (all with concomitant asthma). In controls the median percentage of blood eosinophils was 1.2% (0.5-2.5), whereas increased percentages were

found in NP (3.6%; 2.5-5.9; p = 0.02) and NP with asthma (9.7%; 6.9-19.5; p < 0.001), with a significant difference between the NP groups (p < 0.001).

In supernatants prepared from nasal tissue homogenates median (IQR), concentrations of SOL-IL-5Rα were significantly higher in NP (1504 pg/ml; 1037-3757; p = 0.04) and NP with concomitant asthma (21069 pg/ml; 13177-29784; p < 0.001) compared to control nasal tissue (919 pg/ml; 735-1416). Whereas IL-5 was not detectable in control nasal tissue, increased IL-5 concentrations were found in NP tissue of patients without (56 pg/ml; 43-197; p=0.03) and with concomitant asthma (244 pg/ml; 107-367; p=0.002). Within the NP group, there was a significant further increase of SOL-IL-5Rα (p < 0.001) and IL-5 (p = 0.01) in patients with concomitant asthma compared to patients without asthma. Increased eosinophil percentages were found in NP tissue with (11.3%; 8.1-25.7; p < 0.001) and without concomitant asthma (2.4%; 1.2-5.8; p = 0.006) compared to control tissue (0.2%; 0.1-0.5). Moreover, in NP tissue, SOL-IL-5Rα significantly correlated with concentrations of IL-5 (r = 0.500; p = 0.02), and percentage of eosinophils (r = 0.589; p = 0.003).

Figure 1: *TM-IL-5Rα and CD69 (eosinophil activation marker) expression was evaluated by FACS analysis in blood and in tissue of twelve NP patients. The results are presented as the proportion of TM-IL-5Rα (filled symbols) and CD69 (open symbols) positive eosinophils. Statistical analyses were performed using the Wilcoxon test.*



When peripheral blood and polyp tissue cells were analyzed by flow cytometry the proportion of TM-IL-5Rα positive eosinophils was significantly (p=0.003) lower in polyp tissue eosinophils, 6.1 % (1.3-10.4) when compared to peripheral blood eosinophils, 72.6 % (60.6-86.4) (n=11). The proportion of CD69 positive eosinophils was significantly (p=0.018) up regulated on polyp tissue eosinophils, 63.9 % (63.0-78.4), as compared to peripheral blood eosinophils, 2.7 % (1.9-3.6) (Figure 1).

SOL and TM-IL-5Rα mRNA expression in blood and tissue

Analysis of peripheral blood and nasal tissue samples with quantitative real-time PCR revealed that relative amounts of SOL and TM-IL-5Rα isoforms (copies of mRNA/µl) were significantly higher in samples of patients with NP compared to controls. Within the NP group, SOL and TM-IL-5Rα mRNA levels were increased in peripheral blood of patients with concomitant asthma compared to patients without asthma (Figure 2a). In NP tissue of patients with asthma the SOL-IL-5Rα mRNA was significantly increased whereas the TM-IL-5Rα mRNA was significantly decreased when compared to NP patients without asthma (Figure 2b). The median SOL/TM ratio in blood was 1.6 (1.3-1.8) with no significant differences between groups. In control and NP tissue of non-asthmatics, the median SOL/TM ratio was 0.32 (0.21-0.44) and 0.33 (0.23-0.43) respectively, which was significantly lower (both $p < 0.001$) than the SOL/TM ratio's in NP tissue of patients with asthma 1.12 (0.89-1.63). There were no differences in relative expression of MMP-14 mRNA (β-actin corrected) in peripheral blood ($8.3x10^{-6}$; $5.7x10^{-6}$ - $10.3x10^{-6}$) and nasal tissue samples ($2.0x10^{-3}$; $1.5x10^{-3}$ - $2.9x10^{-3}$) of controls and NP patients. In peripheral blood samples of NP patients, relative amounts of SOL and TM-IL-5Rα mRNA were significantly correlated to eosinophil percentages (Figure 2a). In polyp tissue, protein concentrations of IL-5, SOL-IL-5Rα, and eosinophil percentages correlated positively to SOL-IL-5Rα, but inversely to TM-IL-5Rα mRNA expression (Figure 2b).

SOL and TM-IL-5Rα protein expression after in vitro stimulation with rh IL-5

Purified eosinophils from seven healthy blood donors were incubated with rh IL-5 for 2 and 24 hours. Flow cytometric analysis showed that rh IL-5 induced a significant ($p=0.018$ for all) down-regulation in the proportion of TM-IL-5Rα positive eosinophils at 2 hours, 37.5 % (27.4-61.8) and 24 hours, 13.9 % (8.8-22.0) as compared to the RPMI controls, 78.3 % (68.1-86.0) and 55.0 % (46.9-62.3), respectively (Figure 3a). The SOL-IL-5Rα concentrations in the supernatants were significantly higher after incubation with rh IL-5 compared to RPMI controls at 2 hours ($p=0.002$) and 24 hours ($p=0.03$) (Figure 3b). SOL and TM-IL-5Rα protein expression correlated negatively at 24 hours ($r= -0.612$; $p=0.03$).

SOL and TM-IL-5Rα mRNA expression after in vitro stimulation with rh IL-5

Stimulation of blood eosinophils with rh IL-5 resulted in a significant decrease in TM-IL-5Rα mRNA expression after 2 and 24 hours compared to baseline, which is at both time points significantly lower when compared to the RPMI controls (Figure 3c). SOL-IL-5Rα mRNA expression decreased significantly after 2 and 24 hours, without difference between rh IL-5

incubation and RPMI controls (Figure 3d). Since IL-5Rα cleavage by membrane-associated metalloproteinases was suggested, we investigated the relation to MMP-14 expression. There was no difference in relative MMP-14 mRNA expression (β-actin corrected) between different time points or between stimulation with rh IL-5 and RPMI controls. MMP-14 expression was positively correlated with SOL-IL-5Rα (protein & mRNA) ($r=0.354$; $p>0.05$ & $r=0.786$; $p=0.005$) and TM-IL-5Rα mRNA expression ($r=0.638$; $p=0.02$), whereas a negative correlation was shown with TM-IL-5Rα surface expression ($r= -0.750$; $p=0.007$).

Biological activity analysis of SOL-IL-5Rα on FDC-P1-CA1 cells

The biological role of SOL-IL-5Rα is still uncertain, therefore we analyzed the behavior of SOL-IL-5Rα in the hIL-5-driven FDC-P1-CA1 proliferation assay[20;21]. At physiologic concentrations of hIL-5 (here 10 pg/ml), SOL-IL-5Rα was able to neutralize the hIL-5 induced growth of FDC-P1-CA1 cells only at high concentrations (> 500 ng/ml) (Figure 4). This implicates that at least 18500 times more SOL-IL-5Rα than IL-5 is needed to block its activity in this assay system.

DISCUSSION

Our results demonstrate differential expression of IL-5Rα isoforms in blood and tissue eosinophils. In blood, SOL and TM-IL-5Rα mRNA and protein expression is up regulated in NP versus controls and correlated to eosinophil percentages, whereas in polyp tissue TM-IL-5Rα levels showed an inverse relation to eosinophilia and SOL-IL-5Rα expression. Blood eosinophils express high levels of surface-anchored TM-IL-5Rα and are not activated as judged by a low CD69 expression. In contrast, tissue eosinophils are activated and demonstrate a low level of surface receptors to IL-5. These results extend studies in mice showing that tissue eosinophils have a low or undetectable expression of TM-IL-5Rα [22], and recent studies on human peritoneal fluid [18] and BAL fluid eosinophils [16] that indirectly suggest a low expression of TM-IL-5Rα in tissue versus blood eosinophils.

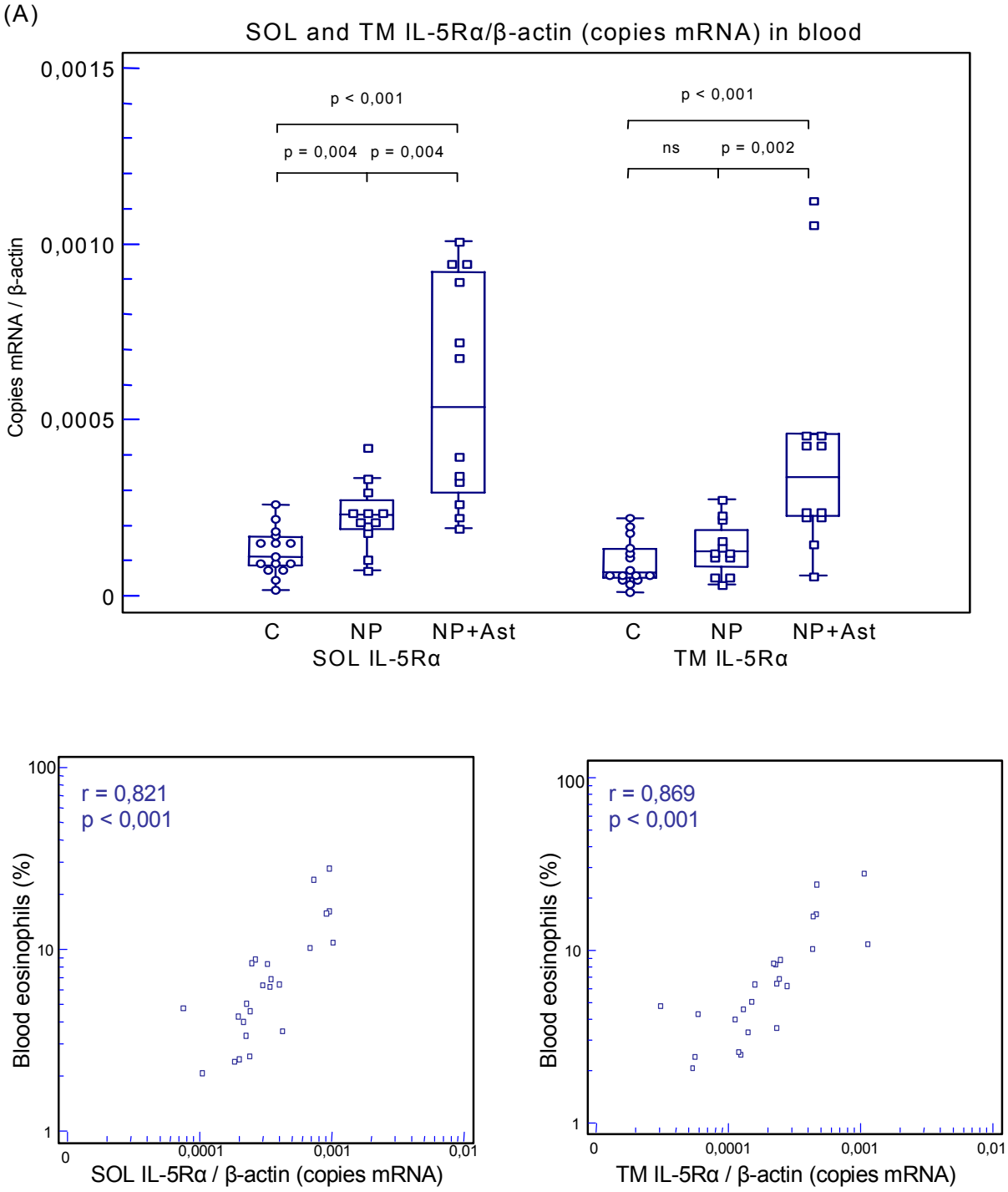


Figure 2: *Relative amounts of SOL and TM hIL-5Rα / β-actin (copies mRNA/µL) were determined by a isoform specific real-time PCR in peripheral blood (A) and in nasal tissue (B) of 16 controls (C), 17 nasal polyps (NP) and 17 nasal polyp patients with concomitant asthma (NP+Asth). The Box-and-whisker plot represents the median, the lower to upper quartile, and the minimum to the maximum value, excluding "far out" values. Statistical analyses were performed using the Mann Whitney U-test (ns = not significant). Spearman rank correlation coefficient (r) was used to assess the relationships between the parameters.*

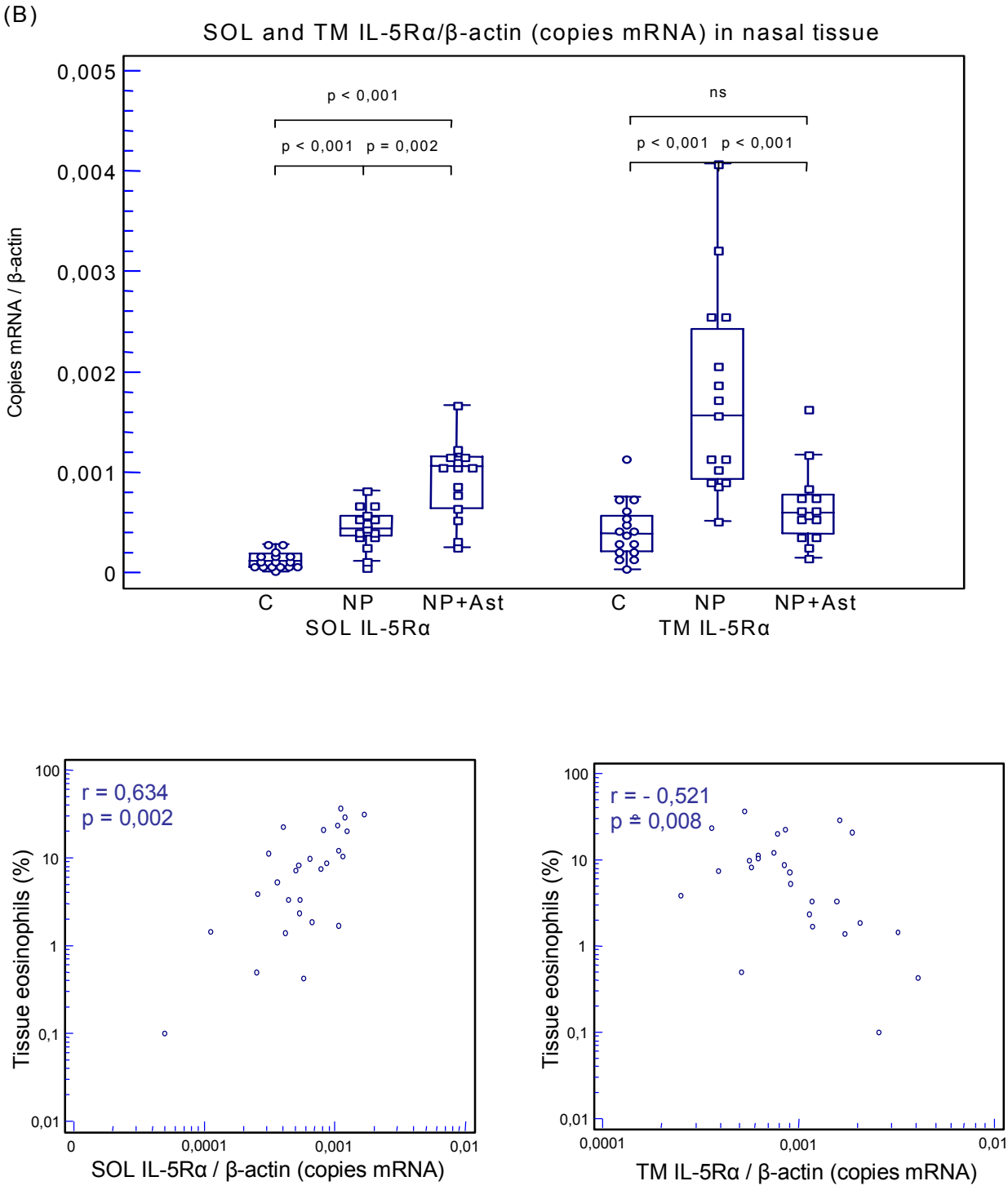


Figure 2 (Continued) *Relative amounts of SOL and TM hIL-5Rα / β-actin (copies mRNA/µL) were determined by a isoform specific real-time PCR in in nasal tissue (B).*

Also, Liu et al. demonstrated that following airway antigen challenge of atopic subjects, BAL fluid eosinophils showed a markedly reduced TM-IL-5Rα and βc-chain mRNA and did not release EDN when exposed ex vivo to IL-5 compared with circulating eosinophils[16].

