Molecular Signatures as a New Classification Scheme for Chronic Rhinosinusitus

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July 2012

A thesis submitted to McGill University

in partial fulfillment of the requirements of the degree of

Masters of Science

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List of Abbreviations

ALI	Air–liquid interface
APC	Antigen Presenting cell
ARS	Acute Rhinosinusitis
AD	Atopic Dermatitis
CF	Cystic Fibrosis
CCL2	Chemokine (C-C motif) ligand 2
ClCa4	Calcium-activated chloride channel regulator 4
CRS	Chronic Rhinosinusitis
CRSwNP	Chronic Rhinosinusitis with Nasal polyp
CRSsNP	Chronic Rhinosinusitis without Nasal polyp
CT	Computed Tomographic
DAB	Diaminobenzidine (peroxidase substrate)
DC	Dendritic cell
FC	Fold Change
FESS	Functional Endoscopic Sinus Surgery
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-22	Interleukin-22
IL-22R	Interleukin-22 Receptor
INF-γ	Interferon Tumor Necrosis Factor- alpha

IPA	Ingenuity Pathways Analysis
KRT4	Keratin 4
KRT13	Keratin 13
MCP-1	Monocyte chemoattractant protein-1
NFkB	Nuclear Factor-KappaB
PAMP	pathogen-associated molecular patterns
PAR	sprotease-activated receptors
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate Buffer Saline
PCA	Principal components analysis
PRRs	pattern recognition receptors
RAST	Radioallergosorbent test
S. Aureus	Staphylococcus Aureus
Sag	Superantigen
SE	Staphylococcus aureus enterotoxin
T-reg	T-regulatory lymphocyte cell
DAB	Diaminobenzidine (peroxidase substrate)

Abstract

Chronic Rhinosinusitis (CRS), an inflammation of the paranasal sinus cavities, is a common disorder of uncertain etiology that affects the upper airways and paranasal sinuses. Biopsy specimens had taken from CRS patients document disruption of the normal epithelial architecture, in addition to an intense infiltration of inflammatory cells, consisting mainly of eosinophils. Current clinical classification of CRS is based on the presence or absence of nasal polyposis; however, no consistent difference in histological aspect characterizes these two groups. Recently, we have identified distinct gene expression patterns in cultured epithelial cells obtained from surgical CRS subjects. These molecular signatures, which differ from clinical phenotype, may help better differentiate this disorder than clinical phenotype. In our current study we investigated the histological pattern associated with these two different molecular signatures in surgical biopsies obtained from CRS patients and control subjects. Cellular infiltrates were identified using immunohistochemistry (IHC) staining using three markers: neutrophil elastase (NE), CD68, Major basic protein (MBP). Macrophage activation status into classical and alternatively activated macrophages was verified by double-staining for CD68 and CD 206 markers. Results were reported both according to standard clinical criteria (CRSwNP and CRSsNP) and also according to their expression signature into two groups (CRS1, CRS2) and control subjects. Expression signatures were validated using immunohistochemical staining for the highest differentially expressed marker, CCL2.

Results showed differences in the number of eosinophils, macrophages and neutrophils cells in CRS patients compared to the control subjects. Using conventional

criterion, eosinophilia was higher in the CRSwNP group, but not greatly different for neutrophils or macrophages between the two groups. Using the molecular signatures to assign groups, eosinophilia was similar between both groups, however, there was a significant increase in the number of neutrophils and macrophages in CRS1 comparing to CRS2. The CRS2 group had a higher incidence of alternatively activated macrophages, supporting the concept of a less inflammatory, immunotolerant CRS2 phenotype. Validity of the molecular signature was supported by demonstration of increased levels of protein product of CCL2 expression in CRS1 compared to CRS2.

Taken together, these results identify a molecular phenotype of CRS that is characterized by a marked neutrophilic infiltration, and a second one that is markedly less inflammatory, accompanied by alternative macrophage activation. This suggests that these expression signatures may identify novel mechanism-based phenotypes, which differ from the clinical phenotype, and can help in providing a better understanding of pathophysiologic mechanism and phenotypes of CRS.

Résumé

La rhinosinusite chronique (RSC), une inflammation des sinus paranasaux, est un trouble commun avec une étiologie incertaine, qui affecte les voies respiratoires supérieures et les sinus paranasaux. Les biopsies des échantillons prélevés sur des patients atteints de RSC documentent la perturbation de l'architecture normale épithéliale, en plus d'une infiltration de cellules inflammatoires intense constituée principalement par des éosinophiles. La classification clinique actuelle de la RSC est basée sur la présence (CRSwNP) ou l'absence (CRSsNP) de polypose nasale, mais aucune différence consistente de l'aspect histologique caractérise ces deux groupes. Récemment, nous avons identifié des profils d'expression génique distincts dans des cultures de cellules épithéliales provenant de sujets atteints de la RSC ayant subis une chirurgie des sinus. Ces signatures moléculaires, qui diffèrent du phénotype clinique, peuvent aider à mieux différencier ce trouble que le phénotype clinique. Dans notre étude, nous avons étudié l'aspect histologique associé à ces deux différentes signatures moléculaires à partir de biopsies chirurgicales obtenus chez des patients atteints de la RSC et les sujets témoins. Les infiltrats cellulaires ont été identifiés par immunohistochimie (IHC), une coloration à l'aide de trois marqueurs: l'élastase de neutrophile (NE), le CD68 et la protéine basique majeure (MBP). L'état d'activation des macrophages dans les formes classiques et alternativement activés a été vérifié par une double-coloration pour les marqueurs CD68 et CD206. Les résultats ont été rapportés à la fois selon les critères cliniques habituels (CRSwNP et CRSsNP) et aussi en fonction de leur signature d'expression en deux groupes (CRS1, CRS2) et les sujets témoins. Les signatures d'expression ont été validées à l'aide de coloration immunohistochimique pour le marqueur le plus différentiellement exprimé, le CCL2.

Les résultats ont montré des différences dans le nombre d'éosinophiles, macrophages et les cellules de neutrophiles chez les patients atteints de la RSC par rapport aux sujets témoins. Selon le critère classique, l'éosinophilie était plus élevée dans le groupe CRSwNP, mais pas très différent entre les deux groupes pour les neutrophiles ou les macrophages. En utilisant les signatures moléculaires pour assigner des groupes, l'éosinophilie était similaire entre les deux groupes, cependant, il y avait une augmentation significative du nombre de neutrophiles et de macrophages dans CRS1 comparativement à CRS2. Le groupe CRS2 avait une incidence plus élevée des macrophages alternativement activés, supportant le concept d'une inflammatoire basse, phénotype CRS2 immunotolérant. La validité de la signature moléculaire a été supportée par la démonstration du niveau accru de la protéine produite par l'expression de CCL2 dans CRS1 par rapport à CRS2.

En somme, ces résultats mettent en évidence un phénotype moléculaire de la RSC qui se caractérise par une infiltration neutrophilique marquée, et une seconde qui est nettement moins inflammatoire, accompagnée par l'activation alternative des macrophages. Ceci suggère que ces signatures d'expression peuvent identifier de nouveaux mécanismes basés sur des phénotypes, qui diffèrent du phénotype clinique, et peuvent aider à fournir une meilleure compréhension du mécanisme physiopathologique et les phénotypes de la RSC.

Acknowledgments

I would like to thank my supervisor, Dr. Martin Desrosiers for giving me the chance and opportunity to do my master's research at his lab, and most importantly for believing in me. Thank you for sharing with me your knowledge and vast experience, your stimulating guidance, and generous support.

I would like to thank my Co-supervisor Dr. Sam Daniel for his academic support.

I would also like to thank the members of my advisory committee: Dr. J. Mort, Dr. Q. Hamid, and Dr. J. Lapointe for their time and scientific inputs.

I would like to thank Dr. Celine Bergeron for allowing me to use her lab facility at the CRHCUM at the Hotel-Dieu Hospital site of the CHUM. .

I would like to thank the wonderful members of our lab for their assistance and support that helped me in expanding my scientific knowledge. I would like to thank Ms. Leandra Mfuna-Endam for her help in organizing and recruiting the patients, Dr. Ali Filali-Mouhim for his help in statistical analysis, and Mrs. Valerie Tardif and Mrs. Céline Divoy for their technical assistance in the lab.

Contribution of the Author

The genomics core of the McGill University and Génome Québec Innovation Centre performed RNA amplification and chip hybridization. Mrs. Valerie Tardif performed the cell culture and RT-PCR works. The author performed all other experiments and procedures presented in this thesis.

Dedication

I would like to express my deepest gratitude to my husband for all his help in making this thesis happens. His endless support means a lot to me.

I would also like to thank my dear children Areej, Hassan, and Tala for their appreciation of the circumstances of my studies, especially the youngest one Tala, who shared most of the experience with me.

I am grateful to my parents for their moral and continuous support, and to my dear friends for their presence in my life, which helped me in reaching my goal.

CHAPTER ONE: INTRODUCTION

Chronic rhinosinusitis (CRS) is a frequent inflammatory disorder of the upper airways characterized by intense inflammatory cell infiltrate and bacterial colonization. Characterization of the cellular infiltrate identifies a pronounced Th2 skewing, with variable degrees of neutrophilia and altered lymphocyte subpopulations. More recently, alteration of the Th17 axis has also been suggested (Van Crombruggen, Zhang, Gevaert, Tomassen, & Bachert, 2011; Zhang et al., 2008). Host factors conferring susceptibility to this disorder, and infectious and environmental factors contributing to pathogenesis remain elusive (Van Crombruggen et al., 2011).

Studies of bacteriology in CRS patients have identified an over-representation of Staphylococcus aureus and gram-negative organisms, with the presence of bacterial biofilms or intracellular forms of Staphylococcus aureus possibly facilitating persistence (Cohen et al., 2009; Stephenson et al., 2010).

Evidence shows that bacteria may act as biological modulator; Staphylococcus aureus can modulate the course of CRSwNP by producing various enterotoxins (Stephenson et al., 2010). These toxins stimulate T cells by their superantigenic activities and stimulate release of cytokines and eotaxin, which lead to a Th2 biased inflammation (Cohen et al., 2009; Van Crombruggen et al., 2011).

CRSwNP is characterized by high rates of S. aureus colonization as compared to controls. The mechanisms of this increased bacterial colonization in CRS are not clear, but may be due to a defect in the defense system in CRSwNP involving the epithelial barrier and/or phagocytosis.

Mucosal macrophages, in the sinus mucosa phagocytosis, are located in the

epithelium, at the interface is assumed by the external environment. Depending on the factors the macrophages encounter, macrophages become polarized into a classically activated pro-inflammatory (M1) phenotype or into an alternatively activated (M2) phenotype (Martinez, Helming, & Gordon, 2009). M1 macrophages express high levels of pro-inflammatory cytokines, as well as high levels of effector molecules, including nitric oxide. M1 macrophages participate in the induction of a Th1 response and prevent pathogen persistence. M2 macrophages producing anti-inflammatory cytokines [e.g. non-opsonic receptor, and mannose receptor (CD206)] (Fairweather & Cihakova, 2009), have a counter regulatory role to phagocytosis, are immunosuppressive and may support intracellular survival of bacteria and viruses (Benoit, Desnues, & Mege, 2008). M2 polarization of macrophages has been linked to the persistence of allergic disease and asthma (Gordon & Martinez, 2010; MacKinnon et al., 2008).

While several aspects of innate immune defenses have been analyzed in CRS patients, the phenotype of macrophages in the nasal tissue of CRS patients is not known, and it is also unclear whether there is any alteration in macrophage function in any CRS patients. A recent study (Krysko et al., 2011) demonstrates, for the first time, the presence of alternatively activated macrophages (M2) and deficient phagocytosis of S. aureus in CRSwNP. Krysko et al.'s (2011) data suggest that a defect in the phagocytic system in CRSwNP might contribute to the increased colonization by S. aureus in this condition. This observation supports the hypothesis that the cytokine milieu in the nasal mucosa could modify macrophage polarization and alter efficiency of the host defense mechanisms.

Dysfunction of the sinus mucosa at the epithelial level has also been suggested as

a factor; Tieu, Kern, and Schleimer (2009) implicate alterations in the epithelial barrier as key to the development of CRS, showing that a defect in the expression of a broad set of epithelial derived genes might lead to barrier compromise and subsequently a dysfunctional host immune response to environmental agents in patients with CRS, while Lane, Truong-Tran, and Schleimer (2006) have suggested impaired innate immunity at the epithelial level in surgery-unresponsive CRS patients.

Population association genetic studies have identified several genetic polymorphisms associated with chronic rhinosinusitis. These implicate candidate genes in potential biological pathways possibly implicated in susceptibility to the development of chronic rhinosinusitis. Interestingly, these suggested dysregulated immune detection and regulation as genetic factors in CRS (Mfuna-Endam, Zhang, & Desrosiers, 2011).

Adding to the difficulty in understanding the pathophysiology of CRS remains the difficulty in accurately phenotyping patients. Currently, classification of CRS is based upon the presence of hypertrophic changes within the sinus cavity extending into the nasal passages (nasal polyps) (Meltzer et al., 2004). However, it has long been appreciated that nasal polyps probably represent the end stage of a number of various pathophysiologic mechanisms. A practical illustration of this is that on histology, nasal polyps are classically described as having a predominantly eosinophilic pattern. However, nasal polyps recovered from patients with cystic fibrosis, despite having an identical macroscopic appearance, most frequently have a predominantly neutrophilic infiltrate (Sobol et al., 2003). Additionally, anecdotal reports from Asian countries on patients of Chinese or Japanese origin have suggested that neutrophilia, as opposed to eosinophilia predominates in nasal polyps from Asian patients in Asia (Zhang et al.,

2010).

Based on these identified racial differences, Bachert et al. (2009) have attempted to identify differences in pathophysiologic mechanisms in nasal polyposis. By comparing samples obtained from populations of Caucasian patients from Belgium and Chinese patients in China, they suggest that a differential activation of the Th17 axis, as documented by differential presence of FOX-P3 T regulatory (T-reg cells) and the TGF-Beta1 (cytokine) serve as markers of different populations. Developping on this disease, they suggest that these identified differences reflect differential responses to staphylococcal super antigens within the tissue (Van Crombruggen et al., 2011). In support of this study, (Fokkens et al., 2012) also demonstrate a down regulation of T-reg activity in CRSwNP.

It is thus clear that current clinical classifications of NP may not accurately reflect underlying pathophysiologic mechanisms. This is of concern as there is a true need for a better means of differentiating patients with airway inflammation according to underlying pathophysiologic mechanisms in order to direct therapy accordingly. Examples of this abound in the lower airway, where patients with “steroid-resistant” asthma present hard-to-manage disease with increased patient morbidity in healthcare expenditures. While this phenomenon has not yet been specifically studied in nasal polyposis, differential responses to therapy have been identified in clinical trials of alternate therapies for CRS (Desrosiers et al., 2011; Kowalski, Cieslak, Perez-Novo, Makowska, & Bachert, 2011).

There thus exists a real need for an improved classification method of CRS, based on pathophysiologic mechanisms rather than clinical aspect. However, we believe that studies to date, which have relied upon sinus tissue obtained at the time of surgery, may

not appropriately represent the initial events of underlying pathophysiological process. Tissue recovered at time of surgery does not adequately represent the early stages of disease development, but instead represents the common end product occurring from the unique combinations of host susceptibility and environmental factors. Biopsy material obtained from severely diseased patients undergoing surgery incorporates modifications occasioned by previous therapy and the inflammatory milieu generated by the cellular infiltrate, rather than the early precipitating events we would like to target.

Hypothesis

We believe that molecular mechanism underlying different forms of CRS are independent of polyp/ non-polyp phenotype and could be better understood by classifying these according to molecular mechanism of pathogenesis, rather than by macroscopic appearance.

In order to better classify CRS patients according to underlying molecular mechanism, we believe it would be important to identify molecular mechanisms contributing to the development of CRS at an early stage by studying tissue free of the influences of inflammatory cells and bacterial products. