UNIVERSITA' DEGLI STUDI DI PALERMO

Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi

Corso di Dottorato di Ricerca in Biopatologia, ciclo XXII

Identification of inflammatory molecules with an important role in the pathogenesis of the Chronic Rhinosinuitis.

Tesi di: Dott.ssa Valeria Scafidi

Tutor: Prof. Calogero Caruso

Coordinatore: Prof. Calogero Caruso

TRIENNIO 2008-2010

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Se non puoi essere una via maestra, sii un sentiero

Se non puoi essere il sole, sii una stella

Sii sempre il meglio di cio' che sei.

Martin Luther King

Alla mia famiglia

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Introduction

1. Chronic Rhinosinusities

Rhinosinusitis is a significant health problem which seems to mirror the increasing frequency of allergic rhinitis and which results in a large financial burden on society (1–3). The clinical presentation of CRS in individual patients covers a spectrum of severity, and CRS is probably best considered as a syndrome with persistent characteristic symptoms rather than a discrete disease entity. Until recently, research has been hampered by many factors, including most prominently the lack of a universally accepted definition.

1.1 Definition and Diagnosis of rhinosinusitis/nasal polyps

Rhinitis and sinusitis usually coexist and are concurrent in most individuals; thus, the correct terminology is now rhinosinusitis.

Because rhinitis and sinusitis are so closely linked the definition of CRS/NP in the EPOS document is developed from the ARIA classification of rhinitis and based on symptomatology, duration and severity of disease.

The diagnosis of rhinosinusitis is made by a wide variety of practitioners, including allergologists, otolaryngologists, pulmonologists, primary care physicians and many others.

Recently, the definition of rhinosinusitis has been revisedas an inflammation of the nose and the paranasal sinuses characterized by two or more symptoms (blockage, discharge, facial pain, reduction of smell) and either endoscopic signs (polyps, discharge from middle meatus, oedema/mucosal obstruction in the middle meatus) and/or CT changes within ostio meatal complex and/or sinuses (4,5). The presence of nasal polyps distinguishes two subgroups: CRS with or without polyps.

1.2 Severity of disease

The disease can be divided into MILD and MODERATE/SEVERE based on total visual analogue scale (VAS) score (0–10 cm): MILD ¼ VAS 0–4, MODERATE/SEVERE ¼ VAS 5-10.

Duration of disease. The disease can be divided into Acute/Intermittent (<12 weeks with complete resolution of symptoms) and Chronic/Persistent (>12 weeks symptoms with no complete resolution of symptoms).

1.3 Rhinosinusitis and Allergy

Acute rhinosinusitis. Review articles on sinusitis have suggested that atopy predisposes to rhinosinusitis (6). This theory is attractive given the popularity of the concept that disease in the ostiomeatal area contributes to sinus disease in that the mucosa in an individual with allergic rhinitis might be expected to be swollen and more liable to obstruct sinus ostia, reduce ventilation, lead to mucus retention that might be more prone to become infected. Furthermore there has been an increase in the body of opinion that regard the mucosa of the nasal airway as being in a continuum with the paranasal sinuses and hence the term rhinosinusitis (7). The number of studies determining the occurrence of acute rhinosinusitis in patients with and without allergy is very limited.

Chronic rhinosinusitis. It has been postulated (8) that swelling of the nasal mucosa in allergic rhinitis at the site of the sinus ostia may compromise ventilation and even obstruct sinus ostia, leading to mucus retention and infection. Furthermore, there has been an increase in the body of opinion that regard the mucosa of the nasal airway as being in a continuum with the paranasal sinuses and hence the term _rhinosinusitis_ was introduced (7).

However, critical analysis of the papers linking atopy as a risk factor to infective rhinosinusitis (chronic or acute) reveal that whilst many of the studies suggest a higher prevalence of allergy in patients presenting with symptoms consistent with sinusitis than would be expected in the general population, there may well have been a significant selection process, because the doctors involved often had an interest in allergy (9-14). A number of studies report that markers of atopy are more prevalent in populations with chronic rhinosinusitis. Benninger reported that 54% of outpatients with chronic rhinosinusitis had positive skin prick tests (15). Among CRS patients undergoing sinus surgery, the prevalence of positive skin prick tests ranges from 50 to 84% (16, 17, 18), of which the majority (60%) have multiple sensitivities (18). As far back as 1975, Friedman

reported an incidence of atopy in 94% of patients undergoing sphenoethmoidectomies (19).

However, the role of allergy in CRS is questioned by other epidemiologic studies showing no increase in the incidence of infectious rhinosinusitis during the pollen season in pollen-sensitized patients (20). In a small prospective study, no difference in prevalence of purulent rhinosinusitis was found between patients with and without allergic rhinitis (21). Newman et al. reported that whilst 39% of patients with CRS had asthma, raised specific IgE or an eosinophilia, only 25% had true markers to show they were atopic (22). Finally, Emanuel et al. (18) found relatively lower percentages of allergic patients in the group of patients with the most severe sinus disease on CT scan and Iwens et al. (23) reported that the prevalence and extent of sinus mucosa involvement on CT was not determined by the atopic state.

Not with standing the lack of hard epidemiologic evidence for a clear causal relationship between allergy and CRS, it is clear that failure to address allergy as a contributing factor to CRS diminishes the probability of success of a surgical intervention (24).

1.4 Lower airway involvement in CRS

Recent evidence suggests that allergic inflammation in the upper and lower airways coexist and should be seen as a continuum of inflammation, with inflammation in one part of the airway influencing its counterpart at a distance. Rhinosinusitis and lower airway involvement are also frequently associated in the same patients, but their interrelationship is poorly understood.

2. Tissue remodeling in upper airways

Several years ago, Bousquet et al. proposed as definition of remodelling : model again or differently, reconstruct_ following the Oxford Dictionary (25).

Remodelling is a critical aspect of wound repair in all organs representing a dynamic process which associates matrix production and degradation in reaction to an inflammatory insult leading to a normal reconstruction process (model again) or a pathological one (model differently). However, it also appears that some components of remodelling are constitutional and may be genetically driven (26).

Finally, this definition stresses the close relationship between the initial inflammation and the subsequent tissue damage.

2.1 Remodelling in the nose

Remodelling in the nose is more complex. In allergic rhinitis, nasal remodelling may exist, but, even if the links between rhinitis and asthma are evident (27, 28), it is far less extensive than in the bronchi of asthmatics. These differences between upper and lower airways could be considered as a consequence of different embryologic origin (29).
After trauma or surgery in airways, a scar may be observed as a pathological consequence of abnormal remodelling. Finally, there are other nasal or paranasal sinus conditions presenting some degrees of remodeling which can significantly interfere with the normal functions of the respiratory mucosa.

2.2 Normal nasal and paranasal mucosa

The nasal mucosa is the first physical barrier to foreign materials and a conditioner for inhaled air. The nose and, also probably the paranasal sinuses, also assume largely other

functions mainly necessary for conditioning the inspired airflow (30). The nasal epithelium is separated from the lamina propria by a continuous basement membrane (Fig. 1A). The pseudostratified columnar epithelium (respiratory epithelium) is composed of four major types of cells: ciliated, non ciliated, goblet and basal cells. A functional nasal mucosa can assure mucus production and transport, resorption of surface materials, and formation of new epithelial cells. The proportion of goblet cells was higher in inferior turbinate and lower in the nasal septum (31). Just beneath the basement membrane, lymphocytes and plasma cells forms the lymphoid layer of the lamina propria. The supporting connective tissue is of loose type and its extracellular matrix (ECM) component plays an essential role in inflammatory reactions. The subepithelial region also contains two layers of sero-mucous glands: the superficial layer is situated just underneath the epithelium, and the deep layer under the vascular layer.

Besides resistant vessels, such as arteries, arterioles or capillaries, the vasculature of the nose is characterized by capacitance vessels, designated as _erectile tissue_. This vascular component is mainly concentrated on the middle and inferior turbinates, the septum and may extend posteriorly to

the choanal orifices. With these vascular specificities, the nasal mucosa can regulate the airflow, adapt the nasal resistance, filter and condition the inspired air by producing nasal mucus, sustaining a mucociliary transport and serving as support for immune response.

The mucosa isolated from maxillary sinuses is slightly different, mainly thinner and less specialized than nasal mucosa (Fig. 1B). The respiratory epithelium is lower and contains less goblet cells (32). The epithelium contains fewer cilia than the nasal mucosa (33). The basal lamina is always easily identifiable. The glands are fewer and smaller and the venous erectile plexus is absent (34).

The differences in mucosal structures could be explained by differences in flow of inspired air (35) or in extent of mesodermisation during the formation of the mid-face and nasal cavities from in utero weeks 4–12. It is probable that other hypotheses will appear when the intrinsic functions of the paranasal sinuses can be better defined.

The inflammation occurring in this region is frequent and many pathological processes could induce chronic types of inflammation. These situations lead regularly to structural

changes in epithelium or lamina propria, but the precise relation remains unclear (36).

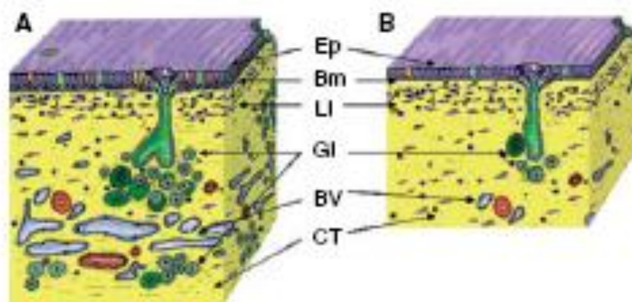


Figure.1 Histomorphological view of normal (A) nasal and (B) paranasal mucosa (Ep: epithelial cells; Bm: basement membrane; L1: lymphoid layer; Gl: glands; BV: blood vessels; CT: connective tissue).

2.3 Tissue Remodelling in Chronic rhinosinusitis with or without nasal polyps

In humans, tissue remodelling in the upper airways cannot be restricted to the very limited tissue damage occurring during allergic rhinitis. Depending on the initial induction (inflammation, trauma), the upper airways can present different types of remodelling: allergic inflammation in allergic rhinitis,

persistent oedema and in chronic rhinosinusitis (CRS) with nasal polyps, purely fibrotic tissue like nasal adhesions, or mixed oedema and fibrosis as CRS without nasal polyps.

Chronic rhinosinusitis without nasal polyps. The mucosallininn CRS without polyp is characterized by basement membrane thickening (37), goblet cell hyperplasia, limited subepithelial oedema, prominent fibrosis and mononuclear cell infiltration (38 Fig. 2A, B). Interestingly, the fibrosis is usually limited to collagen deposition mostly in subepithelial regions. In this disease, the MMP and TIMP ratio seems to be balanced (39).

Chronic rhinosinusitis with nasal polyps. In contrast to CRS without nasal polyps, CRS with polyps reveals frequent epithelial damage, a thickened basement membrane and mostly oedematous to sometimes fibrotic stromal tissue, with a reduced number of vessels and glands but virtually no neuronal structure (38; Fig. 2C,D). Recent hypotheses suggested that mammaglobulin may contribute to epithelial proliferation in polyp formation (40).

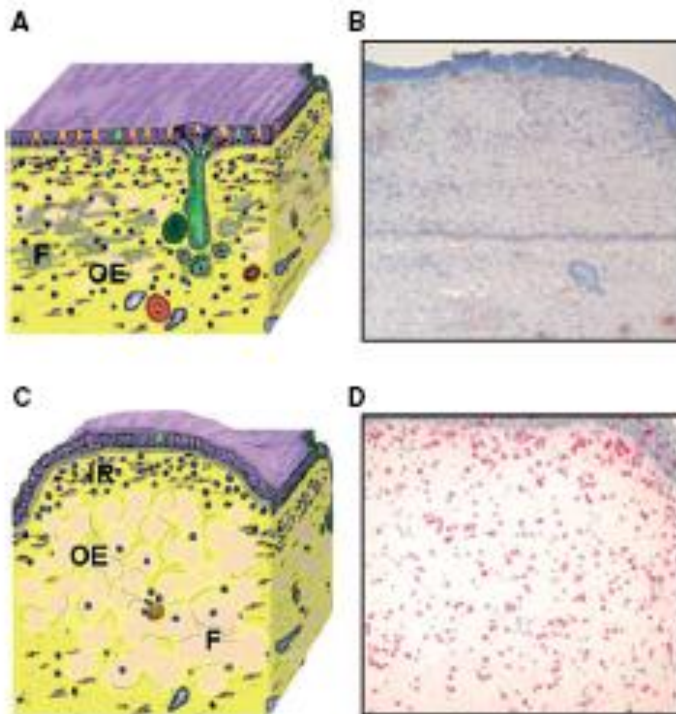


Figure 2. Major types of extracellular matrix remodeling in paranasal diseases. Schemes illustrating the major changes in lamina propria (left column) and corresponding pathological views (right column). (A) Chronic rhinosinusitis without polyps: transforming growth factor-β1 staining (initial magnification x100). (C) Chronic rhinosinusitis with polyps: severe subeithelial inflammation (IR) and massive oedema (OE) in lamina propria with fields of fibrotic foci (F). (D) Chronic rhinosinusitis with polyps: EG2 staining for eosinophils (initial magnification x100)

Polyps demonstrate increased exudation from vessels, oedema of the lamina propria and bulging of the nasal mucosa (Fig.3).
Nasal polyps have been histologically classified into oedematous, glandular and fibrous types (41) according to their stroma remodelling. Among the inflammatory cells, EG2+ (activated) eosinophils, usually located around vessels and glands, are a prominent characteristic in about 80% of patients with nasal polyps.

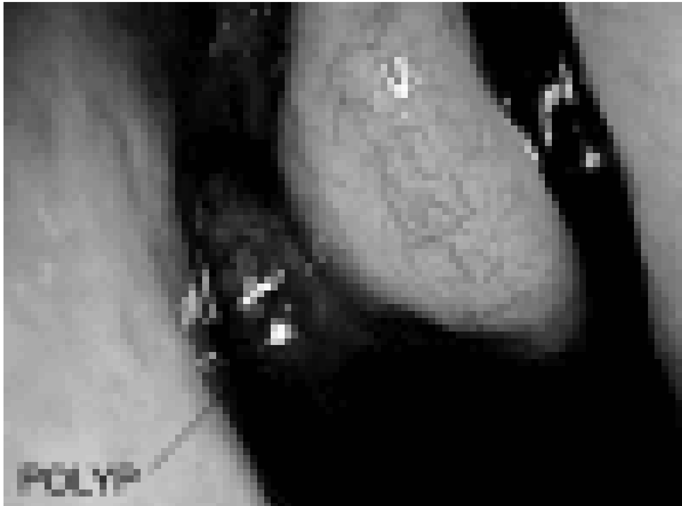


Figure 3: Typical appearance of nasal polyposis in right middle meatus.

The oedematous nature of nasal polyps consists of fibroblasts and infiltrating inflammatory cells localized around pseudocyst formations. These pseudocysts contain albumin and other plasma proteins, this active exudation being supported by the subepithelial eosinophilic inflammation.

Finally, increased aquaporin-1 water channel expression in polyp tissue could influence oedema formation (42). Mostly, in CRS with polyps and aspirin sensitivity, epithelial thickening and eosinophilia was found in inferior turbinates at distance from the polyps (43).

Recently, an imbalance between MMPs and their natural inhibitor TIMP-1 has been reported in CRS with nasal polyps. This imbalance could lead to a local increase of ECM degradation and formation of pseudocysts (44).

Like neutrophils and macrophages, mast cells can express MMPs and interact with ECM (45). This view was recently supported by evidence of participation of other MMPs, such as MMP-2 (46–48) in pathogenesis of CRS with nasal polyps. Finally, this clinical entity must be distinguished from the antro choanal polyp, which presents microscopic similarities with maxillary cyst (49).

2.4 Histology of nasal polyps

Some theories consider polyps a consequence of conditions which cause chronic inflammation in the nose and nasal sinuses characterized by stromal edema and variable cellular infiltrate (50). They consist of loose connective tissue, edema, inflammatory cells, and some capillaries and glands. They are covered with different types of epithelium, most commonly pseudostratified respiratory epithelium with goblet cells and ciliated cells. It presents thickening of the basement membrane, a loose of a vascular edematous stroma and an infiltrate of plasma cells and eosinophils. Eosino phils are found in 85% of NP with the remaining cells being predominantly neutrophils (51) (**Fig.4**).

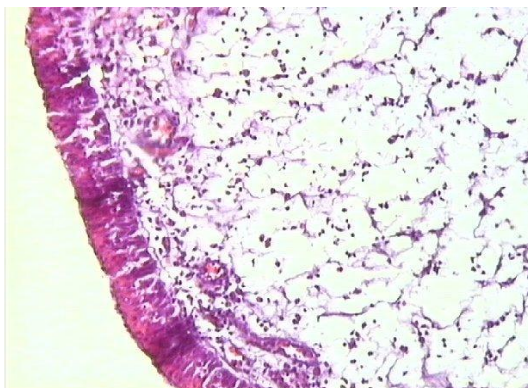


Figure 4: Typical histological apparence of nasal polyposis. Ematossiline/eosine staining for subepithelial inflammatory cells (initial magnification x40).

3. Interleukin-19: Multiple roles in immune regulation and disease

3.1 Introduction

Despite being first published in 2000, no clear role for interleukin-19 has yet been defined. Scientific interest in it is high, but actual hard information is thin on the ground for a cytokine discovered over 10 years ago, indeed, IL-19 has the dubious distinction of being a cytokine which has appeared in almost more review articles than in primary scientific investigations (e.g. 52).

IL-19 is a member of the IL-10 family of cytokines, forming a subfamily with IL-20 and IL-24, and more broadly with the Type-I, -II and -III interferons (Fig. 5). The earliest member of the IL-19/20/24 subfamily, and the only one with a known viral paralog, is IL-24 (53). Like the members of the IFN-l family (54), IL-10 family members'structures are conserved tightly between species. The gene encoding IL-19 comprises seven exons and is located on human chromosome 1.

Secreted IL-19 is composed of 159 amino acids that form an a-helical structured protein. IL-19 is produced by activated monocytes, and to a lesser extent, by B-cells (55), but also by

non-immune cells such as keratinocytes and foetal membranes (56). As known so far, IL-19 functions through a receptor complex composed of IL-20Ra and IL-20Rb that is also utilized by IL-20 and IL-24. High levels of both receptor chains are present in several tissues including the skin, lungs and tissues from the reproductive organs, but the alpha chain appears to be absent from immune cell populations.

Nonetheless, many effects of the IL-19 described to date concern immune cells. IL-19 has been shown to enhance the production of Th2 cytokines in T cells and to be elevated in asthma patients. Furthermore, it induced IL-6, IL-8 and IL-10 expression in monocytes. Additionally, it has been implicated in a range of diseases and disorders, including (but not limited to): aging (57), Type-I diabetes (58), endotoxic shock (59), periodontal disease (60), vascular disease (61) and rheumatoid arthritis (62,63) These sporadic encounters between IL-19 and human disease stand as testimony to there being no clearly defined role for this cytokine in human biology (other than the potential role in atopic and allergic responses and disorders; see below). It also seems to be involved in the pathogenesis of the Th1-type skin disease psoriasis, where both ligand and receptor are present. IL-19 therefore represents a cytokine with

apparently contradictory functions and whose receptor is not always apparent on its target cells; its immunoregulatory functions are far from determined.

G. Gallagher / Cytokine & Growth Factor Reviews 21 (2010) 345–352

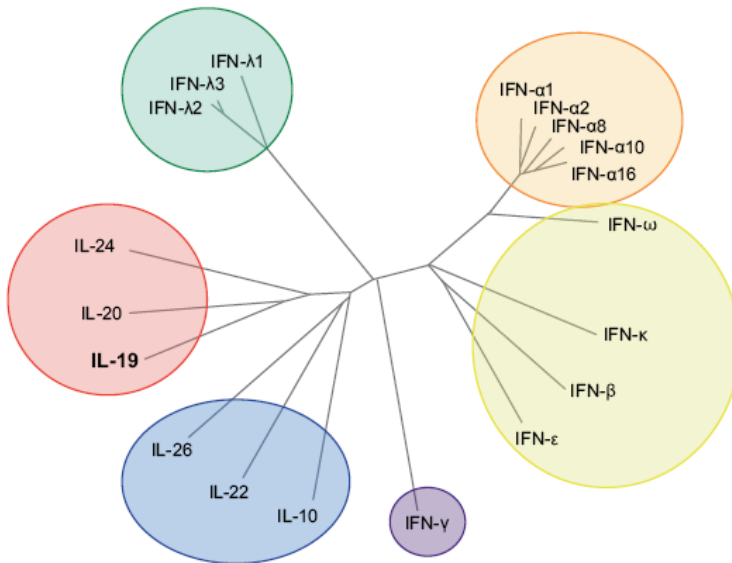


Figure 5: Evolutionary relationship of members of the IL-107IFN clan. Evolutionary relationships between IL-19 and the other subfamily members (shared red), the IL-10 subfamily (blue), the Type –III (lambda) IFNα members (orange) and the other Type –II IFNs (pink) are shown. Relationships are based on protein sequence, excluding putative leader sequences.

3.2 The human IL-19 protein, receptor and signaling

IL-19 is a member of the IL-10 family of cytokines (52, 64). In original analysis of IL-19 (64), was described 30 identical amino acids, including those analogous to the four cysteine residues necessary to correctly fold the α-helices in the IL-10 protein. In addition, a further 36 were similar. Strikingly, 41 of the 50 amino acids previously identified as being essential to the hydrophobic core of IL-10 were conserved in IL-19.

The subsequent crystallization of IL-19 has revealed a very similar structure to that of IL-10, except that IL-19 has seven helices rather than six and is capable of folding back on to itself to forma stable monomer in solution.

The ‘‘*IL-10 family F-helix motif*’’ (K.x.x.x.E.x.D), found in all human IL-10 family members, is located on the IL-19 G-helix, as ‘‘K.S.L.G.E.L.D’’. Unlike IL-10, IL-19 is not a homodimer in solution; it forms a stable, functional monomer.

Structurally, IL-19 is a smooth, compact seven a-helical structure (A–G), held together by two disulphide bonds (Cys57–Cys109 andCys58–Cys111) that parallel those found in IL-10, and an extensively hydrophobic core (65) **(Fig.6)**.

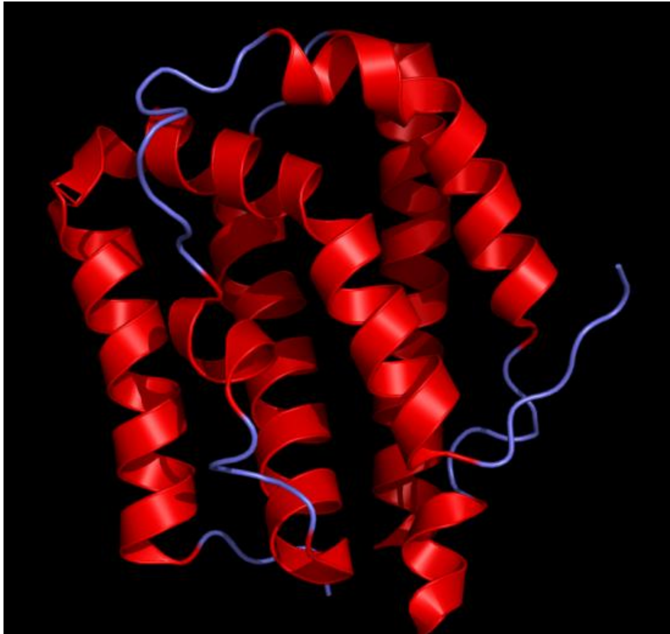


Figure 6 Crystallization of IL-19. IL-19 is a smooth, compact seven a-helical structure(A–G), held together by two disulphide bonds (Cys57–Cys109 andCys58–Cys111) that parallel those found in IL-10, and an extensively hydrophobic core.

IL-19 mRNA expression was first demonstrated in primary human monocytes following stimulation with LPS or granulocyte–macrophages colony stimulating factor; other cytokines (IFN-g, IL-10, IL-4 or IL-13) could not them selves stimulate IL-19 transcription (64). The data also showed that

LPS induction of IL-19 was enhanced by IL-4 but not by IFN-g. It was also demonstrated that IL-19, in common with many monocyte-derived cytokines, is subject to feedback inhibition by IL-10 (66); stimulation of IL-19 secretion with LPS could be inhibited by the addition of IL-10 to the culture.

In complementary experiments, addition of monoclonal anti-IL-10 antibody to the cultures caused an increase in IL19 transcription, indicating that even endogenous IL-10 exerted measurable control over IL-19 production.

IL-19 is similar in sequence and structure to IL-10, but much more so to IL-20 and IL-24 (67), and we have described these three cytokines as the ‘‘IL-19 subfamily’’. While others prefer ‘‘IL-20 subfamily’’ (68), their evolutionary relationship suggests that the honour should in fact fall to IL-24. An additional element of similarity comes from their sharing a common element to their receptor—the IL-20Rb chain (69). IL-19, IL-20 and IL-24 can all bind to a hetero dimeric receptor complex comprising the IL-20Ra and IL-20Rb chains, and signal thephosphorylation of STAT3 efficiently.

Rather, IL-19 may well utilize some alternative receptor on lymphoid cells. Of interest in this regard is the observation that it is the IL-20Rb chain and not the Ra chain, that binds IL-19

with high affinity (stable 20Ra:IL-19:20Rb complexes) are only formed after IL-19 binds 20Rb (70).

Early reports addressing IL-19-induced signaling demonstrated clearly that the main pathway is the tyrosine phosphorylation (69,71) and nuclear translocation (71) of STAT3. Both reports utilized cells transfected with various combinations of receptor chains. Moreover, these authors showed that, while STAT1 was also activated, this required a 100-fold greater ligand concentration.

They also showed that IL-20 effects required 10-fold less IL-20 when mediated via the ‘‘alternative’’ IL-20 receptor (IL22Ra/IL-20Rb) than that of either IL-19 or IL-20 when using the ‘‘original’’receptor pair (71). In a more recent study using the HaCaT human keratinocyte cell-line, IL-19-induced STAT3 tyrosine phosphorylation was clearly detected, although at lower levels than that induced by IL-20 and IL-24 (72). Interestingly, the ability of IL-19 to inhibit the growth of the OvCar-3 cell line (which expresses the20R1/2 heterodimer) is accomplished in the absence of STAT3 or STAT1 phosphorylation or translocation, suggesting some alternative signaling mechanism may be triggered by IL-19 binding to these cells (71).

3.3 IL-19 and inflammatory disorders

Two interesting studies have addressed the subject of vascular disease. In the first, Tian et al. (73) describe the induction of IL-19 in human vascular (arterial) smooth muscle. Proliferation of these cells was markedly inhibited by IL-19 in vitro and this was accompanied by SOCS5 induction (74). Adenoviral administration of IL-19 to rats effectively reduced injury, and MAP kinase activation. More recently, inflammatory cytokines were shown to induce IL-19 in these same cells (61), while administration of IL-19 reduced the inflammatory response in vascular smooth muscle by diminishing the stability of mRNA species encoding proinflammatory proteins.

This work correlates well within patients suffering from septic shock (59). These patients display elevated IL-19 peripheral titres and human lung and liver cells were activated to undergo apoptosis or release reactive oxygen species by IL-19, in vitro. In addition, significant activation of neutrophils was observed. In LPS challenged mice, administration of soluble IL-19 receptor reduced these pro-inflammatory indices of IL-19's activity. Conversely, ininflammatory bowel disease, endogenous IL-19 appears to be protective. Azuma et al. (75) examined the DSS colitis model in IL-19_/_ mice and showed

that these mice were more susceptible to colitis than their immunologically intact cousins. Here, it was macrophages and their associated pro-inflammatory TNF, IL-1 andIL-6 whose accumulation was enhanced in the absence of IL-19 (these cytokines were also enhanced following LPS stimulation in vitro).

3.4 IL-19, the Th2 response and asthma

The majority of the data implicate IL-19 as a component of the Th2 system and clinically, as having a clear role in asthma.
In an original description of IL-19 (64), was noted that IL-19 expression in LPS-stimulated monocytes was enhanced by preincubation of the monocytes with IL-4 and, to a lesser extent, with IL-13. These data were strengthened by experiments on human T-cells, where the presence of IL-19 elevated the proportion of IL-4+ve CD4+ve T-cells, to the detriment of the numbers of those that were IFN-g positive (76). A corresponding up regulation of IL-4 mRNA was also observed (67). These observations were recently confirmed and extended by Oral et al. (77), who demonstrated that repeated rounds of stimulation of naive human T-cells in the presence of

IL-19 polarised these cells to a Th2 secretory (elevated IL-4, IL-13 and diminished IFN-g) phenotype. Evidence for direct effects on human B-cells, leading to elevated IgG levels has also been presented (78).

The induction of Th2 cytokine expression by IL-19 has also been confirmed for the murine system (79). Using activated splenic CD4+ve T-cells, this group demonstrated that introduction of IL-19 caused increased secretion of IL-4, IL-5, IL-10 and IL-13, confirming and extending the available human data. In addition, these effects could be manifest in vivo. Introduction of an IL-19-encoding plasmid DNA to immunologically intact mice caused an elevation of serum levels of IL-4, IL-5 and IL-10 (79). It therefore appears that one of the main physiological functions of IL-19 is to participate in the promotion of the Th2 response and in final proof of this is required, there cent cloning and expression of avian IL-19, and the demonstration that it to oup regulates Th2 responses, should suffice (80).

Dysregulated Th2 responses are associated with certain diseases. Some of these, such as uremia, are acute and transient.

Nonetheless, elevated Th2 responses in uraemic patients are accompanied by elevated IL-19 titres (81), and IL-19 is present with other Th2 cytokines in uveitis (82). However, the best-known Th2 disease in man is asthma.

Asthma is a chronic condition characterized by increased bronchial responsiveness and by airway obstruction and inflammation; it is a significant, and growing, problem in the Western World interms o morbidity and economic impact. According to the NHLBI, 15million adults and children have asthma in the USA (one case per300people), causing around 0.5 million hospital admissions annually, over 5000 deaths, and over 15% of all paediatric emergencies (83).

Current treatments only relieve the symptoms, failing to address the underlying immunological mechanisms. Its economic impact has been estimated at over $16B per year. The incidence is highest in children ('75 cases per thousand); numbers have been rising over the last 20 years. Although asthma development is multifactorial, Th2 cytokines are highly associated with the disease, especially IL-4, IL-5 and IL-13, which are secreted following antigen challenge in allergic asthma (84-86). Experimentally, IL-19 mRNA levels were

elevated in the lungs of BALB/c mice, following exposure to allergen; the mice also had elevated serum IL-19 (84).

In human patients, Liao et al. (79) demonstrated that IL-19 levels were elevated 3–4-fold in the serum of children with asthma, over normal children or adults. High IL-19 levels correlated with high IL-4 and IL-13 levels. Huang et al. (91) expanded these results by demonstrating that epithelial cells in theair way of human asthma and COPD (chronic obstructive pulmonary disorder) patients were rich in IL-19. Further, as described above, bronchial epithelial cells in this study responded to synergistic stimuli by IL-17 and IL-13 (both key asthma mediators (87,88) to express IL-19 at high levels, demonstrating positive feedback between the Th2 and pro-inflammatory aspects of asthma, and increased IL-19 expression.

Interestingly, it has also been shown that stimulation of adenosine receptors on human bronchial epithelial cells would induce IL-19 secretion. This could induce TNF secretion from THP-1cells which in turn would elevate the A2B adenosine receptor on the bronchial cells (89), increasing adrenergic responsiveness and elevating the cycle of both inflammation (via TNF) and Th2 responses (via IL-19). The responsiveness

of IL-19 expression to adrenergic stimulation was recently independently confirmed (90).

Thus, IL-19 is very closely associated with the Th2 response, and with human asthma, with important contributions from Th17 cells (91). IL-13 is key to driving many of the asthma symptoms (92-94).

Thus there may be a cycle of IL-19 up regulating Th2 and inflammatory cytokines, which in turn up regulate IL-19. How this cycle contributes to pulmonary host defense and/or asthma susceptibility and progression, remains to be determined (Fig.9).

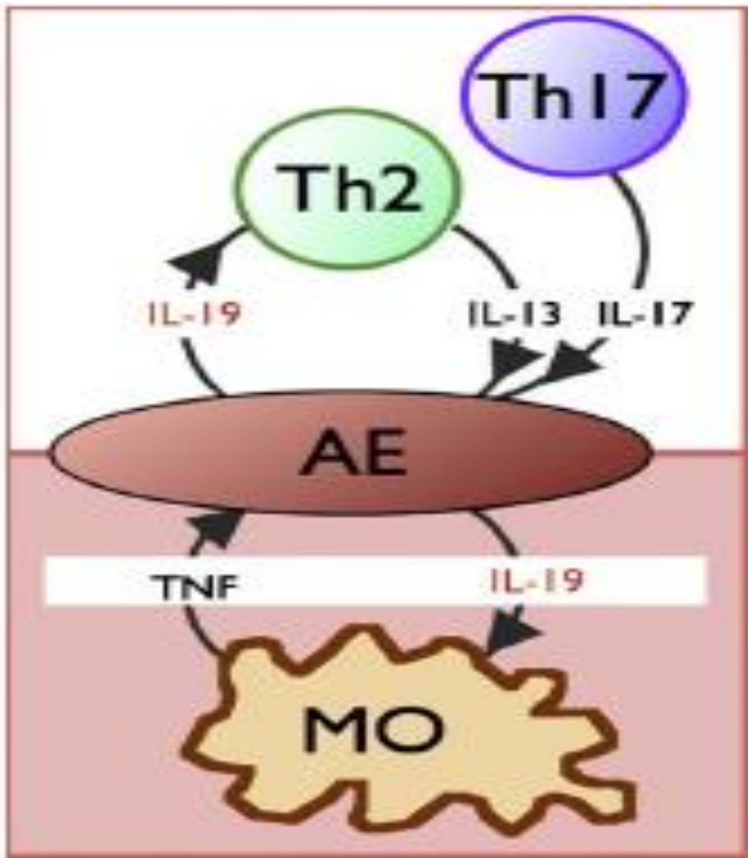


Figure 9. IL-19 drives pathogenic Th2 and inflammatory mechanisms in asthma. As discussed in the text, airway epithelial cells secrete IL-19, and this is stimulated synergistically by IL-17 and IL-13 [39]. IL-19 promotes the development and function of Th2 cells [82]. In addition, monocytes are IL-19 responsive, secreting TNF which also promoted IL-19 secretion from airway epithelial cells [94,95]. Thus ,in the airway, IL-19 may lie at the pivot point of the two key pathogenic pathways that combine to produce asthma in man: the Th2 response and the inflammatory response. Th2, CD4+ve T-helper Type-II cells; Th17, CD4+ T-helper, IL-17 secretor cells.

AIMS

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal and paranasal sinus mucosa. Patients with CRS are symptomatic for longer than 3 months and have associated to inflammatory mucosal thickening and sometimes to polyploid changes.

An improved understanding of mechanisms that lead to the formation of nasal polyps in allergic or non allergic patients affects with Chronic Rhinosinusitis, may foster the development of more effective therapies.

Thus, the aims of the present study are:

- To demonstrate whether a specific biomarker promote tissue remodeling in Chronic Rhinosinusitis.
- To asses the contribute of allergy in these phenomena.

Current methods of research in CRS/NP select and probe for known inflammatory cytokines and chemokines and their corresponding mRNA transcript.

In designing our study, we looked to recent research strategies with potential applicability to nasal polyps and nasal mucosa. Because it has been assumed that nasal polyps from patients

affected by CRS, should share a common cytokine milieu with asthma, sinusitis, or allergic rhinitis, we used Human Asthma Gene Array to study the phenomena.

Gene expression arrays are powerful new tools that can provide important information about the identification of inflammatory molecules which may lead to the identification of specific genes and signaling pathways involved in airway remodeling and inflammation that occour in Chronic Rhinosinusitis disease.

In this study, we'll use a real-time PCR approaches to confirm gene expression, instead we'll use western blot and immunohistochemistry to evaluate protein expression and its localization at level of specific cells types.

RESULTS

We studied 30 patients. Characteristics of patients are shown in **Table 1**. Two groups were formed according to the presence of Chronic Rhinosinusitis (CRS). **Group 1**: patients undergoing surgery for non-CRS pathological conditions (control group) in which CRS, nasal polyps and allergy were excluded based on clinical and radiographic analysis (*n* = 10), **Group 2**: patients with CRS, without nasal polyps, (with or without allergy) (CRSw/oNPt) (*n* = 10). **Group 3**: Nasal polyposis, also referred to as (CRSwNPt), present on CT scan and/or endoscopic examination from CRS patients (with or without allergy) (*n*=10).

Patients smokers, with an established immunodeficiency, a diagnosis of classic allergic fungal sinusitis, or cystic fibrosis were, however, excluded from the study. Patients with allergy were distinguished from those without allergy on the basis of allergy skin tests.

Table 1. Characteristics of patients

	Normal (Group 1)	CRSw/oNPt (Group 2)	CRSwNPt (Group 3)
Patients (*n*)	10	10	10
Age (year) (mean)	38.5 ± 9.8	35.1±5.7	57.6±16
Gender (male/female)	5/5	5/5	5/5
Duration of disease years (mean)	0	8.6	7.2
Total IgE (KU/L) (mean)	0	339	516
Incidence of elevated total IgE (>128 KU/L)	0	50%	50%
Incidence of allergy†	0%	45%	50%

†Defined as a positive skin prick test to at least 1 allergen in a panel of 16 common aeroallergens

Biomarker's identification in CRS patients with nasal polyps: Gene Expression Analysis

Because it has been previously demonstrated that in patients with chronic inflammatory airway diseases can contribute to the pathogenesis of rhinitis, nasal polyps, acute asthma, cystic fibrosis, and COPD (95-99) and that the remodeling of lower airways correlate with those of the upper airways (27, 28), it was first evaluated wether some specific biomarkers in nasal

polyposis promote tissue remodelling in Chronic Rhinosinusitis.

Focus was placed on genes differentially expressed in the normal nasal mucosa and polyps of patients with CRS with or without allergy. During a pilot phase of this study, 20 CRS patients underwent biopsies, 10 were control subjects and 10 were CRS patients with nasal polyps (CRSwNP).

However, only 5 of 10 sample produced RNA in sufficient quantities and quality to undergo analysis with gene array.

This array was unfortunately discontinued by the manufacturer during the study.

Analysis of nasal polyp samples revealed a similar gene expression pattern between patients. Gene analysis revealed changes in expression of several key genes that code for chemokines, cytokines, receptors, and signal transduction molecules. The most significant changes in expression (>2.5 fold) were noted for IL-19, after analysis with the Human Asthma Gene Array **(Fig.10).** It was found to be expressed 5,3 fold higher in the nasal polyps of the patients with CRS independently of allergy, compared with the control subjects **(Fig.11).**

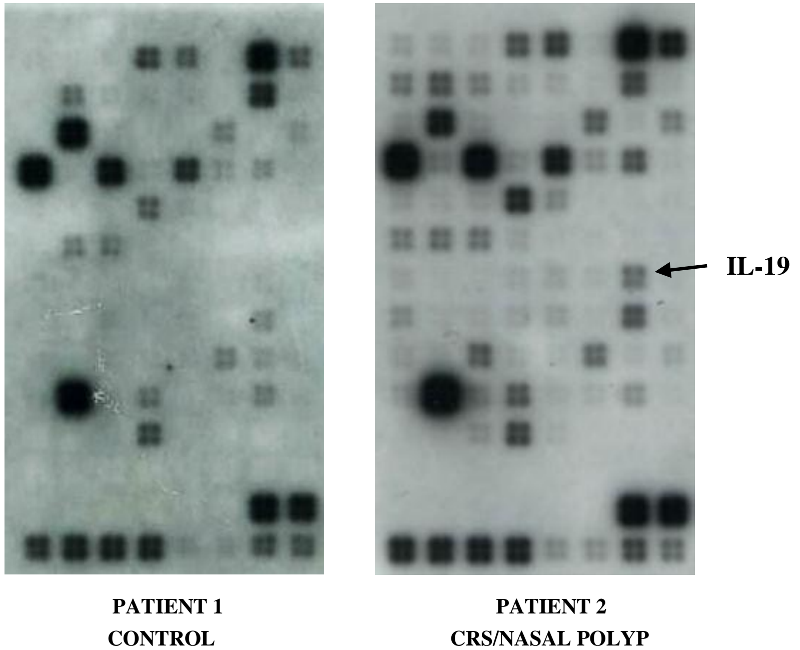


Figure 10. Changes in gene expression in nasal polyps. Note the increase in expression of IL-19 gene in nasal polyps from patients with CRS/NP.

Therefore, gene array analysis demonstrated, that receptor for the costant fragment of immunoglobuline E (FCR1A) was upregulated, instead the expression level of Interferon alpha 1 IFN-a 1 gene was decreased (see table2).

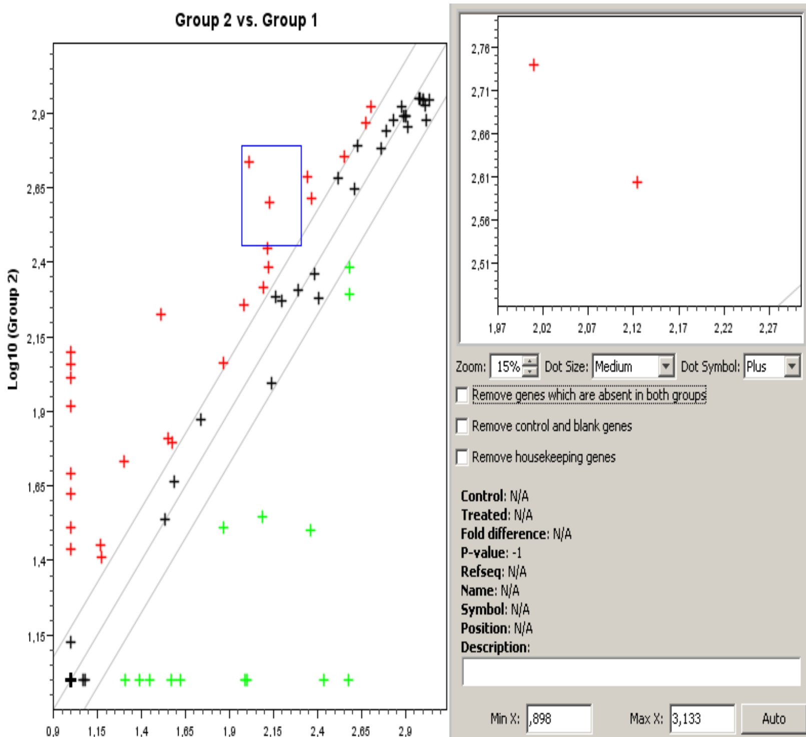


Figure 11. Representative analysis performed by the GEarray analyzer program showing the differences in gene expression between Control and CRS/NP groups.

Table 2
Expression of up- or down-regulated genes. (changes in expression > 2.5 fold)

Gene Bank ID	Gene definition	Genesymbol
NM 013371	IL 10C7MDA	IL-19
NM002001	Fc fragment of IgE	FCR1A
NM024013	Interferon alpha 1	IFL/IFN

Changes in expression of IL-19 gene in patienst with different degrees of Chronic Rhinosinusitis

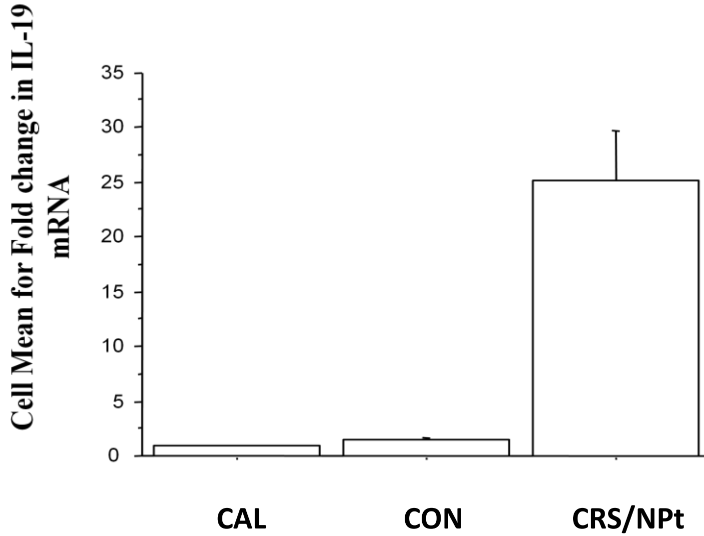
To verify the reliability of findings from the gene array analysis, we analyzed mRNA samples from nasal mucosa and nasal polyps of 15 additional subjects (nasal mucosa from 5 control subjects, nasal mucosa from 5 CRS patients and 5 nasal polyps from CRS patients, (CRS patients with or without allergy).

The quantitative gene expression of the IL-19 gene was determined by Real-time PCR and is displayed in **Fig. 12**.

The IL-19 gene expresion was detectable in all samples, but high levels of expression of mRNA were detected in nasal polyps from CRS patients (**see A, Fig.12**).

For to establish a role of allergy in CRS, we also evaluated, IL-19 mRNA expression in 5 subgroups: control group in which CRS, nasal polyps and allergy were excluded (CON), patients without CRS, without nasal polyps and with allergy (CONwA), CRS patients without nasal polyps, with allergy (CRSwA) and without allergy (CRSw/oA), and nasal polyps from CRS patients with allergy (CRS/NPwA) and without allergy (CRS/NPw/oA).

IL-19 gene expression was detectable in all samples, but high levels of expression of mRNA were detected in nasal polyps from CRS patients indipendently of allergy. Low levels was also detected in nasal mucosa of patients CRS with/without allergy (**see B, Fig.12**).



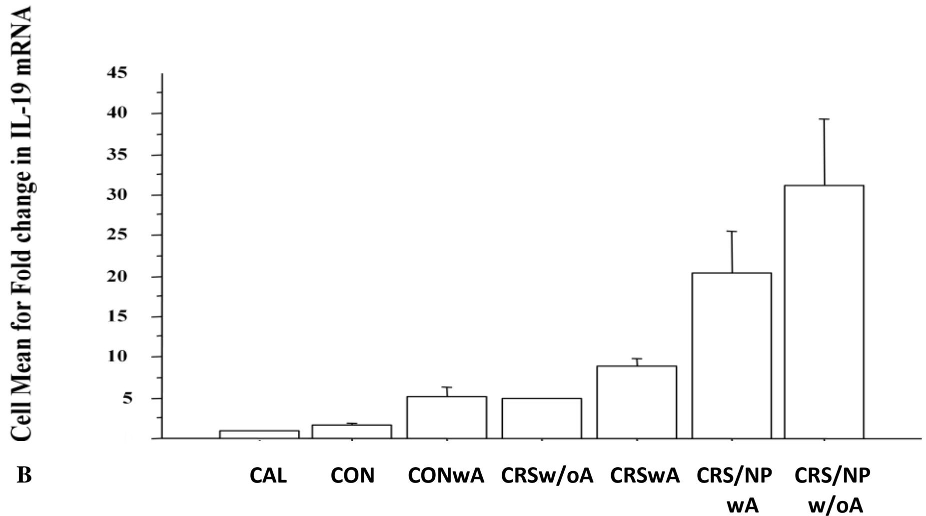
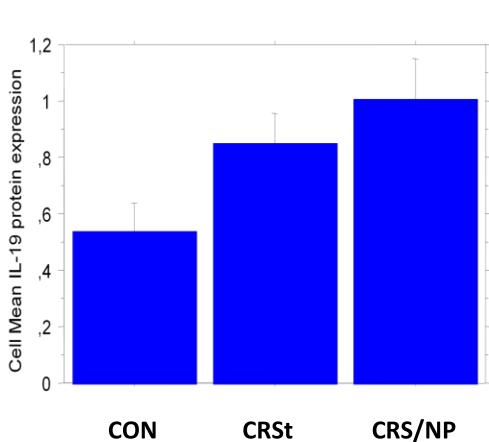
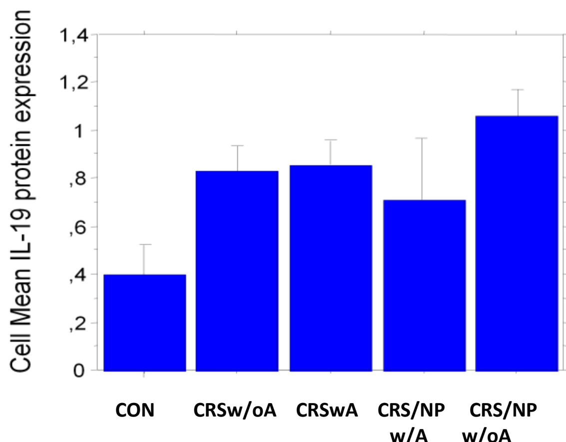


Figure.12: Real-Time confirmed the increased expression of IL-19 mRNA in CRS with Nasal Polyps. (A,B).GAPDH gene expression was used as endogenous control for normalization. Relative quantitation of mRNA was carried out with comparative CT method.

IL-19 proteine expression confirme mRNA levels

Next, we measured the levels of IL-19 protein by immunoblotting after incubation with specific antibody. We observed that IL-19 protein were detectable in lower expression in control subjects, however, a robust increase were detectable in CRS/NP (with or without allergy) and in lower extent in CRS w/o NP (with or without allergy) (**Fig. 14, A**). In second step, to determine whether the allergy play a role in IL-19 expression, we analyzed it in 5 differents subgroups : control (CON), CRS patients without allergy (CRSw/oA), CRS patients with allergy (CRSwA), CRS patients with NP and without allergy(CRS/NPw/oA), and CRS patients with NP and allergy(CRS/NPwA). We seen that there were not detactables change in expression between groups, infact we seen no differences between allergic CRS patients without NP with allergic and non allergic CRS patients with nasal polyps, therefore no difference were present between nasal polyps from allergic and non allergic CRS patients (**Fig.14, B**).
Those IL-19 seems to be specifically a nasal polips marker.





A **B**

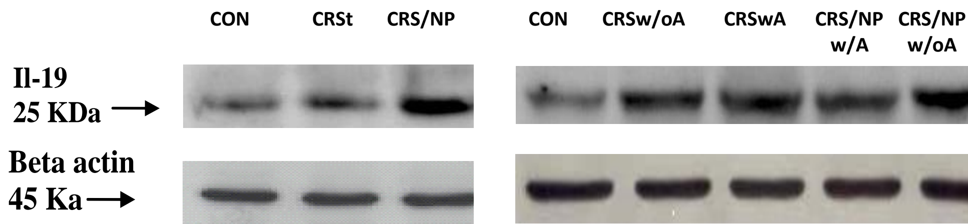


Figure. 14. ± Detection of Interleukine 19 (IL-19) via Western immunoblots. The total protein (20 µg) extracted from nasal mucosas and nasal polyps from CON, CRS patients with or without nasal polyps, with or without allergy and NP from CRS patient with or without allergy, were run on the 12% polyacrilammide gel and a goat polyclonal antihuman IL-19 (Santa Cruz) used for detection. IL-19 was detected in its dimeric 25 kDa form: intensely onNasal polyps and moderately on nasal mucosas from respectly CRS patients with or without allergy. STD: molecular weight standards (kDa).

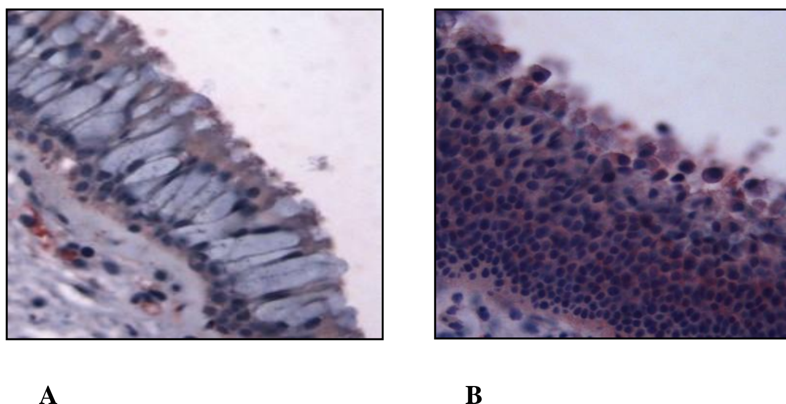
Localization of IL-19 protein in the upper airways

In this initial study, we analyzed overall gene expression with RNA from the entire nasal mucosas and nasal polyps speciments. It is likely that some genes are expressed to a greater degree in the epithelium, whereas others are expressed more in the stroma. It is also likely that gene expression within eosinophils, lymphocytes, dendritic cells, and other cells may differ and hold clues to the specific pathophysiologic mechanisms at play.

Therefore, to investigate the levels expression of IL-19 protein and the role in remodelling mechanisms, we observed the IL-19 protein expression in metaplastic area of nasal polyps ephitelium. On immunohistochemistry staining, the epithelium in CRS/NP sample demonstrated a mucosal hypertrophy, hyperplasia and metaplasia of epithelial goblet cells. A large number of inflammatory cells such as plasma cells, lymphocytes, macrophages and eosinophils was noted.

Interestingly, it has been shown that the major cell type stained specifically positive for IL-19 were ephitelial cells (**Fig.15).** In according with a study, we established a staining IL-19 level score: low intensity (score 1-3) and high intensity (4-9). We

evaluated IL-19 staining in normal, hiperplastic and metaplastic ephitelium from nasal polyps of CRS patients and we observed that the intensity of staining was more evident in metaplastics area than hiperplastic and normal ephitilium.
More staining were the macrophages and glandular cells. (Data not shown).



A **B**

Figure 15. Immunohistochemical staining for Interleukine 19 (IL-19) in epithelial cells of human nasal polyp tissue and nasal mucosa at 400 x magnification. IL-19 immunoreactivity is weak in hypertrophic ephitelium (**A**). Squamous metaplastic epithelium showed higher expression of IL-19 protein in CRS nasal polyps (**B**).

DISCUSSION

Chronic rhinosinusitis (CRS) is an inflammatory disease with distinct cytokine and remodeling patterns. Chronic rhinosinusitis with nasal poyps (CRSwNP) is characterized by a Th2-skewed eosinophilic inflammation, whereas chronic rhinosinusitis without nasal polyps (CRS) represents a predominant Th1 milieu. However, although nasal chronic inflammation can lead to obstruction and subsequent chronic rhinosinusitis (95), not all individuals with chronic nasal inflammation develop CRS or CRSwNP visible on CT imaging.

Previous studies showed the presence of IL-19 in the epithelial cells of the bronchus and small airways, but no data are available to date on IL-19 expression in the upper airways.

In this study we found for the first time an increased expression of IL-19 in patients with chronic rhinosinusitis (CRS). The increase was high in patients with CRS with nasal polyps (NP), less in those without NPs. The increased expression of IL-19 was mainly shown the squamous metaplastic epithelium of nasal polyps, not only in tissue infiltrating inflammatory cells (data not shown), which commonly represent the primary

source of IL-19. These data are consistent with what reported by other studies, showing an increase of IL-19 levels in different epithelia, such as bronchial epithelium under inflammatory conditions.

It was also shown that inflammatory stimuli can induce IL-19 in other non-immune cells such as keratinocytes and foetal membranes (56). IL-19 is produced by activated monocytes, and to a lesser extent, by B-cells (55),

The majority of data implicate IL-19 as a component of the Th2 system and demonstrare a clear role in asthma. Although asthma development is multifactorial, Th2 cytokines are highly associated with the disease, especially IL-4, IL-5 and IL-13, wich are secreted following antigen challenge in allergic asthma (89-91). Il-19 mRNA levels were found elevated in the lung of BALB/c mice, following exposure to allergen. In humans, Liao et al. (84) demonstrated that IL-19 levels were elevated 3-4-fold in the serum of children with asthma, compared to normal children or adults. High IL-19 level correlated with IL-4 and IL-13 levels. Huang et al (39) expanded these results, demonstrating that IL-4 and IL-13 elicit the production of IL-19. On the other hand, it was also shown

that IL-19 is sufficient to induce the production of Th2 cytokine production in vivo (55).

On the basis on these considerations, it is not surprising the finding of an increase of IL-19 in CRS, given the known link between CRS and asthma. The nose and the bronchi belong, in anatomical and physiopathological terms, to the concept of united airways. Associations between upper and lower airways diseases have been demonstrated since allergic rhinitis and CRS are all associated to asthma.

In this context, it could be hypothesized that the increase of IL-19 in patients with CRS, could be considered as a biomarker of subsequent evolution to asthma. This could be in particular for those patients, with CRS with NP, showing the highest expression of IL-19. Indeed, asthma improves after medical or surgical treatment of NPs. A recent study reported that in patients with NPs the incidence of asthma was remarkable (48,9%) and that skin prick tests were positive in 63,2% of these patients.

The involvement of IL-19 in immune response, as a modifier of Th1/Th2 balance in favor of Th2 pathway, suggests a possible role in allergic disease. However, we did not found

any difference of IL-19 expression between allergic and non-allergic NPs, ruling out an association of IL-19 with allergy.

Epithelial damage and aberrant tissue remodeling are common features in NP. Features of nasal remodeling may be considered: squamos metaplasia, basement membrane thickening, collagen deposition, hyperplasia of mucous glands, and globet cells (96). Mucus hypersecretion is a common pathologic change in chronic inflammatory airway disease associated with hyperplasia and metaplasia of secretory cells (97). In this regard, persistent mucoid and mucopurulent rhinorrhea are some of the major clinical features in CRS and CRS/NP. Here, it has been demonstrated that IL-19 is up-regulated at both mRNA and protein level and its higly expressed by the squamous metaplastic epithelium of nasal polyps.

The exact role of IL-19 in goblet cell metaplasia in CRSwNp patients is not clear, but on the basis of previously finding, we suggested that the presence of IL-19 in metaplastic ephitelium may play a role of the upper airways remodeling phenomena in certain people who develop polyps, irrespective of whether or not they are atopic.

Data on a possible role of IL-19 in tissue remodeling in asthmatic patients are still lacking. IL-19 was involved in the pathogenesis of asthma, through its modulatory role on Th2 cytokines. In contrast, we showed that in upper airways the role of IL-19 more related to tissue remodeling, being associated to squamous metaplasia of NP tissue. Considering the close relationship between asthma and CRS, it is possible that also in asthma IL-19 could play a role in tissue remodeling. On the other hand, the lack of correlation with allergy in the present study we seems to suggest that possible different mechanisms may be activated in upper and lower respiratory tract. Ongoing studies are now exploring this complex issue.

CONCLUSIONS

The results of the present study provide evidence on a putative role of IL-19 in NPs tissue remodeling, in patients with CRS independently on their allergic status.

Given the role of IL-19 in asthma and the tight relationships between asthma and CRSwNP, further studies will aim to understand a possible role of IL-19 as predictor of subsequent development of asthma for patients with CRSwNP.

MATERIAL AND METHODS

Subjects group

Patients were recruited at the "Dipartimento di Otorinolaringoiatria, Palermo, Italy" and at the Department of Otorhinolaryngology-Ghent, Belgium. A total of 25 patients and 15 control subjects were included in this study: group 1, control (CON); group 2, CRS patients without NPs (CRSw/oNP) (n=10); the last, group 3, nasal polyps from CRS patients (CRSwNP) (n=15). The diagnosis of CRS was based on history, clinical examination, nasal endoscopy, and CT scanning of the sinuses according to the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines (Fokkens W., Allergy 2005). The patients with Samter's syndrome and systemic diseas (cystic fibrosis, autoimmune systemic diseases, hyper-responsiveness to aspirine) were not included in the study. The control group consisted of inferior turbinate specimens of patients, without polyp and without rhinosinusitis and allergic rhinitis who underwent partial inferior turbinate excision and septoplasty.

All procedures were performed in accordance with the ethical guidelines, and they had approval by the Ethics Committee of our Hospital and was in agreement with the Helsinki Declaration. All subjects had given their informed consent.

Nasal biopsies and tissue handling

Tissue samples from nasal polyps and tissue samples of the normal nasal mucosa from inferior turbinate were performed under local anesthesia. Prior to surgery, 2% tetracaine HCl were applied topically to the turbinates, nasal septum and middle meatus. After decongesting, the polyp was injected with 2-4 ml of 1% lidocaine with 1:1000000 adrenaline. Then, the polyps were removed using gentle traction and snare technique.

Tests for Allergy and Atopy and Determination

Patients were tested for allergy and atopy by using skin print test (SPT) and measuring serum total and specific-IgE levels (DPC-Immulite 2000). A panel of common allergens was used

for SPT and specific IgE including animal, house dust, grass, tree, mold, food, and weed panel allergens. SPTs were performed after histamine provocation. After 15 minutes, interpretation was done as: 0, negative, erythema only or no reaction; 1+, wheal ≤3mm; 2+, wheal 3 to 5 mm with flare; 3+, wheal ≥5 to 7 mm with flare; and 4+, wheal ≥7 mm usually with pseudopods and flare. The patient was considered prick test positive, if at least 1 allergen elicited a weal diameter was at least 3 mm larger than that of the negative control.

Evaluation of RNA extraction yeld, purity and integrity

Total RNA was isolated from the samples using TriZol reagent (Invitrogen, California, USA), as described in manufacture's protocol. RNA quality was evaluated by evaluated by running on a 1% agarose gel and inspecting for distinct 18S, 28S, and tRNA bands, indicating lack of degradation; quantity was determined by spectrophotometry analysis and finally purity of each sample was determined by calculating the 260/230 ratios. Sample were frozen at -80°C until use.

Gene expression Arrays (GEArray Q series Gene Array, SuperArray)

At the time of the surgery, nasal polyps and nasal mucosa of each grups, were immediately separated, kept in RNA*later*© (Ambion, Italy) and place at 4°C until the time of the analysis. Total RNAs were isolated from samples, using TRIzol reagent (Invitrogen). The levels of RNA purity and degradation were assessed spectrophotometrically and evaluated in a 1% agarose gel electrophoresis. Total RNA was used as a template for biotinylated probe synthesis. cDNA probes were created with a standardized validated technique (Super Array, Inc, Bethesda, MD). Hybridization studies were conducted with a focused gene array technique (Human Pathway Finder or Human Asthma gene array (Super Array, Inc). The nylon membranes were prehybridized with heat-denatured sheared salmon sperm DNA and diluted in GEAhyb Hybridization Solution (Super Array, Inc) for 2 hours at 60°C. cDNA probes were then added in a hybridization tube filled with GEAhyb hybridization solution and were hybridized overnight with continuous agitation at 60°C. After blocking with SuperBlock (Pierce, <Rockford, IL) (SuperArray) and incubation with alkaline phosphatase-conjugated streptavidin (1:5000), the membranes

were exposed to CDP-Star chemiolumionescent substrate (Tropix, Inc, Bedford,MA) (SuperArray) for 2 minutes. The images obtained after film exposure were digitized with Adobe Photoshop software according to specications, and analyzed with the GEArray Expression Analysis Suite (SuperArray Bioscence Corp, Frederick, MD). Expression of housekeeping genes such as β-actin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were used for data normalization. The relative expression of each gene in the pretreatment and posttreatment groups was determined by comparing the signal intensity and results were reported in light intensity units.

Statistical Analysis

Statistical analysis was performed with the GEArray Expression Analysis Suite (SuperArray Bioscience Corp, Frederck, MD). Results were considered significant when there was at least a 1.5-fold difference between the pretreatment and post-treatment sample intensities. Data were further analyzed with Microsoft Excel (Microsoft Corp, Redmond, WA) statistical software.

Real-Time Polymerase Chain Reaction (PCR)

Total cellular RNA was extracted from nasal biopsies using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the method of Chomezynski an Sacchi (1), and was reverse-transcribed to cDNA, using ;-MLV-RT and oligo (dT)12-18 primers (Invitrogen). Real –Time quantitative PCR of IL-19 transcript was carried out on ABI PRISM 7900 HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) using specific FAM-labelled probe and primers (Applied B yosistems, TaqMan Assay on Demand). GAPDH gene expression was used as endogenous control for normalization. Relative quantitation of mRNA levels was carried out with comparative Ct method (100) and was plotted as fold-change compared to the normal sample expressing the least level of IL_19 transcript, that was chosen as the reference sample.

Western Blot Analyses of IL-19

Total proteins from human nasal turbinates and nasal polyps were prepared using Paris TM Kit (Ambion) as described in manufacturer's protocol. Cell extracts were transferred in

microcentrifuge tubes, left on ice for 45 min, and centrifuged at 15,000g for 20 min at 4°C. Fourthy micrograms of proteins were subjected to SDS-polyacrylmide, resuspended in 2µl Laemmli buffer 10X and separated by SDS-PAGE gel electrophoresis at 12% gradient gels and blotted onto nitrocellulose membranes. These was blocked overnight at 4°C with PBS containing 5% milk and 0,1% Tween 20 (5%milk-PBST), and then probed with a polyclonal Ab directed against human IL-19 (IL-19(C-20): sc-16729(Santa Cruz) diluited at 1:100 in 5% milk-PBST for 1 hour. The membrane was washed with PBST, and incubated with peroxidase-conjugated secondary antibodies (anti-goat, Invitrogen) diluited at 1:2000 in 5% milk-PBST for 1 hour. Detection was performed with an enhanced chemioluminescence system (GE Healthcare, Chalfont St. Giles, UK). Approximate molecular masse was determinate using calibrated prestained standard (GE Healthcare).

Gel Image Evaluation

Gel images were taken with an EPSON GT-6000 scanner and then imported to a NIH Image analyses 1.61 program to determine band intensity. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

Tissue processing, morphometry and immunohistochemistry

Fragments of polyps and nasal inferior turbinate specimens from each patients were removed surgically, fixed immedediately in NBF (Neutral Buffer Formaline 10%) for 24 hours, and embebbed in paraffine. The sections were cutted at 4-5 μm and stained by haematoxylin and eosin for test the quality. Before deparaffinization in xylene, tissue section slides were baked in oven at 37°C for 24 hours on a vertical rack to melt the extra layer of coated paraffins. The sections were deparaffinised, rehydrated using the following series of washes: two xylen washes, followed by one in 100% ethanol rinses, one in 80% ethanol, and 50% ethanol. The last wash

were in tap water and TBS (Tris-Bufferd Saline containing Tris-HCl 0,5 M *ph*7.6 and NaCl 1,5 M.) on a shaker. Antigen retrieval was optimized by incubating the specimens in a bath at 95°C for 45 minutes. The samples were washed in TBS and incubated in 0,5% Albumin from Bovine Serum (BSA, Sigma-Aldrich) in TBS at room temperature for 20 minutes, before incubation overnight at 4°C, with primary antibody: IL-19 (C-20): sc-16729 (Santa Cruz), diluted at a concentration of 1:50 in TBS. Omission of the Primary antibody was used as negative control. The samples were then incubated in biotinylated secondary antibody reacts with alkaline phosphatase conjugated streptavidin molecules (Universal DakoCytomation LSAB2 System, Alkaline Phosphatase). Staining is completed after a 15 minutes of incubation with the substrate-chromogen solution. Sections were assessed in a random order with no knowledge of the patients clinical details on two occasions.

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