Nasal polyposis is an excellent model for eosinophil-associated diseases, since 80 to 90% of bilateral nasal polyps are characterized by abundant eosinophilic infiltration. Nasal polyps are swellings of the lamina propria mucosa, a pathology that is frequently associated with asthma. Especially in nasal polyp patients with concomitant asthma the highest levels of IL-5 and eosinophil counts are reported [23;24]. In this report we show that the SOL-IL-5Rα expression is increased whereas the TM-IL-5Rα mRNA expression is decreased in NP tissue of patients with asthma compared to those without asthma. Furthermore, TM-IL-5Rα mRNA is inversely correlated to protein levels of IL-5, SOL-IL-5Rα and the proportion of eosinophils. This suggests TM-IL-5Rα mRNA tissue expression is down regulated independently of eosinophil counts and this further supports the finding that tissue eosinophils have a low IL-5Rα surface expression. Hence, in tissue with the highest levels of IL-5 and eosinophils, a regulatory mechanism must operate to control the ongoing eosinophilic inflammation by up-regulation of SOL-IL-5Rα and down-regulation of TM-IL-5Rα. Taken together, our results suggest that the expression of the IL-5Rα isoforms differs according to the eosinophil activation state, maturation and localization in the body.

It has been shown that differential splicing of the IL-5Rα gene can generate different mRNA isoforms: a signaling competent membrane-anchored TM-IL-5Rα isoform, and at least one secreted variant (SOL-IL-5Rα) [9;12]. The expression of the latter depends on the utilization of a specific exon, leading to the loss of the membrane anchor. In human umbilical cord blood derived CD34 cells, a transient switch at the alternative splicing level from predominantly soluble isoform to TM-IL-5Rα messenger RNA (mRNA) expression was found during IL-5-driven eosinophil development [13]. This was accompanied by surface expression of IL-5Rα and acquisition of functional responses to IL-5. These data provide an explanation for the selective requirement of IL-5 for expansion of the eosinophil lineage within the bone marrow.

In contrast, in mature human blood eosinophils Wang et al. demonstrated that IL-5, IL-3 and GM-CSF down-regulate IL-5Rα mRNA while up-regulating IL-3Rα, GM-CSFRα, and β-chain mRNA. This cytokine-induced inhibition of IL-5Rα mRNA accumulation occurs at the IL-5Rα promoter level [14].

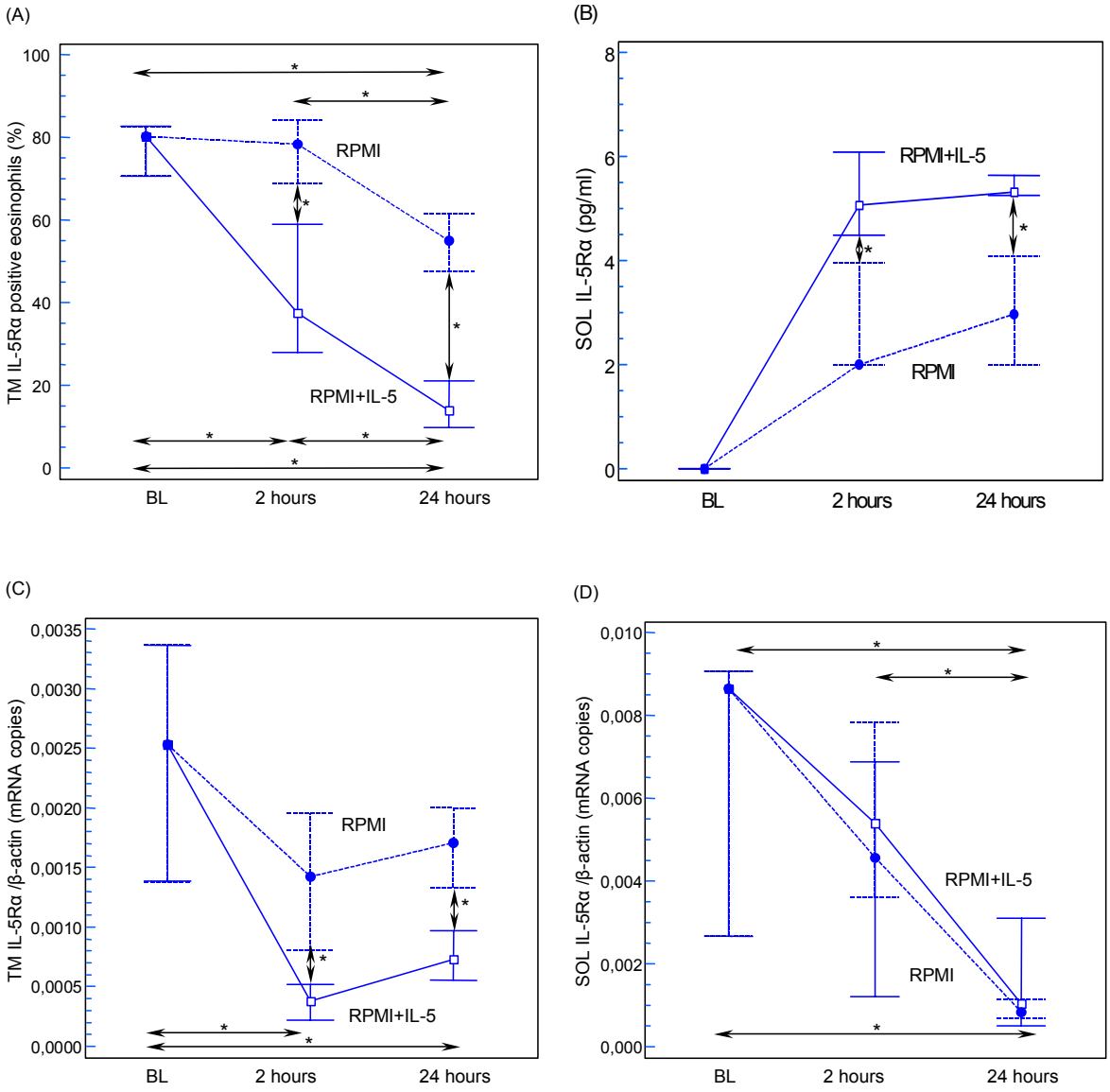


Figure 3: *Purified eosinophils from seven healthy blood donors were incubated with 10ng/ml rh IL-5 for 2 and 24 hours (RPMI+IL-5; full line). As control, eosinophils were incubated in RPMI alone (RPMI; dotted line). TM-IL-5Rα positive eosinophils (%) determined by FACS analysis (A); SOL-IL-5Rα(pg/ml) concentrations in supernatants determined by ELISA (B); TM-IL-5Rα/β-actin (C) and SOL-IL-5Rα/β-actin (copies mRNA) expression detremined by real-time PCR (D). The lines represent the median and the lower to upper quartile (*, $p < 0.05$).*

Recently, Hellman et al. demonstrated that surface TM-IL-5Rα was strongly down-regulated by recombinant IL-5, intermediately with both IL-5 and GM-CSF, and weakly with only GM-CSF, suggesting that GM-CSF binding partially inhibits the surface IL-5 receptor to be down modulated[25]. Furthermore, the proportion of CD69 positive eosinophils was significantly up-regulated to the same level by IL-5, GM-CSF as well as by the combination of both cytokines, which indicate that activation ofeosinophils, judged by CD69 up-regulation, is not mandatory linked to TM-IL-5Rα down-regulation. In the present study we show that freshly isolated mature human blood eosinophils express more SOL than TM-IL-5Rα mRNA expression. *In*

vitro exposure of mature human blood eosinophils with rh IL-5 for 2 and 24 hours induces an extensive down-regulation of IL-5Rα TM protein and mRNA, whereas SOL-IL-5Rα was only significantly up-regulated at the protein level. In contrast to Liu et al. [15] we demonstrated a significant down-regulation of the TM-IL-5Rα specific transcripts probably because of the higher statistical power (seven experiments versus three). Furthermore, they demonstrated that the down-modulation of TM-IL-5Rα from the cell surface and the increased release of SOL-IL-5Rα into culture supernatant fluid were partially inhibited by the MMP-specific inhibitor BB-94 [15]. These data suggest that exposure of peripheral blood eosinophils to IL-5 results in a rapid, sustained loss of TM-IL-5Rα that is, at least in part, dependent on MMP activity. Furthermore, we recently demonstrated increased levels of MMP-7, MMP-9 and TIMP-1 in nasal polyp tissue compared to controls[26]. However, since TIMP-3, but neither TIMP-1 nor TIMP-2, inhibited the action of the proteinase activity on IL-5 receptor modulation, it is suggested that the responsible enzyme may be a membrane-associated metalloproteinase[15]. Although the expression membrane type-1 MMP (MT1-MMP or MMP-14) is not different in polyps and controls and is not influenced by IL-5 stimulation of eosinophils, we found a significant inverse correlation between MMP-14 and IL-5Rα surface expression, which suggests a role in proteolytic cleavage of the receptor. Taken all observations into account we suggest that an IL-5 driven inflammation generates an eosinophil tissue phenotype that is characterized by a low TM but high SOL-IL-5Rα expression and that this process is partially the result of proteolytic receptor modulation and down-regulation of TM-IL-5Rα gene transcription.

Undoubtedly, a primary role of IL-5 in eosinophil hemopoiesis and recruitment from the bone marrow is widely accepted, however its local role in inflamed tissue is much less clear. Eosinophils leave the bone marrow with a so-called “non-primed” phenotype that is refractory to activation. Upon interaction with IL-5 and other cytokines / chemokines these cells become prone for activation by physiologically relevant activators. Virtually all eosinophil responses are under control of this priming mechanism which acts as a safety lock preventing specific activation of this highly cytotoxic cell. The local tissue environment typically shows the highest levels of IL-5, providing sufficient levels of IL-5 into the bone marrow for selective expansion of the eosinophil lineage. Therefore it is not surprising to find an IL-5-dependent differential expression of the IL-5Rα isoforms according to the eosinophil activation state, maturation and localization in the body. Despite drastic reduction of TM-IL-5Rα expression, tissue eosinophils are activated and have a prolonged survival, suggesting that only a limited

number of receptors are sufficient for an IL-5 response. Furthermore, our data on the biological activity of SOL-IL-5Rα in a hIL-5-driven FDC-P1-CA1 proliferation assay suggest that only an excess of SOL-IL-5Rα (18500 times) compared to IL-5 can block its activity, whereas only limited amounts (0.3 times) of a humanized anti-IL-5 monoclonal antibody (SCH55700) are necessary to antagonize IL-5 activity (own data). Indeed, the median concentration of SOL-IL-5Rα in nasal polyp tissue is 21069 pg/ml and can be up to 1200 times higher than local IL-5 concentrations. Cameron and coworkers demonstrated that recombinant SOL-IL-5Rα in high concentrations of 5 µg/ml had antagonistic properties in nasal tissue explants since it almost completely attenuated the ragweed-induced decrease in eosinophil precursors and increase in MBP-immunoreactive cell numbers[3]. Although SOL-IL-5Rα protein has antagonistic properties *in vitro*, it is important to note that the endogenous concentrations might be insufficient to block IL-5 activity *in vivo*. Furthermore, secreted receptors may bind their ligands in circulation, protecting them from proteolytic inactivation and prolonging their serum half-life or facilitating ligand-mediated signaling. Hence, SOL-IL-5Rα may be involved in the fine-tuning of eosinophil homeostasis not only as an antagonist, but possibly also as a carrier.

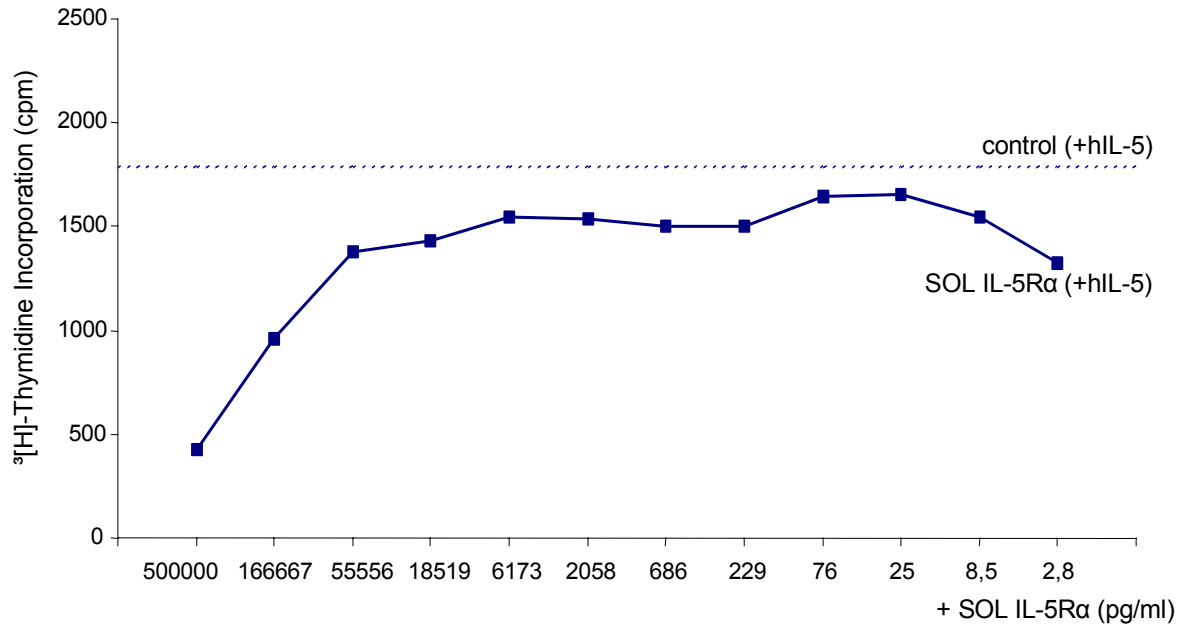


Figure 4: *SOL-IL-5Rα was tested for the ability to inhibit the effect of hIL-5 on FDC-P1-CA1 cells. Proliferation was measured by monitoring ³[H]thymidine incorporation. The full line represent proliferation of FDC-P1-CA1 cells in the presence of hIL-5 (10 pg/ml) and a dilution of SOL-IL-5Rα. The dashed line indicates proliferation in the presence of hIL-5 (10 pg/ml) alone. The experiment was performed in triplicate samples and similar results were obtained in several independent assays.*

CONCLUSIONS:

This report demonstrates differential expression of SOL and TM-IL-5Rα in blood and tissue eosinophils. Interestingly, in polyp tissue SOL-IL-5Rα expression is increased and correlated to disease severity and eosinophil percentages, whereas TM-IL-5Rα levels decreased and were inversely correlated to eosinophils and SOL-IL-5Rα expression. *In vitro* exposure of blood eosinophils to IL-5 reduces the expression of TM-IL-5Rα, but induces SOL-IL-5Rα protein release. SOL-IL-5Rα protein has antagonistic properties *in vitro*, however, endogenous concentrations might be insufficient to block IL-5 activity and thereby act as an IL-5 carrier in vivo. The expression of the IL-5Rα isoforms differs according to the eosinophil activation state, maturation and localization in the body and may therefore be involved in the fine-tuning of the eosinophil homeostasis.

MATERIALS AND METHODS

Patients

Thirty-four subjects with bilateral nasal polyps and 16 controls were recruited at the Departments of Otorhinolaryngology at the Ghent University Hospital, Belgium and the Karolinska Hospital in Stockholm, Sweden. Peripheral blood and nasal polyp samples were collected in 34 NP subjects (mean age 51.4 years, range from 22-79 years; twelve females/ twenty-two males) during routine endoscopic sinus surgery and immediately processed for ELISA and PCR measurements, whereas flow cytometry was performed on blood and tissue samples of eleven NP subjects. Bilateral nasal polyposis was diagnosed based on history, clinical examination, nasal endoscopy and sinus CT-scan. A history of asthma was reported in seventeen nasal polyp patients, with seven subjects additionally demonstrating a history of aspirin hypersensitivity. Patients treated with oral corticoids within the last 4 weeks prior to surgery were excluded.

Blood and nasal tissue (inferior turbinates) from healthy controls were obtained during routine corrective nasal surgery. All controls (mean age 34.5 years, range from 18-71 years; seven females/ nine males) were skin prick test negative, in generally good health, and none had a history of nasal or sinus disease, allergic disease (asthma, rhinitis or dermatitis), upper respiratory tract infection in the previous month, use of any intranasal medications, decongestants, antihistamines or oral steroids. The ethical committees of both universities approved this study and a written informed consent was obtained from all subjects before inclusion in the study.

Preparation of nasal tissue

Removed polyps were transferred to +4°C HEPES (10mM)-buffered RPMI 1640 medium (Gibco, Paisley, UK). The polyp tissue was either homogenized or prepared to single cell suspension. To obtain tissue homogenates, nasal tissue specimens were weighed, and 1 ml of 0.9% NaCl solution was added for every 0.1 g of tissue and then homogenized with a mechanical homogenizer (B. Braun Melsungen, Germany) as described previously [23].

For single cell suspensions, the tissue was cut into small pieces, passed through a fine wire mesh by a syringe stamper and rinsed with RPMI. The cell suspension was centrifuged at 200g for <1 minute at +4°C and remaining tissue elements were sedimented. The cell supernatant was further centrifuged at 300g for 12 minutes at +4°C. Twenty up to fifty million cells were obtained in a single cell suspension of nasal polyps and the cells were separated (per one million cells). One part was lysed and stored at –80°C until RNA purification. The other part was resuspended in 5 ml PBS and used for FACS analysis.

Preparation of blood and serum

Peripheral blood was collected in tubes containing EDTA (Vacutainer, 5 ml, with 50 µl of 21% EDTA) (Terumo, Leuven, Belgium). Leukocytes were isolated by hemolysing 150 µl portions of blood in 3 ml +4°C isotonic NH_4Cl-EDTA lysing solution (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) for 5 minutes at +15°C. Cell suspensions were then centrifuged at 300g for 6 minutes at +4°C and washed in PBS. The cells were used for RNA preparations and FACS analysis. Serum was collected tubes with no additives, was allowed to coagulate for 30 minutes and centrifuged at 1500g for 15 minutes at +4°C.

Preparation of purified peripheral blood eosinophils

Peripheral blood eosinophils from healthy blood donors (age 18-64 years) were purified by the magnetic cell separation system MidiMacs (Miltenyi, Biotec, Bergisch Gladbach, Germany) [27]. Briefly, blood was layered onto Percoll solution (Pharmacia-Upjohn, Uppsala, Sweden) and centrifuged (30 minutes, 1000g, 20°C). The mononuclear cell layer was removed and the remaining cell suspension was hemolysed in isotonic lysing solution (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). Eosinophils and neutrophils were washed in PBS and anti-CD16 magnetic beads were added for 20 minutes at +4°C. The eosinophils were obtained by negative selection using a separation column in a magnetic field where magnetically labeled cells (CD16+ neutrophils) were trapped and unlabelled cells (eosinophils) were collected.

In vitro incubation of purified eosinophils with recombinant human IL-5,

Purified eosinophils ($1.0x10^6$/ml) were incubated with recombinant human (rh) IL-5 (10 ng/ml) (Immunokontact, Frankfurt, Germany) diluted in HEPES (10mM)-buffered RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (RPMI). As control, eosinophils were incubated in RPMI alone. The cell suspensions were incubated in 24-well plates for 2 or 24 hours at +37°C in 5% CO_2. Supernatants were collected and stored at –30°C until ELISA measurements. The cells were washed twice in PBS (300g for 5 minutes at +4°C) and used for FACS.

Immunofluorescence staining of eosinophils and flow cytometry

Polyp tissue cells ($0.5\text{-}1.0 \times 10^6$), isolated blood leukocytes or purified eosinophils (0.1×10^6) were incubated with non-conjugated mAb to IL-5Rα (10 μg/ml, Clone: α16, non-neutralizing; gift by Prof J Tavernier). Secondary immunostaining was performed with fluoroscein isothiocyanate (FITC)-conjugated Rabbit Anti Mouse immunoglobulin, $F(ab')_2$ (50 μg/ml, Code: F313) (DAKO A/S). Polyp tissue cells and blood leukocytes were double stained with phycoerythrin (PE)-conjugated mAb to CD16 (Immunotech) or stained with FITC-conjugated mAb to CD69 (5μg/ml, Clone: L78) (Becton Dickinson, Meylan-Cedex, France) together with mAb to CD16 (Immunotech). Cells and antibodies were incubated for 30 minutes at +4°C, and then washed in PBS. Non-specific binding was determined with isotype-matched control antibodies in corresponding concentrations. Cells were finally diluted in 0.5 ml PBS and a minimum of 1000 eosinophils was analyzed in an EPICS XL-MCL (Beckman Coulter Inc., Fullerton, CA, USA) flow cytometer. Polyp tissue and peripheral blood eosinophils were detected and analyzed as a separate CD16 negative population by using depolarized light [28]. The flow cytometer was calibrated daily with Flow Check and Flow Set (Beckman Coulter).

Measurement of IL-5 and SOL IL-5 Rα protein concentrations

A SOL-IL-5Rα specific sandwich ELISA was developed by combining two monoclonal antibodies (Innogenetics, Gent, Belgium). A detailed description and characterization of this ELISA was previously published[19]. Serum, tissue homogenates and eosinophil supernatants were assayed by a research ELISA for SOL-IL-5Rα (Innogenetics, Gent, Belgium) and IL-5 (R&D Systems, Minneapolis, USA).

SOL and TM IL-5 Rα Real time PCR

As a standard we used PCR products generated from plasmids containing the cDNA sequences for SOL and TM hIL-5Rα. The cDNA sequences of the SOL and TM isoforms

were amplified using a forward primer complementary to a common sequence of hIL-5Rα 5'GTGTCTGCTTTTCCAATCCATTGC 3' and reverse specific primers: TM hIL-5Rα reverse primer: 5' TTTTGGTGCTGGAATTGGTGG 3' and SOL hIL-5Rα reverse primer: 5' TCAGATACGGTGTGGGGCAG 3'. All primers were designed using the Primer Express 2.0 Software (Applied Biosystems, Foster City, US) and purchased from Invitrogen. The PCR resulted in a unique band that was purified by using the PCR Gel extraction QIAquick (Qiagen, Hilden, Germany) spin columns. The purified fragments were quantified using PicoGreen reagent (Molecular Probes, Leiden, The Netherlands). Equimolar 10 fold dilutions of both PCR products were used to generate standard curves for each spliced variant.

RNA was isolated from snap frozen blood leukocytes and nasal tissue cells of 16 controls and 34 NP patients using the Rneasy Kit (Qiagen, Hilden, Germany), whereas RNA from eosinophil cell pellets was extracted using the TriReagent method (Sigma, Bornem, Belgium). Total RNA was quantified using RiboGreen Kit (Molecular Probes) and 0.5 μg was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen), oligo dT and random hexamers (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacture's protocol. cDNA equivalent to 25 ng total RNA was used to perform the Real Time PCR. The Real Time amplifications were performed using the 1X SYBR Green I Mastermix (Qiagen, Hilden, Germany) and a set of primers including a common forward primer 5'-GCAGCAGTGAGCTCCATGTG-3' (nts 1177 to 1196, Genbank accession number M96652) and specific reverse primers for SOL hIL-5Rα 5'-TGGATGTTATCTCCTTTATCTTGAGAAC-3' (nts 1248 to 1272) and TM hIL-5Rα isoforms 5'-AGGGCTTGTGTTCATCATTTCC-3' (nts1245 to1264). All the PCRs were performed in a 5700 SDS Thermal Cycler (Applied Biosystems, Foster City, US). Each sample was tested in duplicate. The quantity of each amplicon was calculated from the values of each standard curve and normalized by the quantities obtained for β-actin transcripts [29].

MMP-14 RT-PCR was done with Assay-on-Demand Gene Expression products (Applied Biosystems, Fosteer City, CA). PCR was performed in a 25 μl reaction mixture composed of cDNA (equivalent to 25 ng total RNA), primers and FAM-labeled probes and TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, US). Reactions were incubated for 10 min at 95°C, followed by 40 cycles of a two-step amplification procedure composed of denaturation for 15 seconds at 95°C and annealing/extension at 60°C for 1 minute. A standard curve of pooled cDNA from tonsils was used for calculation of relative quantities.

Biological activity analysis of SOL-IL-5Rα on FDC-P1-CA1 cells

FDC-P1-CA1 cells are derived from murine, early myeloid FDC-P1 cell-line. hIL-5 responsiveness was obtained by stable transfection of a chimeric IL-5Rα-chain, consisting of the human extracellular part fused to the transmembrane and intracellular parts from the mouse IL-5Rα-chain (mIL-5Rα)[20;21]. Cells were cultured in RPMI 1640 medium containing 10% FBS and 1 ng/ml hIL-5. Baculo virus expressed human SOL-IL-5Rα was purified (Innogenetics, Ghent, Belgium) and tested for the ability to inhibit the effect of hIL-5 on FDC-P1-CA1 cells. hIL-5 (10 pg/ml) was pre-incubated with or without indicated dilutions of SOL-IL-5Rα for 6h. Cells were washed to remove hIL-5 and resuspended in medium depleted of growth factors for 3h. Then, FDC-P1-CA1 cells were added to the hIL-5/SOL-IL-5Rα mixture. After four days, growth was measured by monitoring ^{3}[H]thymidine incorporation (0,5μCi per well; 4-6h; 37°C). The experiment was performed in triplicate samples and similar results were obtained in three independent assays.

Statistical analysis

Data are expressed as median and interquartile range (IQR). When comparisons were made between groups, significant between-group variability was first established using Kruskal-Wallis test. The Mann Whitney U-test was then used for between-group (unpaired) comparison. Differences between the paired data were calculated by using the Wilcoxon test. Spearman rank correlation coefficient (r) was used to assess the relationships between the parameters.

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CHAPTER 9

NASAL INTERLEUKIN-5 LEVELS DETERMINE THE RESPONSE TO ANTI-INTERLEUKIN-5 TREATMENT IN NASAL POLYP PATIENTS.

NASAL INTERLEUKIN-5 LEVELS DETERMINE THE RESPONSE TO ANTI-INTERLEUKIN-5 TREATMENT IN NASAL POLYP PATIENTS.

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ABSTRACT

Background: Antagonizing the effect of Interleukin-5 (IL-5) is a potential new treatment strategy in nasal polyposis (NP), as polyp tissue is characterized by an eosinophilic inflammation and high IL-5 levels.

Methods: In a double-blind, placebo controlled, randomized, two-center, safety, and pharmacokinetic study, 24 subjects with bilateral NP were randomized to receive a single intravenous infusion of Reslizumab, a humanized anti-human IL-5 monoclonal antibody, at 3 mg/kg or 1 mg/kg, or placebo. We evaluated safety and pharmacokinetics of reslizumab, whereas biological activity was assessed by nasal peak inspiratory flow, symptoms, endoscopic evaluation of NP size, peripheral eosinophil counts, peripheral and local IL-5, soluble IL-5 receptor and ECP levels.

Results: We demonstrated that a single injection of Reslizumab up to 3mg/kg is safe and well tolerated. Blood eosinophil numbers and concentrations of ECP and SOL-IL-5Rα were reduced up to eight weeks after treatment in serum and nasal secretions. Individual nasal polyp scores improved only in half of the treated NP patients for four weeks. Responders had increased IL-5 concentrations in nasal secretions at baseline compared to non-responders, and logistic regression analysis revealed that increased nasal IL-5 levels (> 40 pg/ml) predict the response to anti IL-5 treatment. Only responders had a decrease in nasal IL-5 levels after treatment, which sustained for two weeks.

Conclusion: A single dose of anti IL-5 treatment reduces the size of nasal polyps in half of the patients up to four weeks after intravenous injection, and nasal IL-5 levels predict the response to anti IL-5 treatment.

INTRODUCTION

Bilateral nasal polyps (NP) are characterized by an abundance of eosinophils in more than 80 % of cases and are frequently associated with asthma[1;2]. The role of eosinophils in this disease is not clear, and its clarification awaited the availability of a specific drug approach that would not, like topical or systemic glucocorticosteroids, affect a wide range of cells and mediators. Interleukin (IL)-5 is essential for terminal differentiation of the committed eosinophil precursor, but also activates and prolongs survival of the mature cell in the tissues, and represents such a specific therapeutic target[3;4]. IL-5 was found to be significantly increased in NP, compared to healthy controls and other forms of sinusitis, independent of the atopic status of the patient[5;6]. The highest concentrations of IL-5 were found in NP tissue of subjects with non-allergic asthma and aspirin sensitivity, conditions linked to severe tissue eosinophilia. The key role of IL-5 was supported by the finding that treatment of eosinophil-infiltrated polyp tissue with neutralizing anti-IL-5 monoclonal antibody (mAB), but not anti-IL-3 or anti-GM-CSF mAbs, resulted in eosinophil apoptosis and decreased tissue eosinophilia *in-vitro*[7]. However, as in asthma, only antagonizing the effect of IL-5 in nasal polyposis *in vivo* would be the ultimate test of the IL-5 / eosinophil hypothesis[8].

Several clinical trials with humanized monoclonal antibodies against IL-5 in mild to severe asthmatics demonstrated depletion from blood and sputum eosinophils, but no effect on airway hyperresponsiveness or the late asthmatic reaction to inhaled allergen challenge[9-11]. Kips et al. described a dose rising study with a humanized monoclonal anti-IL5 antibody (Reslizumab; SCH55700) in steroid-treated patients with severe persistent asthma[12]. A single administration of Reslizumab at a maximum dose of 1mg/kg seems to be safe and well tolerated, but despite the profound long-lasting reduction in blood eosinophil counts, the significant increase in FEV1 was short lived and only persisted as a trend at subsequent time points. This rapid but short improvement in FEV1 may have been due to a direct blockade of the actions of IL-5 on airway smooth muscle, which in turn improved airway caliber[12].

At the local tissue level, strategies to antagonize IL-5 may have to face unexpected difficulties since it was demonstrated that in BAL derived eosinophils from asthmatics [13;14]and in nasal polyp tissue, the membrane-anchored (TM) IL-5Rα isoform is down-regulated whereas the secreted (SOL) IL-5Rα variant (antagonistic) is up-regulated compared to peripheral blood[6]. As a target tissue for measuring the effect of anti-IL5 treatment on eosinophilic inflammation, nasal polyps have the advantage of being easily visualized by nasal endoscopy and are accessible for the measurement of IL-5, SOL IL-5Rα and ECP levels. Furthermore, it is

possible to determine if anti-IL5 treatment affects tissue eosinophilic inflammation without the confounding effects of systemic corticosteroids required by most subjects with persistent asthma.

The primary objective of this study was to determine the safety and pharmacokinetics of Reslizumab (SCH55700) given as a single intravenous dose of 3 mg/kg to subjects with severe nasal polyposis. In addition, the activity of Reslizumab on the clinical course of severe nasal polyps, on peripheral and nasal eosinophilic inflammation was evaluated.

METHODS

Patients

24 subjects with massive bilateral nasal polyps (Grade 3 or 4, Table 1) or recurrent nasal polyps after surgery were included. The baseline characteristics were comparable between treatment groups (Table 2), with exception of sex, where males outnumbered females. The exclusion criteria specified that the use of systemic corticosteroids was not allowed during the last month prior to treatment, whereas subjects were not permitted to use systemic and nasal corticosteroids, nasal antihistamines, nasal atropine, nasal cromolyn, nasal saline, and antibiotic treatment up to two months after dosing. The study was conducted at the Departments of Otorhinolaryngology of the University Hospitals in Ghent, Belgium and in Graz, Austria. The local ethics committees approved the study and all volunteers gave a written informed consent before participation in the study.

Table1: Baseline characteristics

		Reslizumab (SCH55700)	
	Placebo (n=8)	***1mg/kg (n=8)***	***3mg/kg (n=8)***
Age, yr (range)	48 (21-59)	43.6 (22-63)	48.5 (18-57)
Female/male	2/6	2/6	4/4
Asthma in history	6	7	5
Nasal surgery in history	4	2	6
Total nasal polyp score (range)	6.00 (2-8)	5.75 (2-8)	4.25 (2-6)
BLOOD/SERUM *(mean±SEM)*			
Blood eosinophils (10^3/ml)	0.21 ± 0.03	0.31 ± 0.09	0.32 ± 0.06
ECP (μg/l)	20.6 ± 8.4	26.6 ± 9.4	20.7 ± 5.9
SOL IL-5Rα (pg/ml)	606.6 ± 112.9	521.6 ± 80.6	1306.7 ± 488.3
NASAL SECRETION *(mean±SEM)*			
ECP (μg/l)	567.5 ± 244.6	876.6 ± 451.9	512.2 ± 153.1
IL-5 (pg/ml)	74.4 ± 26.2	126.9 ± 52.0	55.7 ± 17.7
SOL IL-5Rα (pg/ml)	2122.3 ± 730.2	3758.9 ± 1375.9	4104.8 ± 1764.2

Study Design

This is a Phase I, single dose, randomized, double-blind, placebo controlled, three-arm, parallel group, two-center, safety, and pharmacokinetic study of Reslizumab in patients with nasal polyps. Following a 1-2 week run-in period, subjects were randomized to receive treatment with Reslizumab (Schering-Plough Research Institute, Kenilworth, NJ, US) at 3 mg/kg or 1 mg/kg or placebo. A single dose was given as an intravenous infusion over 30 minutes in a double-blind fashion. Subjects were confined to the study site for 24 hours after dosing for safety evaluations and collection of samples for pharmacokinetic analyses. Follow up visits were scheduled 48 hours after dosing and 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, and 36 weeks after dosing.

Outcome measures

Safety was assessed by adverse event reporting, vital signs, symptom check, physical examination, ECG, blood and urine analysis. The key efficacy variable was the nasal polyp score evaluated for each nostril by nasal endoscopy. Polyps were graded from 0 to 4, based on polyp size: 0 = no polyps; 1 = small polyps in the middle meatus not reaching below the inferior border of the middle concha; 2 = polyps reaching below the lower border of the middle turbinate; 3 = large polyps reaching the lower border of the inferior turbinate or polyps medial to the middle concha; 4 = large polyps causing complete congestion/obstruction of the inferior meatus. Throughout the manuscript, the total nasal polyp score is used, which is the sum of the right and left nostril scores. Furthermore, daily nasal peak inspiratory flow (nPIF) measurements and symptoms (anterior rhinorrhea, nasal obstruction, postnasal drip, and loss of sense of smell) were recorded daily on subjects' diary cards. Biological activity was evaluated by peripheral blood eosinophil counts, peripheral blood and nasal secretion (local) IL-5, SOL IL-5Rα and ECP levels.

Blood eosinophil counts were automated on a 2ml heparinized blood sample. Nasal secretions were obtained by placing sinus packs (IVALON® 4000 plus) in both nasal cavities for exactly 5 minutes. All freshly obtained nasal secretion and serum samples were immediately processed, separated and stored in aliquots at – 20°C until analysis as previously described[6]. Serum and nasal secretions were assayed by ELISA for SOL IL-5Rα (Innogenetics, Ghent, Belgium) and IL-5 (R&D Systems, Minneapolis, USA)[6]. ECP concentrations were measured by the Uni-CAP system (Pharmacia & Upjohn, Upsala, Sweden).

Statistical analysis

Analysis of safety and efficacy were based on the data collected from all subjects randomized in the study (intention-to-treat population). Statistical analysis of efficacy was based on all randomized subjects. However, two subjects, both in the placebo group, had undergone sinus surgery due to unbearable symptoms, and their polyps removed during the treatment period. Therefore, additional analysis, excluding post nasal surgery data, was performed where applicable. Missing observations were replaced by the last non-missing observation carried forward. Results are expressed as means ± SEM. Baseline variables were analyzed by an unpaired T-test. Pair-wise treatment comparisons were obtained from a two-way ANOVA model with treatment and site effects. Changes in clinical and biological parameters were evaluated by repeated measure analysis of variance. We performed logistic regression analysis to identify independent predictors of rebound and response after anti-IL-5 treatment. Differences were regarded as statistically significant if $p < 0.05$.

RESULTS

Safety and adverse events

Twenty-three of the 24 subjects (95.8%) reported at least one adverse event. The one subject who did not report an adverse event was in the 3.0 mg/kg treatment group. In two subjects, both in the placebo group, nasal surgery with removal of the nasal polyps was indicated during the treatment period. The most common adverse event was upper respiratory tract infection, reported by a total of 14 subjects (58.3%): 5 in each of the treatment groups and 4 in the placebo group. Examination of other adverse events did not reveal any major difference between treatment groups. Administration of a single dose of Reslizumab was well tolerated and no clinically meaningful changes were observed in laboratory parameters, vital signs or physical examinations in any of the treatment groups.

Table 2: Clinical course of nasal polyps

				Reslizumab (SCH55700)					
	Placebo (n=8)			***1mg/kg (n=8)***			***3mg/kg (n=8)***		
total NP score compared to baseline score is	worse	no change	better	worse	no change	better	worse	no change	better
Week 1	3	4	1	1	4	3	0	5	3
Week 2	3*	4	1	0	5	3	0	4	4
Week 4	3	3	2	0	4	4	0	5	3
Week 8	3*	4	1	1	2	5	3	4	1
Week 12	3	2	3	1	2	5	4	3	1

Number of subjects who developed a better, an unchanged or a worse total nasal polyp score compared to individual baseline score. * nasal surgery with removal of the nasal polyps was indicated in two subjects

Clinical efficacy analysis

At no individual time point, there was a significant difference in the total nasal polyp score, in the symptom scores or in the nPIF in both treatment groups compared to placebo (Figure 1). However, this study was not designed and not powered to detect treatment differences in efficacy variables. Therefore, the individual clinical course was evaluated and expressed as the number of subjects who had a better, an unchanged or a worse total nasal polyp score compared to the individual baseline score (Table 2). Treatment with 1mg/kg Reslizumab improved the NP scores up to twelve weeks in 5 out of 8 subjects. In the 3mg/kg treatment group, 4 out of 8 patients had a better NP score up to 4 weeks after treatment, however, with a deterioration of the NP score in 4 subjects after 12 weeks after receiving Reslizumab. In the placebo group, three patients had worse nasal polyp scores of whom two patients needed

nasal surgery at 2 and 11 weeks, whereas one patient had a better NP score. Systemic and nasal corticosteroids were permitted after 8 weeks, and were used in 7 and 20 out of 24 patients, respectively, with an equal distribution over the three groups and follow-up visits (from 12 to 36 weeks). To avoid bias by concomitant treatment, further analysis was mainly focused on the first 12 weeks after dosing.

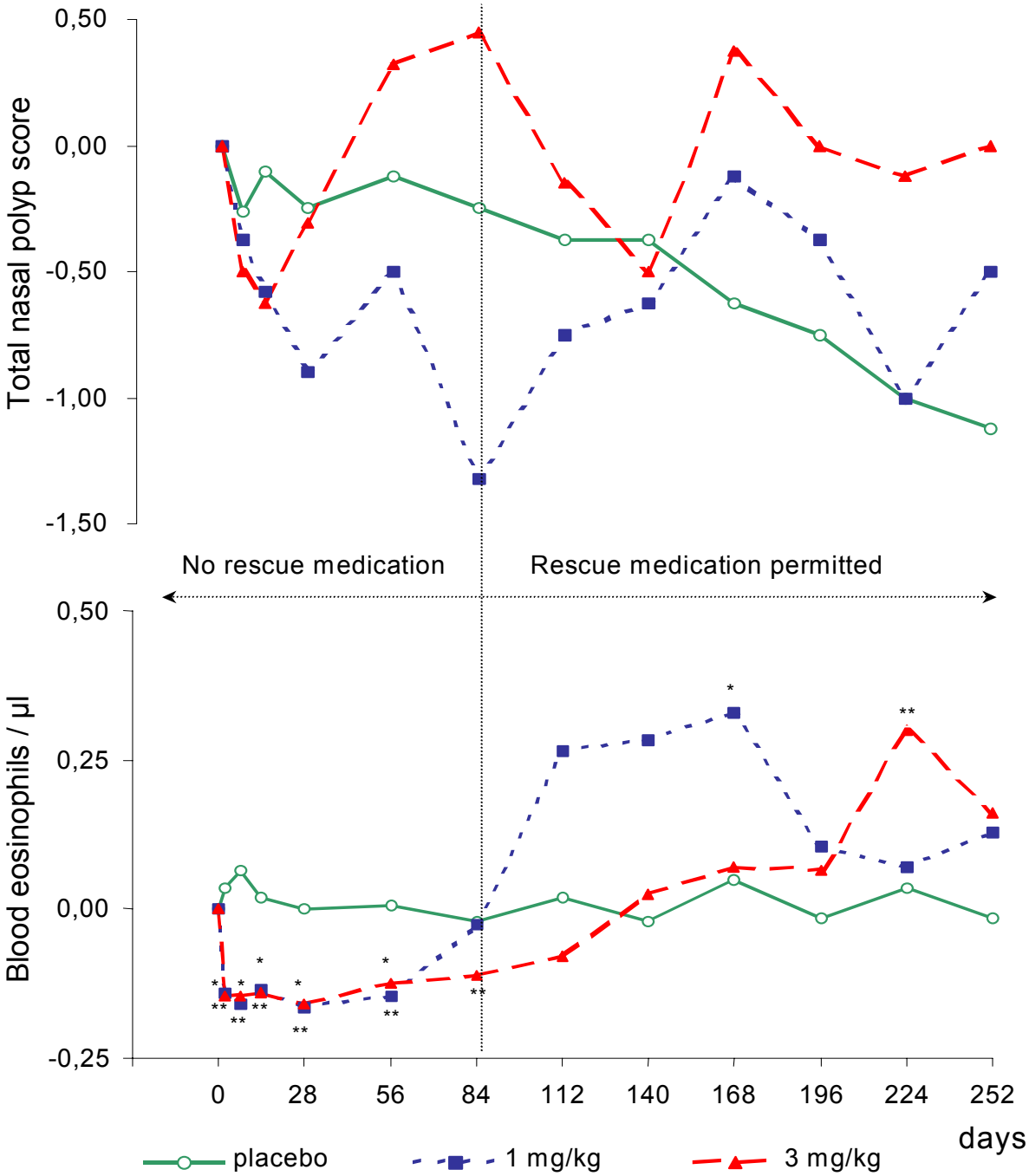

Figure 1: *Change from mean baseline total nasal polyps score (Panel A) and blood eosinophil counts (Panel B) after intravenous administration of placebo (n = 8; open circle), or a single dose of reslizumab, an antihuman IL-5 antibody, at 1 mg/kg (n = 8; solid square) or 3 mg/kg (n = 8; solid triangle). The changes from baseline were significantly different in the 1 mg/kg group (*) and in the 3 mg/kg group (**) compared to placebo (p < 0.05).*

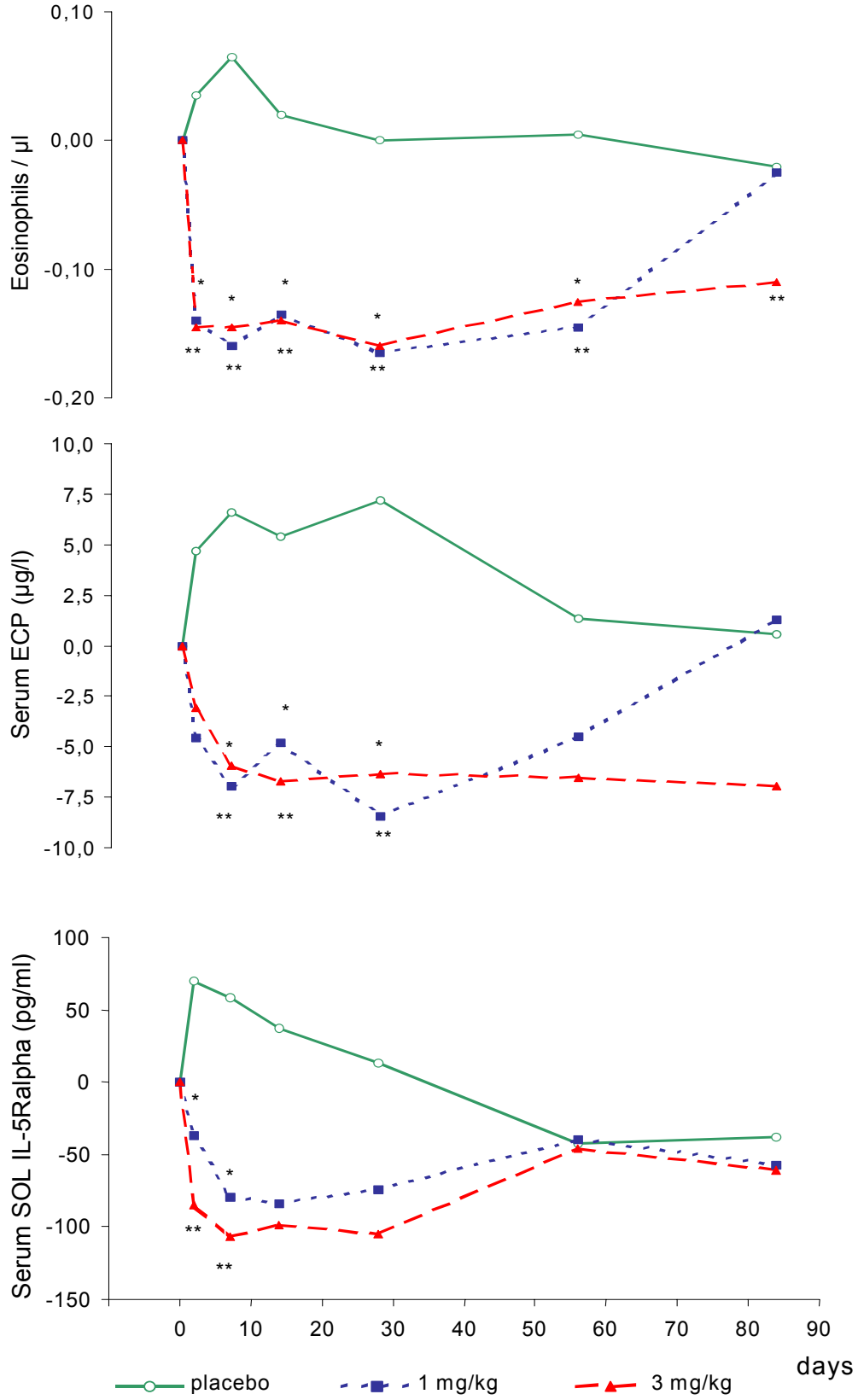

Figure 2:*Panel A shows the effect of a single dose of reslizumab, at 1 mg/kg (n = 8; solid square) or 3 mg/kg (n = 8; solid triangle) versus placebo (n = 8; open circle), on blood eosinophil counts, concentrations of ECP and SOL IL-5Rα in serum.*

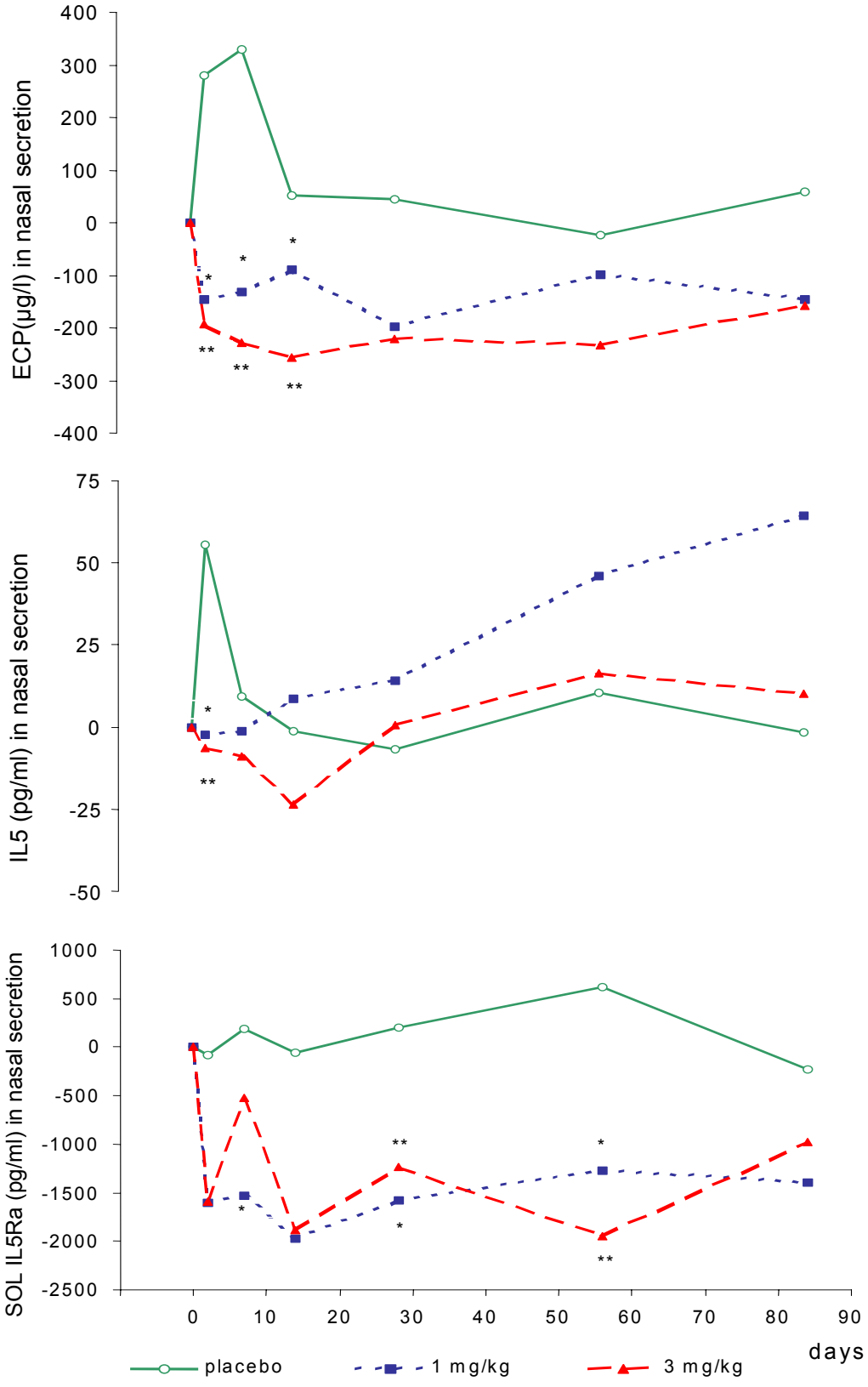

Figure 2 (continued): *Panel B shows the effect on the concentrations of ECP, IL-5 and SOL IL-5Rα in nasal secretions. The mean changes from baseline were significantly different in the 1 mg/kg group (*) and in the 3 mg/kg group (**) compared to placebo ($p < 0.05$).*

Biological activity analysis

Reslizumab induced a significant decrease in blood eosinophils in both treatment groups compared to placebo as early as 12 hours post dose, which sustained through week 8 after dosing (Figure 1). Blood eosinophils returned to baseline levels at week 12. However, a significant rebound eosinophilia was noted at week 24 and week 32 after 1mg/kg and 3mg/kg treatment, respectively (Figure 1). Rebound eosinophilia with a more than 100% increase of baseline eosinophil numbers was present in 6 and 4 patients of the 1mg/kg and 3mg/kg treatment group, respectively. Serum ECP and SOL IL-5Rα levels decreased significantly in both treatment groups compared to placebo and paralleled the blood eosinophil counts, but did not show a significant rebound at later time points (Figure 2a).

Nasal secretion levels of ECP and SOL IL-5Rα decreased in both groups receiving Reslizumab compared to placebo and sustained through week 8 (Figure 2b). Nasal IL-5 concentrations were only significantly suppressed at day 2 in both treatment groups versus placebo. In the 1mg/kg group, nasal IL-5 levels exceeded baseline levels at 4 weeks, and reached its maximal level at 16 weeks post dose, without statistical significance. The evolution of the nasal IL-5 levels in the 3mg/kg was similar but less pronounced than in the 1mg/kg treatment group.

Responders versus Non-responders

Based on the individual course of the total nasal polyp score (all treated patients with an improvement of one unit within 4 weeks post dosing), were sorted in responders (n=8) and non-responders (n=8). The total nasal polyp score was significantly decreased in responders compared to non-responders from 1 to 4 weeks post treatment (Figure 2). At baseline, the nasal scores and biological markers were comparable between both groups, with the exception the IL-5 levels in nasal secretion, which were significantly higher in the responders than in the non-responders ($p = 0.03$; Table 3). Logistic regression analysis revealed that increased nasal IL-5 levels (> 40 pg/ml) predict the response to anti IL-5 treatment (odds ratio = 21.0; 95% confidence interval = 1.5 to 293.3; $p = 0.009$), whereas no other variables could be retained in the model. Nasal IL-5 concentrations decreased significantly within 2 days post dose and sustained through week 2 after dosing in the responders (Figure 2), whereas it increased in the non-responders with reaching significance within 12 to 20 weeks after active treatment. The decrease in nasal ECP levels was only significant for 1 week and was more pronounced in responders compared to non-responders. There was a significant decrease in blood eosinophil counts and SOL IL-5Rα concentrations in serum and nasal secretions in

both treated groups compared to placebo (similar as in Figure 1), without significant difference between responders and non-responders (data not shown).

Table 3: Comparison of baseline characteristics responders versus non-responders.
Based on the individual course of the total nasal polyp score all treated patients (with an improvement of one unit within 4 weeks post dosing) were sorted in responders and non-responders.

	Reslizumab (SCH55700) at 1mg/kg or 3mg/kg		
	Non-responders (n=8)		*Responders (n=8)*
Age (years)	48 (33-63)	ns	42 (18-57)
Sex (female/male)	4/4	ns	2/6
Asthma in history	6	ns	6
Sinus Surgery in history	5	ns	3
Total nasal polyp score (max. 8)	4.12 (2-8)	ns	5.87 (2-8)
SERUM			
Blood eosinophils (10^3/ml)	0.38 ± 0.09	ns	0.24 ± 0.03
ECP (µg/l)	30.3 ± 9.1	ns	17.1 ± 5.6
SOL IL-5Rα (pg/ml)	870.4 ± 80.6	ns	957.9 ± 461.9
NASAL SECRETION			
ECP (µg/l)	876.6 ± 451.9	ns	512.2 ± 153.1
IL-5 (pg/ml)	32.8 ± 4.4	p = 0.03	149.9 ± 48.8
SOL IL-5Rα (pg/ml)	4123.7 ± 1802.8	ns	3740.0 ± 1324.2

DISCUSSION

This study demonstrates that administration of a single dose of Reslizumab, a humanized anti-human IL-5 monoclonal antibody, at 3mg/kg is safe and well tolerated in subjects with nasal polyposis. Individual nasal polyp scores improved only in half of the verum-treated patients for up to four weeks. When carefully analysing responders and non-responders, only those nasal polyps with increased levels of IL-5 (> 40pg/ml) in nasal secretions before treatment seemed to benefit from anti-IL5 treatment. Furthermore, nasal IL-5 decreased only in the responders, whereas it increased in the non-responders. The decrease in circulating blood eosinophils was as pronounced in responders as in non-responders and sustained for 8 weeks, whereas the decrease in nasal ECP was stronger in responders, but lasted for one week only. These data show that at least in 50% of the nasal polyps, IL-5 and eosinophils play a key role (IL-5-dependent) in sustaining polyp size, whereas in the other group, eosinophilia may be dependent on other factors such as eotaxin (IL-5-independent)[15].

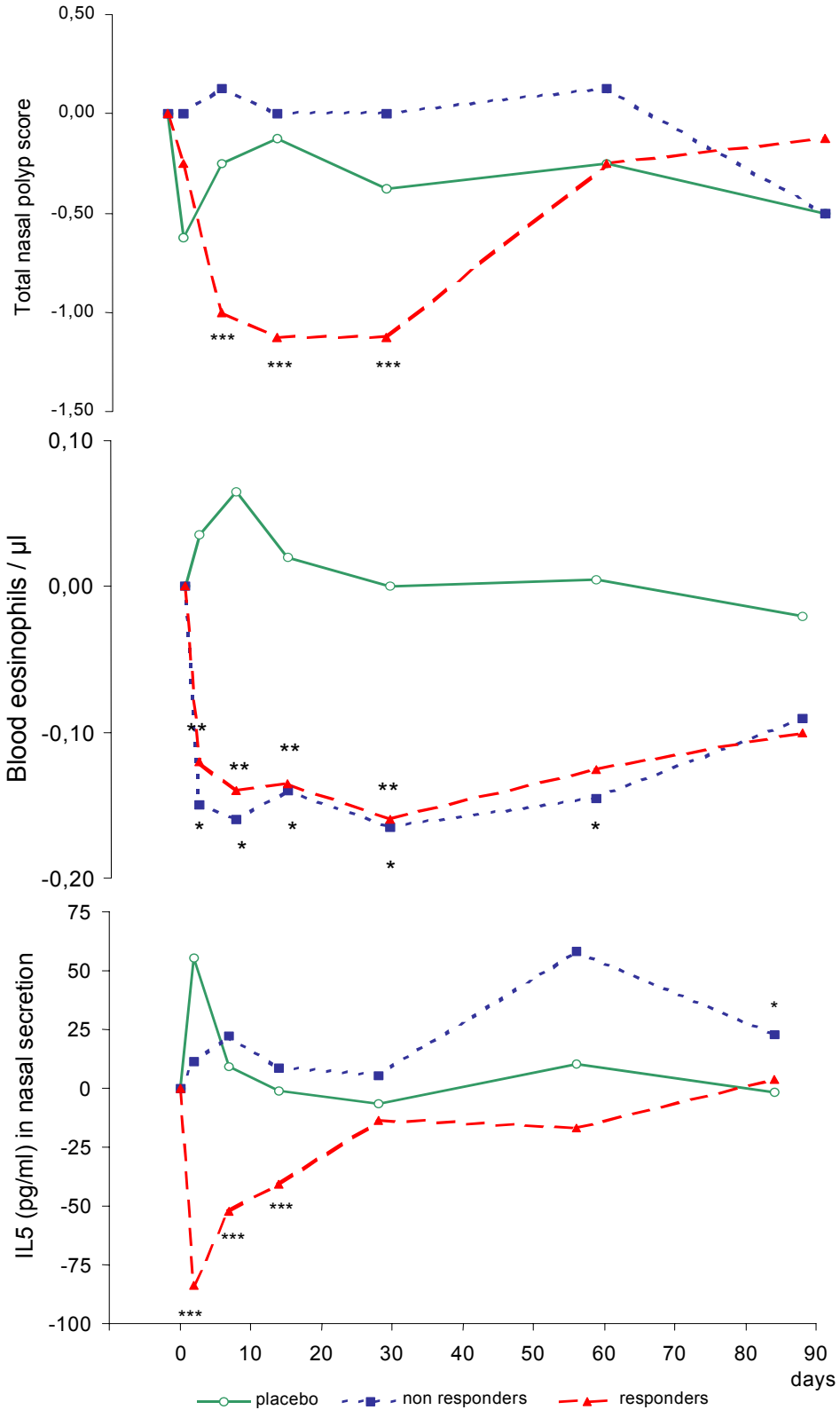

Figure 3: *Based on the individual course of the total nasal polyp score all treated patients with an improvement of one unit within 4 weeks post dosing, were sorted in responders (n = 8; solid triangle) and non-responders (n = 8; solid square). The total nasal polyp score and concentrations of IL-5 in nasal secretions were significantly decreased in responders (***) compared to non-responders. The mean changes from baseline in blood eosinophil counts were significantly different in the non-responders (*) and in the responders (**) compared to placebo ($p < 0.05$).*

Analysis of anti-IL-5 studies in animal models of allergic disease shows that, although it has been observed that eosinophil trafficking to the allergic mucosa is markedly attenuated in IL-5-/- mice or those treated with anti-IL-5 antibodies in comparison with wild-type responses, a marked residual tissue eosinophilia can persist in these mice after allergen inhalation[16-19]. Indeed, in a clinical trial, Flood-Page et al. observed a significant differential effect of IL-5 blockade on eosinophil counts in various body compartments.[9;10] After multiple dosing with mepolizumab, another anti-IL-5 mab, the authors found 100% reduction in blood eosinophils, but only 52% reduction in the bone marrow and a 55% decrease in the bronchial mucosa[10]. A possible reason for a different effect of anti-IL-5 in one compartment as opposed to another may be due to poor penetration of anti-IL-5 into the tissues or a varying IL-5 sensitivity due to a different expression of the IL-5Rα isoforms according to activation state, maturation and localization in the body. Liu et al. showed that IL-5 receptor expression on airway eosinophils was down-regulated *in vivo* after inhaled allergen challenge, associated with a loss of IL-5 responsiveness[13;14]. In addition, Gregory et al. have demonstrated that exposure of blood eosinophils to IL-3, IL-5, or granulocyte-macrophage colony-stimulating factor *in vitro* leads to sustained down-regulation of SOL IL-5Rα expression and reduced responsiveness to IL-5, but no sustained changes in CCR3 expression[20]. Analysis of nasal tissue samples revealed that SOL IL-5Rα protein and mRNA levels were significantly increased in polyp versus control tissue, whereas the TM IL-5Rα was down-regulated in polyp tissue with severe eosinophilia[6]. Here we demonstrate that anti-IL-5 treatment of NP patients reduced SOL IL-5Rα levels for several weeks, especially in nasal secretions. Since SOL IL-5Rα shows antagonistic activity *in vitro*[21-24], it could theoretically be expected that assessment of endogenous SOL IL-5Rα levels may distinguish responders from non-responders, or help in the titration of anti-IL-5 mAb therapy[6]. However, SOL IL-5Rα levels did not show any relation to the individual response in this safety study in NP patients. In contrast, local IL-5 concentrations predicted the response: only NP patients with increased nasal IL-5 showed a reduction of nasal polyp scores after a single dose of reslizumab.

Recently, Foster et al. have shown that local chemokine networks in the allergic lung regulate eosinophil accumulation independently of IL-5, and that this mechanism plays an important role in disease processes[15]. In the absence of IL-5 and eotaxin, tissue eosinophilia is abolished in BALB/c mice and so is AHR[16;17]. Furthermore, inhibition of eotaxin alone does not abolish eosinophilia and AHR, thus targeting both pathways is required[17]. These studies indicate that pathways operated by local chemokine systems (in particular those which

involve CCR3, the eotaxin receptor) play an important role in regulating the recruitment of eosinophils into tissues independently of IL-5, and that this mechanism is linked to the induction of disease. Importantly, this mechanism also operates in the absence of blood eosinophilia[15].

A heterogeneous group of disorders characterized by the presence of unexplained blood eosinophilia is hypereosinophilic syndrome (HES). Two reports showed successful treatment of patients with mepolizumab and reslizumab [25;26]. Anti-IL5 therapy effectively controlled eosinophilic dermatitis with a drop in eosinophil counts, IL-5, eotaxin and ECP levels in serum[26]. However, one report described an exacerbation of symptoms and a rebound eosinophilia as drug levels waned[25]. Reinstitution of monthly anti-IL-5 treatment led to decreased eosinophilia and symptomatic improvement[25]. Here we describe a rebound eosinophilia in 10 out of 16 NP patients after anti-IL-5 treatment, but without major exacerbation of symptoms. In the 3mg/kg treatment group, the rebound was less dramatic and later than in the 1mg/kg, suggesting that higher concentrations and/or repeated dosing of anti-IL-5 mabs might overcome rebound effects. Currently, none of the present studies in asthma, HES or nasal polyps were large enough to detect different subgroups and to differentiate between patients with IL-5 dependent disease from those mainly dependent on other mediators. Long-term studies in well characterized patients are therefore needed and should include analysis of local IL-5 and other cytokines or chemokines such as eotaxin.

In summary, we here show that anti-IL-5 treatment results in a decrease in volume of nasal polyps only in patients with increased nasal IL-5 levels. It is therefore suggested to select appropriate patients before conducting further clinical trials with IL-5 antagonists. Furthermore, a combination therapy with anti-IL-5 and a CCR3 antagonist may be another successful approach, as this would have the advantage of inhibiting both bone marrow maturation (primarily IL-5 dependent) and tissue accumulation (mainly a CCR3-dependent effect).

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CHAPTER 10

DISCUSSION - PERSPECTIVES

DISCUSSION - PERSPECTIVES

In view of the prominence of eosinophilic inflammation associated with the vast majority of nasal polyps and the well recognised potential of eosinophils in eliciting tissue damage and subsequent re-modelling, it is likely that a better understanding of the mechanisms underlying the migration, activation and maintenance of eosinophils in nasal polyp tissue will be key to understanding the aetiology and pathogenesis of nasal polyps. Especially the regulation of interleukin-5 and the interleukin-5 receptor α isoforms were studied with emphasis on future therapeutic strategies in NP.

NASAL POLYPOSIS: AN EOSINOPHIL MEDIATED DISEASE?

In **chapter 4** we aimed to identify the most important factors in polyp growth. How eosinophilic inflammation leads to polyp formation remains largely unclear. Histomorphologic characterisation of two early stage manifestations of NP showed the presence of eosinophils, forming a subepithelial cap over a pseudocyst area, which was filled with albumin. In mature NP, a large pseudocyst area containing albumin was surrounded by subepithelial eosinophilia. The stroma of mature polyps is mainly characterised by its oedematous nature and consists of supporting fibroblasts and infiltrating inflammatory cells, localized around "empty" pseudocyst formations. These observations suggest a central deposition of plasma proteins (albumin), regulated by the subepithelial, mainly eosinophilic inflammation, as pathogenetic principle of polyp formation and growth. The extravasated plasma - for reasons of distance, binding force, or by extracellular matrix abnormality – may not find its way to the airway surface. Furthermore, the subepithelial accumulation of eosinophils points to the epithelium as probable location of a stimulating factor (such as antigens or an infection) for polyp etiogenesis.

Second, in an approach to quantify and to study possible relations between eosinophilic inflammation and changes in the concentrations of extracellular tissue components, we measured IL-5, eotaxin, ECP, LTC4/D4/E4, TGF-β_1, fibronectin, hyaluronic acid and albumin in nasal tissue homogenates of control and NP patients. The comparison between the untreated polyp group and controls showed significantly higher concentrations of IL-5, eotaxin, ECP and albumin in polyp supernatants, whereas TGF-β1 was significantly lower. Thus, the overproduction of IL-5 and the lack of TGF- β_1 would favor eosinophil survival and facilitate degradation of tissue matrix, both characteristics of polyp formation.

Third, in oral GCS treated polyps, IL-5, ECP and albumin were significantly reduced compared to untreated nasal polyps, whereas TGF-β_1 was increased. These data indicate that GCS affect nasal polyps by reducing eosinophil inflammation and albumin retention, leading to the shrinkage of NP (Figure 5). The major drawback is that it is a retrospective, descriptive finding in a limited number of subjects. On the other hand, it is surprising that little evidence or studies are present on treatment with oral corticosteroids of nasal polyps, although these treatment courses are daily practice in the ENT clinic.

Perspectives

- We are currently performing the first double-blind, placebo controlled, multi-centre study with oral administration of methylprednisolone or placebo in nasal polyp patients. The above studied markers and mediators will be measured in serum and nasal secretion.
- A paper on the evolution and impact of eosinophilic inflammation after sinus surgery is in preparation (in cooperation with Dr. JB. Watelet).
- In this thesis we focused exclusively on bilateral nasal polyposis, however currently we are comparing these findings with other chronic sinus diseases such as chronic rhino-sinusitis and nasal polyps in cystic fibrosis (in cooperation with Dr. S. Claeys).

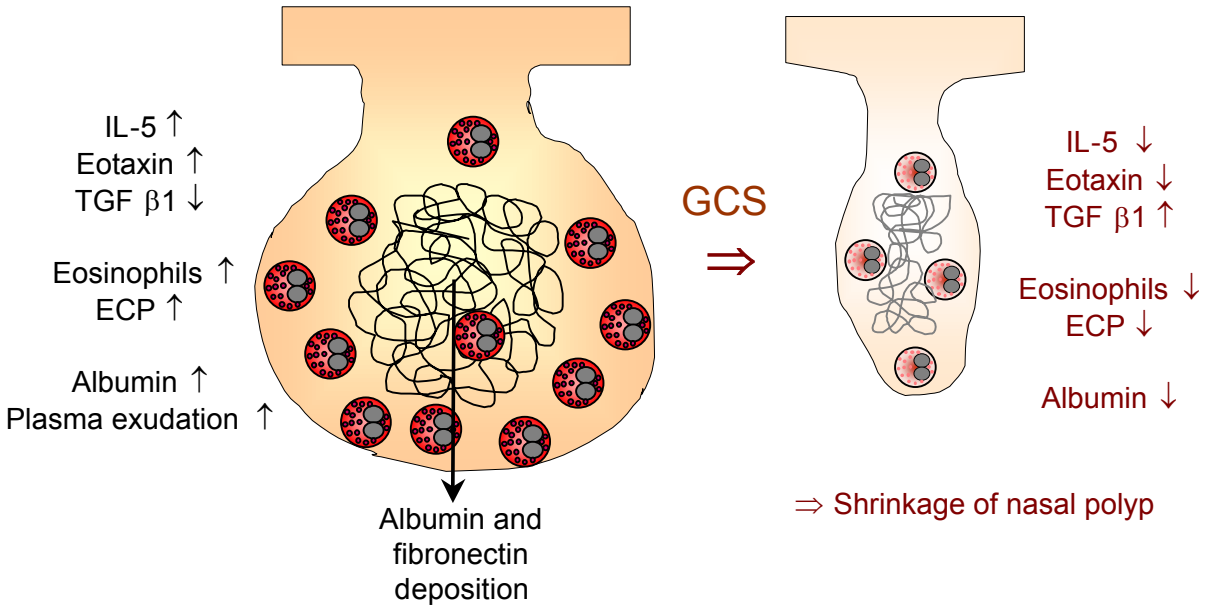

Figure 1: *Eosinophils in the pathophyiology of nasal polyposis and the impact of oral corticosteroid treatment (GCS)*

EOSINOPHILIC INFLAMMATION AND IGE FORMATION IN NASAL POLYPS

Another hallmark of bilateral nasal polyposis are the elevated total IgE levels in polyp fluid that accompanies tissue eosinophilia. Therefore, an allergic aetiology of nasal polyps has been presumed, but never firmly demonstrated. **In chapter 5** we could demonstrate that skin prick tests do not predict total IgE levels in polyp homogenates. In 10 out of 20 non-polyp and in 13 out of 20 NP patients, the skin prick test was positive for at least one allergen. As expected, increased total IgE and specific IgE in the nasal tissue of subjects allergic rhinitis was found in correspondence with the skin prick test results. In marked contrast, atopy based on positive skin prick tests to inhalant allergens was not related to IgE concentrations or IgE antibodies in NP tissue. Furthermore, within the NP group no impact was found on the levels of IgE, IL-5, IL-4, eotaxin, LTC4/D4/E4, sCD23, ECP and number of tissue eosinophils, questioning the role of common allergens in nasal polyp pathophysiology. Strikingly, IL-5, eotaxin, ECP and number of eosinophils in NP tissue were significantly correlated with tissue total IgE.

In chapter 6 we demonstrated the organisation of secondary lymphoid tissue in nasal polyp tissue and a polyclonal hyper-immunoglobulinemia E associated with the presence of IgE specific to *S. aureus enterotoxins (*SAEs), colonization with *S. aureus,* and increased eosinophilic inflammation in a relevant subgroup of nasal polyp patients. Furthermore, the dissociation in IgE levels and IgE antibodies between nasal polyp tissue and serum suggests that IgE in NP are mainly due to a local production. Detailed analysis of IgE expression in serum and tissue of NP patients reveals two patterns: “the allergic type” and “the polyclonal type” that can be found either isolated or combined. The “allergic” type of IgE expression is characterized by increased concentrations of total IgE and IgE antibody specificities in nasal tissue that correspond to those in serum and to the skin prick test results. In contrast, the “polyclonal” IgE expression is a local process and IgE antibodies found in polyp tissue are only partially reflected in serum of the same patients and are independent of the skin prick results. Notably, this polyclonal expression is associated with a hyper-immunoglobulinemia and only a small fraction of the total can be explained by IgE antibodies. Polyclonal expression was described in 16/24 NP tissues and was associated with IgE antibodies to SAEs in 12 cases, indicating that other than the classic enterotoxins might have acted as superantigens. Additionally we found a higher incidence of *S. aureus* colonisation (17/24) and IgE antibodies to SAEs in NP tissue (12/24) compared to controls (resp. 3/12 and 0/12).

An augmented local synthesis of IgE may, under appropriate circumstances, increase allergic reactivity, but when it is excessive, it could suppress specific reactivity by saturation of Fcε receptors on mast cells through polyclonal IgE and/or an inhibition of specific antibody

synthesis to envorimental allergens by the polyclonal response. These mechanisms could explain why nasal symptoms and markers of nasal mucosal inflammation did not increase in relation to natural seasonal allergen exposure in highly ragweed-allergic patients with polyps and why nasal provocation in nasal polyps is largely unsuccessful although elevated IgE levels are present. However, the backside of this local polyclonal IgE production may be the permanent triggering of the IgE-mast cell-FcεRI cascade that maintains polyp growth.

We illustrated binding of biotinylated SAE to follicular structures and in lymphoid accumulations in NP tissue. The follicular structures were mainly characterized by an accumulation of B-cells and T cells, whereas lymphoid accumulations showed diffuse plasma cell infiltration. These data suggest an organization of secondary lymphoid tissue with polyclonal B-cell activation in nasal polyps due to chronic microbial colonization and release of enterotoxins. In this context staphylococcal toxins can act as both superantigens and "conventional" allergens and therefore play a role in modulating chronic inflammatory airway disease, via both non-IgE and IgE-mediated mechanisms.

Relevance to other diseases

Studies of patients predominantly suffering from atopic dermatitis have increasingly demonstrated associations between incidence of disease and colonisation with *S. aureus* or presence of staphylococcal superantigens, thus proposing a putative causative role for *S. aureus* in the pathogenesis of atopic dermatitis. Recently, we demonstrated that IgE antibodies to SAE were more often found in serum from patients with severe asthma than in those with mild asthma [1]. These antibodies were linked to the severity of inflammation, concentrations of IgE antibodies, and corticosteroid dependence. Similarly to severe asthma, we found significantly elevated IgE to SAE in COPD patients [2].

Finally, these findings on local IgE formation in NP that is independent of skin prick tests or serum RAST data are of particular interest in the understanding of other so called non atopic diseases such as intrinsic asthma or NARES (nonallergic rhinitis with eosinophilia syndrome).

Perspectives

- The evidence for the role of S. aureus superantigens in airways disease is preliminary and, at best, circumstantial. Eradication of S. aureus may provide insights leading novel therapeutic strategies for these disorders. Currently, we are performing a double-blind, placebo controlled, multi-centre trial comparing oral doxycyline to placebo in NP patients.
- Considering the marked local production of IgE-antibodies in nasal polyps and its relation to severity of disease, strategies to antagonize IgE antibodies could be of relevance (an application with the FWO is submitted to study the effect of anti-IgE-treatment on NP).

INTERLEUKIN-5 RECEPTOR α ISOFORM EXPRESSION IN NASAL POLYPOSIS

Given the key role of interleukin-5 in processes leading to differentiation, recruitment and activation of eosinophils, we investigated IL-5Rα expression in human nasal polyposis. Regulated alternative splicing of the IL-5Rα-subunit leads to the generation of either a signaling, membrane-anchored (TM) isoform, or a soluble (SOL) variant with antagonistic properties *in vitro*. It is suggested that eosinophils are able to control their responsiveness to IL-5 by regulated expression of these IL-5Rα isoforms. Therefore, a better understanding of the biology of IL-5 and the regulation of its receptors seems to be mandatory to understand the effect of anti-IL-5 treatment in eosinophil-associated diseases.

In chapter 7 we described a sensitive, specific and reliable ELISA that enables determination of SOL-IL-5Rα concentrations in various human body fluids such as serum, nasal secretion and nasal tissue homogenates. In nasal secretion, SOL-IL-5 Rα concentrations were well detectable and there was a strong correlation to the SOL-IL-5 Rα concentrations in nasal tissue confirming that collection of nasal secretion is a valuable alternative for biopsies. Furthermore, we demonstrated an abundant SOL-IL-5Rα expression in NP with concomitant asthma often dramatically exceeding the IL-5 concentrations. Indeed, in nasal tissue SOL-IL-5Rα levels up to 1200 times higher than IL-5 concentrations can be found. This finding questions the physiologic role of SOL-IL-5Rα. On one hand it could be that SOL-IL-5Rα is up-regulated to antagonise the massive eosinophilic inflammation. However, our data in a hIL-5-driven proliferation assay show that only an excess of SOL-IL-5Rα (18500 times) compared to IL-5 can block its activity. Although SOL-IL-5Rα protein has antagonistic properties *in vitro*, it is important to note that the endogenous concentrations might be insufficient to block IL-5 activity *in vivo*. Moreover, secreted receptors may bind their ligands in circulation, protecting them from proteolytic inactivation and prolonging their serum half-life or facilitating ligand-mediated signaling. Hence, SOL-IL-5Rα may be involved in the fine-tuning of eosinophil homeostasis not only as an antagonist, but possibly also as a carrier.

In chapter 8 we examined SOL and TM-IL-5Rα expression and regulation at both protein and transcript level *in vitro* and *in vivo* by FACS, ELISA and real-time PCR. In blood, SOL and TM-IL-5Rα mRNA and protein expression was up-regulated in NP versus controls and correlated to eosinophil percentages, whereas in polyp tissue, TM-IL-5Rα levels showed an inverse relation to eosinophilia and SOL-IL-5Rα expression. Blood eosinophils express high levels of surface-anchored TM-IL-5Rα and are not activated as judged by a low CD69

expression. In contrast, tissue eosinophils are activated and demonstrate a low level of surface receptors to IL-5. Hence, in tissue with the highest levels of IL-5 and eosinophils, a regulatory mechanism must operate to control the ongoing eosinophilic inflammation by up-regulation of SOL-IL-5Rα and down-regulation of TM-IL-5Rα.

In vitro exposure of mature human blood eosinophils with rh IL-5 for 2 and 24 hours induces an extensive down-regulation of IL-5Rα TM protein and mRNA, whereas SOL-IL-5Rα was only significantly up-regulated at the protein level. It is suggested that the down-modulation of TM-IL-5Rα from the cell surface and the increased release of SOL-IL-5Rα into culture supernatant fluid dependent on MMP activity. Although the expression membrane type-1 MMP (MT1-MMP or MMP-14) is not different in polyps and controls and is not influenced by IL-5 stimulation of eosinophils, we found a significant inverse correlation between MMP-14 and IL-5Rα surface expression. In addition, Gregory (in cooperation with us) have demonstrated that exposure of blood eosinophils to IL-3, IL-5, or granulocyte-macrophage colony-stimulating factor *in vitro* leads to sustained down regulation of surface IL-5Rα expression and reduced responsiveness to IL-5, but without sustained changes in CCR3 expression [3].

Taken all observations into account we suggest that an IL-5 driven inflammation generates an eosinophil tissue phenotype that is characterized by a low TM but high SOL-IL-5Rα expression and that this process is partially the result of proteolytic receptor modulation and down-regulation of TM-IL-5Rα gene transcription. Furthermore, the expression of the IL-5Rα isoforms differs according to the eosinophil activation state, maturation and localization in the body and may therefore be involved in the fine-tuning of the eosinophil homeostasis. Since IL-5Rα surface expression is down-regulated in NP tissue, which probably causes IL-5 responsiveness, our findings indicate that strategies to antagonize IL-5 may have to face unexpected difficulties.

Relevance to other diseases

In serum of asthmatics we found increased SOL IL-5Rα levels compared to controls, whereas within the asthma group, SOL IL-5Rα levels were significantly higher in severe versus mild asthma *(in cooperation with P Howarth, Southampton, UK).* Remarkably, serum levels of SOL IL5Rα are increased in chronic obstructive pulmonary disease (COPD) with the highest levels in virus-associated exacerbations of COPD *(in cooperation with G. Rohde, Bochum, Germany)* [4].

In atopic dermatitis patients serum levels of SOL IL-5Rα are increased compared to healthy controls and correlated with the SCORAD, which represents severity of disease *(in cooperation with A Kapp, Hannover, Germany).*

Perspectives

In human serum, we demonstrate that SOL IL-5 Rα concentrations are detectable in all serum samples and with a median concentration of 210 pg/ml in healthy controls. Despite absent to low eosinophil counts in controls, a baseline expression of SOL IL-5Rα is found in all samples (serum, tissue, nasal secretion) without any relation to eosinophil numbers. Certainly, this finding needs further investigation, especially because it points again to other cellular sources of IL-5 Rα next to eosinophils. With reliable methods now in place to determine SOL IL-5Rα protein and mRNA expression, detailed studies can be performed to detect other cell populations expressing IL-5Rα.

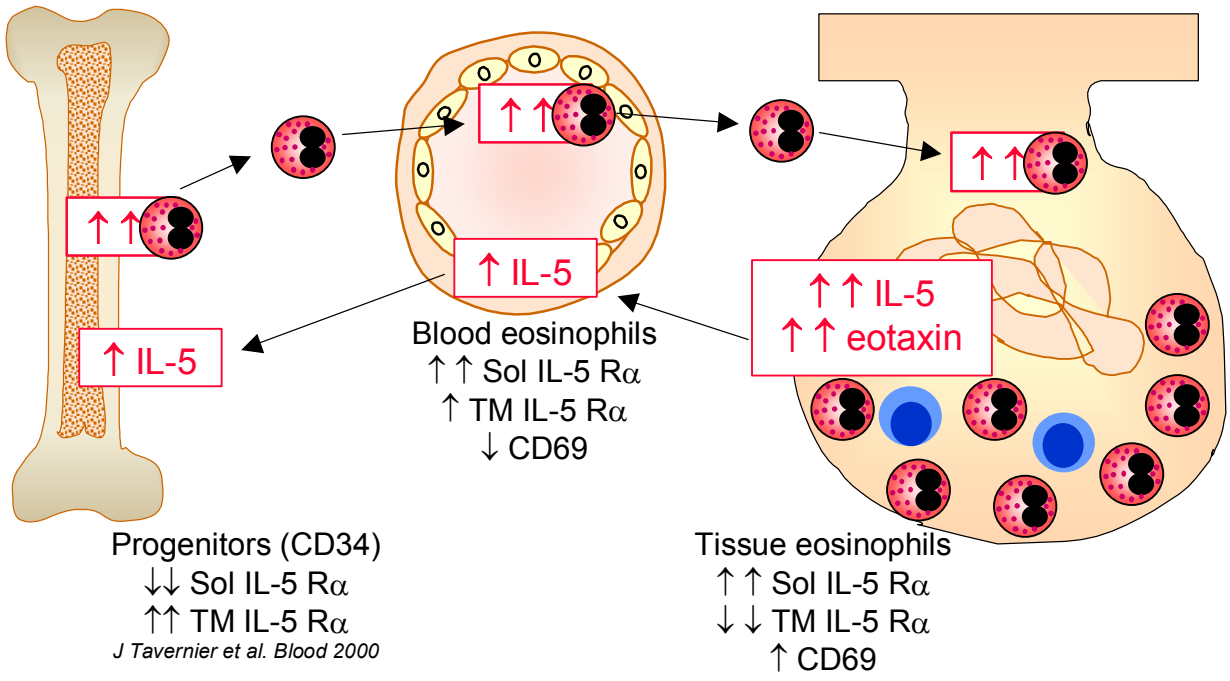

Figure 2: *Regulated interleukin 5 receptor α isoform expression in blood and tissue eosinophils.*

ANTAGONISING THE IL-5 FUNCTION IN NASAL POLYPS

The key role of eosinophils in the pathogenesis of nasal polyposis is strongly suggested by the previous studies, however never firmly proven since the findings are observational. Indeed, the increased concentrations of IL-5 and severe tissue eosinophilia were found in NP tissue and treatment with oral corticosteroids affect nasal polyps by reducing eosinophil numbers, IL-5 levels, and albumin retention, leading to the shrinkage of NP. Systemic glucocorticosteroids affect a wide range of cells and mediators and therefore, these finding only may be circumstantial. Given the key-role of IL-5 in the eosinophil biology, treatment strategies antagonizing IL-5 offers the ultimate opportunity to test the IL-5 / eosinophil hypothesis in nasal polyposis.

In chapter 9 we described a double-blind, placebo controlled, randomized, two-center, safety, and pharmacokinetic study, 24 subjects with bilateral NP were randomized to receive a single intravenous infusion of Reslizumab, a humanized anti-human IL-5 monoclonal antibody, at 3 mg/kg or 1 mg/kg, or placebo. It is important to note that the primary objective of this study was to determine the safety and pharmacokinetics of Reslizumab (SCH55700) and that it was not designed and not powered to detect treatment differences in efficacy variables. Nevertheless, we assessed biological activity by nasal peak inspiratory flow, symptoms, endoscopic evaluation of NP size, peripheral eosinophil counts, peripheral and local IL-5, soluble IL-5 receptor and ECP levels.

Administration of a single dose of Reslizumab, a humanized anti-human IL-5 monoclonal antibody, at 3 mg/kg is safe and well tolerated in subjects with nasal polyposis. Individual nasal polyp scores improved only in half of the verum-treated patients for up to four weeks. When carefully analysing responders and non-responders, only those nasal polyps with increased levels of IL-5 (> 40pg/ml) in nasal secretions before treatment seemed to benefit from anti-IL-5 treatment. Furthermore, nasal IL-5 decreased only in the responders, whereas it increased in the non-responders. Remarkably, blood eosinophil numbers and ECP levels in serum and nasal secretion were not different at baseline between responders and non-responders. The decrease in circulating blood eosinophils after anti-IL-5 treatment was as pronounced in responders as in non-responders and sustained for 8 weeks, whereas the decrease in nasal ECP was stronger in responders, but lasted for one week only. Anti-IL-5 treatment reduced SOL IL-5Rα levels for several weeks, especially in nasal secretions. As regulatory properties (antagonist/carrier) of SOL IL-5Rα were proposed, it could theoretically be expected that assessment of endogenous SOL IL-5Rα levels may distinguish responders

from non-responders, or help in the titration of anti-IL-5 mAb therapy. In contrast to local IL-5, concentrations of SOL IL-5Rα did not show any relation to the individual response.

Although a single injection of anti-IL-5 treatment resulted in shrinkage of NP in half of the patients, nasal polyps never disappeared completely. Furthermore, it is clear from this and other studies that anti-IL-5 treatment failed to completely inhibit eosinophilia at the tissue level. Completely blocking IL-5 activity *in vivo* therefore appears to be much more difficult than *in vitro*. A possible reason may be the poor penetration of anti-IL-5 into the tissues or a varying IL-5 sensitivity due to a different expression of the IL-5Rα isoforms according to activation state, maturation and localization in the body. Nevertheless, the ultimate judgement on the role of eosinophils in nasal polyps cannot be made based on the current therapeutic anti-IL-5 approach. However, we may conclude that at least in 50% of the nasal polyps, IL-5 and eosinophils play a key role (IL-5-dependent) in sustaining polyp size, whereas in the other group, eosinophilia may be dependent on other factors such as eotaxin (IL-5-independent).

Relevance to other diseases

Asthma was, indeed, for long the main target of anti-IL-5 treatment. Currently, the first reflections on the disappointing clinical results are the subject of many debates. The role of IL-5 and eosinophils in asthma is even more complex than in nasal polyposis, since there exist many features of asthma, including airflow obstruction, bronchial hyperresponsiveness and airway remodelling, each likely to be regulated by distinct processes. In this respect comparison of the findings between asthma, nasal polyps or atopic dermatitis are of interest.

A heterogeneous group of disorders characterized by the presence of unexplained blood eosinophilia is hypereosinophilic syndrome (HES). Two reports showed successful treatment of patients with mepolizumab and reslizumab. Anti-IL5 therapy effectively controlled eosinophilic dermatitis with a drop in eosinophil counts, IL-5, eotaxin and ECP levels in serum.

Perspectives

This study re-opens therapeutic perspectives in nasal polyposis based on eosinophil-selective targets. Since anti-IL-5 treatment resulted only in a decrease in volume of nasal polyps in patients with increased nasal IL-5 levels, further clinical trials with IL-5 antagonists should be conducted in appropriately selected patients. Furthermore, a combination therapy with anti-IL-5 and a CCR3 antagonist may be another successful approach, as this would have the advantage of inhibiting both bone marrow maturation (primarily IL-5 dependent) and tissue accumulation (mainly a CCR3-dependent effect).

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CHAPTER 11

CURRICULUM VITAE

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Cellular and biochemical approach to the extracellular matrix in nasal polyps and its relation to eosinophils, BOF, Ghent University

Expression of human interleukin 5 receptor α subunit isoforms in nasal polyposis, IWT-Innogenetics - Ghent University

Eosinophilic inflammatoin and expression of isoforms of the human interleukin 5 receptor α subunit in nasal polyposis, BOF mandate, Ghent University

Phase I study: Safety, Tolerance, and Pharmacokinetics of Reslizumab (Anti IL-5) in Patients With Nasal Polyposis, Schering-Plough Research Institute

Congress presentations

Poster presentations:
Comparison of specific IgE to Staphylococcus aureus enterotoxins in polyp tissue and serum of patients with nasal polyposis. Basic Immunology for the Allergist and the Clinical Immunologist, DAVOS, Switzerland, 1-4th February 2001

Are bacterial superantigens involved in the pathogenesis of nasal polyposis? XIth EuroCellPath Course, Gent, Belgium, June 13-18th 2001

Soluble Interleukin 5 Receptor Alpha: a novel marker for eosinophilic diseases of skin and airways. 24th Collegium Internationale Allergologicum (CIA) Symposium, Bermuda, November 1-6th 2002

Oral presentations:
Effect of corticosteroids on release of inflammatory mediators and plasmatic exudation in nasal polyps. XVIIIth Congress of the European Academy of Allergy and Clinical Immunology (EAACI), Brussels, Belgium, July 4th 1999

Eosinophilic inflammation and IgE production in nasal polyps. Royal Belgian Society for Otorhinolaryngology, Head and Neck Surgery, Brussels, Belgium, February 19th 2000

The role of IL-5 and TGF-beta1 in eosinophilic inflammation in nasal polyps. 56th Annual Meeting of the American Academy of Allergy, Asthma and Immunology (AAAAI), San Diego, California, March 4th 2000

Eosinophilic inflammation in nasal polyposis is related to tissue IgE. XIXth Congress of the European Academy of Allergy and Clinical Immunology (EAACI), Lisbon, Portugal, July 5th 2000

Comparison of specific IgE antibodies to Staphylococcus aureus enterotoxins in polyp tissue and serum of patients with nasal polyposis. 4th International Symposium on Experimental Rhinology & Immunology of the Nose (SERIN), London, UK, 14th February 2001

Local IgE formation in nasal polyps. Royal Belgian Society for Otorhinolaryngology, Head and Neck Surgery, Brussels, Belgium, February 17th 2001

Interleukin (IL)-18 and IL-1β in seasonal and perennial allergic rhinitis. 57th Annual Meeting of the American Academy of Allergy, Asthma and Immunology (AAAAI), New Orleans, Louisiana, March 19th 2001

Local IgE production to Staphylococcus aureus enterotoxins in Nasal Polyps. XXth Congress of the European Academy of Allergy and Clinical Immunology (EAACI), Berlin, Germany, May 12th 2001

New Insights in the pathogenesis of nasal polyps: Van Cauwenberge P, Bachert C, Gevaert P. XIth EuroCellPath Course, Gent, Belgium, June 15th 2001

Differential expression of human interleukin 5 receptor alpha isoforms in eosinophilic inflammation. 2nd Basic Immunology on Allergy and Clinical Immunology meeting, Davos, Switzerland, February 1st 2003

Expression of human interleukin 5 receptor alpha isoforms in nasal polyposis. Royal Belgian Society for Otorhinolaryngology, Head and Neck Surgery, Brussels, Belgium, February 22th 2003

Eosinophilic inflammation and Interleukin 5 receptor alpha isoform expression in Eosinophilic Airway Diseases. 60th Anniversary Meeting of the American Academy of Allergy, Asthma and Immunology (AAAAI), Denver, US, March 7-12th 2003

Effect of a single dose of a humanized anti-human IL-5 antibody, in nasal polyp patients. Royal Belgian Society for Otorhinolaryngology, Head and Neck Surgery, Brussels, Belgium, February 7th 2004

Invited Lectures:
Diagnosis and treatment of sinusitis and nasal polyposis. Setting New Standards in Allergy IV, Ghent, Belgium, 20th April 2002

Soluble IL-5 receptor alpha: a new marker of disease. BELSACI–meeting, Gent, Belgium, 25th April 2002

Interleukin 5 and it's receptor in nasal inflammatory diseases. XXIth Congress of the European Academy of Allergy and Clinical Immunology (EAACI), Naples, Italy, 3rd June 2002

Il punto di vista del giovane otorinolaringoiatra. Congresso Interannuale del Società Italiana Allergologia ed Immunologia Clinica. Rappalo, Italy, March 27-29th, 2003

Interleukin 5 receptor regulation and anti-IL5 treatment in nasal polyposis. XXIIth Congress of the European Academy of Allergology and Clinical Immunology (EAACI), Paris, France, 7-11th June 2003

The soluble and transmembrane IL-5 receptor alpha in eosinophil-associated disease. EAACI ENT-section-meeting, Ghent, Belgium, 15th November 2003

Entorotoxins: a new concept in nasal polyposis. 3rd International Consensus Conference on Nasal Polyposis. Brussels, Belgium, 23th of April 2004

Future therapeutic possibilities: from anti-IL-5 therapy onwards. 3rd International Consensus Conference on Nasal Polyposis. Brussels, Belgium, 24th of April 2004

Chairman:
Allergy in different organs – Eosinophils: Hallmark or Epiphenomenon? XXIth Congress of the European Academy of Allergy and Clinical Immunology (EAACI), Naples, Italy, 3rd June 2002

EAACI Junior member Poster session. XXIIth Congress of the European Academy of Allergology and Clinical Immunology (EAACI), Paris, France, 7th June 2003

Oral abstract session: Rhintis Treatment. XXIIth Congress of the European Academy of Allergology and Clinical Immunology (EAACI), Paris, France, 7-11th June 2003

Plenary session: Interaction between infection and allergic inflammation. EAACI ENT-section-meeting, Ghent, Belgium, 17th November 2003

Surgical therapy. 3rd International Consensus Conference on Nasal Polyposis. 25th of April 2004 - Brussels - Belgium

Publications **** Equal Contribution***

Bachert C, Gevaert P, van Cauwenberge P. Nasal polyposis: A new concept on the formation of polyps. ACI international 11(4), 130-135. 1999.

Bachert C. and Gevaert P. Effect of intranasal corticosteroids on release of cytokines and inflammatory mediators. Allergy 54 (Suppl 57), 116-123. 1999.

Bachert C*, Gevaert P*, Holtappels G, Cuvelier C, van Cauwenberge P. Nasal polyposis: From cytokines to growth. Am J Rhinol 14(5), 279-90. 2000.

Bachert C, Gevaert P, Holtappels G, van Cauwenberge P. Nasal Polyposis; Is There a Link between Eosinophils and IgE. Int Arch Allergy Immunol 124, 315-7. 2001

Bachert C*, Gevaert P*, Holtappels G, Johansson S.G.O, van Cauwenberge P. Total and specific IgE is related to local eosinophilic inflammation in nasal polyposis. J Allergy Clin Immunol 107 (4), 607-614. 2001

Bachert C, Gevaert P, van Cauwenberge P. Staphylococcus aureus superantigens and airway disease. Curr Allergy Asthma Rep 2(3), 252-8. 2002

Bachert C, Gevaert P, Van Cauwenberge P. Staphylococcus aureus enterotoxins: a key in airway disease? Allergy 2002; 57(6):480-7.

Watelet JB, Bachert C, Gevaert P, Van Cauwenberge P. Wound healing of the nasal and paranasal mucosa: a review. Am J Rhinol 2002; 16(2):77-84.

Watelet JB, Gevaert P, Bachert C, Holtappels G, Van Cauwenberge P. Secretion of TGF-betal, TGF-beta2, EGF and PDGF into nasal fluid after sinus surgery. Eur Arch Otorhinolaryngol 2002; 259(5):234-8.

Verhaeghe B*, Gevaert P*, Holtappels G, Lukat KF, Lange B, Van Cauwenberge P et al. Up-regulation of IL-18 in allergic rhinitis. Allergy 2002; 57(9):825-30.

Bachert C, Gevaert P, Holtappels G, Van Cauwenberge P. Mediators in nasal polyposis. Curr Allergy Asthma Rep 2002; 2(6):481-7.

Bachert C, Gevaert P, Howarth P, Holtappels G, Van Cauwenberge P, Johansson SG. IgE to Staphylococcus aureus enterotoxins in serum is related to severity of asthma. J Allergy Clin Immunol 2003; 111(5):1131-2.

Gevaert P, Bachert C, Holtappels G, Novo CP, Van der Heyden J, Fransen L et al. Enhanced soluble interleukin-5 receptor alpha expression in nasal polyposis. Allergy 2003; 58(5):371-9.

Gregory B, Kirchem A, Phipps S, Gevaert P, Pridgeon C, Rankin SM et al. Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. J Immunol 2003; 170(11):5359-66.

Claeys S, de Belder T, Holtappels G, Gevaert P, Verhasselt B, Van Cauwenberge P et al. Human beta-defensins and toll-like receptors in the upper airway. Allergy 2003; 58(8):748-53.

Perez C, Vandesompele J, Vandenbroucke I, Holtappels G, Speleman F, Gevaert P et al. Quantitative Real Time Polymerase Chain Reaction for measurement of human Interleukin - 5 receptor alpha spliced isoforms mRNA. BMC Biotechnol 2003; 3(1):17.

Bachert C, van Zele T, Gevaert P, De Schrijver L, Van Cauwenberge P. Superantigens and nasal polyps. Curr Allergy Asthma Rep 2003; 3(6):523-31.

Watelet JB*, Gevaert P*, Holtappels G, Van Cauwenberge P, Bachert C. Collection of nasal secretions for immunological analysis. Eur Arch Otorhinolaryngol 2003.

Zabeau L*, Gevaert P*, Bachert C, Tavernier J. Interleukin-5, eosinophilic diseases and therapeutic intervention. Curr Drug Targets Inflamm Allergy 2003; 2(4):319-28.

Perez-Novo CA, Kowalski ML, Kuna P, Ptasinska A, Holtappels G, van Cauwenberge P, Gevaert P, Johannson S, Bachert C. Aspirin sensitivity and IgE antibodies to Staphylococcus aureus enterotoxins in nasal polyposis: studies on the relationship. Int Arch Allergy Immunol. 2004 Mar;133(3):255-60.

Bachert C, Vignola AM, Gevaert P, Leynaert B, Van Cauwenberge P, Bousquet J. Allergic rhinitis, rhinosinusitis, and asthma: one airway disease. Immunol Allergy Clin North Am. 2004 Feb;24(1):19-43.

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Gevaert P, Bachert C, van Cauwenberge P. Effect of corticosteroids on release of inflammatory mediators and plasmatic exudation in nasal polyps. Allergy 1999; 54:12

Gevaert P, Bachert C, van Cauwenberge P. The role of IL-5 and TGF-beta1 in eosinophilic inflammation in nasal polyps. J Allergy Clin Immunol 2000; 105(1): S72

Gevaert P, Bachert C, van Cauwenberge P. Eosinophilic inflammation and IgE production in nasal polyposis. Acta Otorhinolaryngol.Belg. 2000 ;51:65.

Gevaert P, Bachert C, van Cauwenberge P. Eosinophilic inflammation in nasal polyposis is related to tissue IgE. Allergy 2000;S63;55: 71

Van Kempen M, Gevaert P, Bachert C, van Cauwenberge P. Accumulation of dendritic cells subsets in nasal polyps. Allergy 2000;S63;55: 40

Gevaert P, Bachert C, Holtappels G, van Cauwenberge P. Local IgE formation in nasal polyps. Acta Otorhinolaryngol.Belg. 2001;55:8.

Verhaeghe B*, Gevaert P*, Bachert C, van Cauwenberge P. Comparison of local steroid and oral anti-histamine treatment on nasal inflammation in seasonal allergic rhinitis. Acta Otorhinolaryngol.Belg. 2001;55:11.

Gevaert P, Bachert C, Verhaeghe B, van Cauwenberge B. Interleukin (IL)-18 and IL-1β in seasonal and perennial allergic rhinitis. J Allergy Clin Immunol 2001; 107(2): S240.

Verhaeghe B*, Gevaert P*, Watelet JB, Bachert C, van Cauwenberge P. Allergic sensitization and co-morbidity in patients suffering from chronic nasal symptoms. Allergy 2001; S68; 56: 176.

Gevaert P, Bachert C, van Cauwenberge P. Local IgE production to Staphylococcus aureus enterotoxins in Nasal Polyps. Allergy 2001; S68; 56: 68.

Awards

Prijs van de Vereniging der Geneesheren, Oud-studenten der Universiteit Gent (Alumni), 1998

Glaxo Wellcome Award for fundamental research in ENT, Brussels, February 29th 2000, for lecture titled: Eosinophilic inflammation and IgE production in nasal polyposis.

The Pharmacia Allergy Research Foundation (PARF) Poster Award 2001, Berlin, May 9th 2001, for the poster titled: Local IgE production to Staphylococcus aureus enterotoxins in Nasal Polyps.

The EAACI Exchange Research Fellowship 2001 of 20.000 Euro for the research project in collaboration with Prof Dr SGO Johansson and Prof Lars Lundblad at the Karolinska Hospital in Stockholm, Sweden.

GlaxoSmithKline Award for clinical research in ENT, Brussels, February 7th 2004, for the lecture entitled: Effect of a single dose of a humanized anti-human IL-5 antibody, in nasal polyp patients.

Grants

The UCB Institute of Allergy Travel Grant for the oral presentation titled: The role of IL-5 and TGF-beta1 in eosinophilic inflammation in nasal polyps. 56th Annual Meeting of the American Academy of Allergy, Asthma and Immunology (AAAAI), San Diego, California, March 4th, 2000

The European Academy of Allergy and Clinical Immunology (EAACI) Travel Grant for the oral presentation titled: Eosinophilic inflammation in nasal polyposis is related to tissue IgE. XIXth Congress of the EAACI, Lisbon, Portugal, July 5th, 2000

The American Academy of Allergy, Asthma and Immunology (AAAAI) Fellows-In-Training International Travel Grant for the oral presentation titled: The Interleukin (IL)-18 and IL-1β in seasonal and perennial allergic rhinitis. 57th Annual Meeting of the AAAAI, New Orleans, Louisiana, March 19th 2001

The European Academy of Allergy and Clinical Immunology (EAACI) Travel Grant for the oral presentation titled: Local IgE production to Staphylococcus aureus enterotoxins in Nasal Polyps. XXth Congress of the EAACI, Berlin, Germany, May 12th 2001

Memberships

Royal Belgian Society for Otorhinolaryngology, Head and Neck Surgery, 1999-2004

European Academy of Allergology and Clinical Immunology, 1999-2004

Miscellaneous

Stichtend voorzitter van de StudentenWerkgroep OPleiding (SWOP), 1996-1998

Lid van de Opleidingscommissie Geneeskunde van de Universiteit Gent, 1994-1998

Lid van de Faculteitsraad Geneeskunde van de Universiteit Gent, 1996-1998 en 2000-2002

EAACI Junior Member (JMA) representative for the ENT section, 2000-2003

EAACI board member of the ENT section, 2001-2003

EAACI Junior Member (JMA) Chairman 2003-2005

EAACI Executive committee adjunct member 2003-2005

Contributor to the EAACI Newsletter 1999-2004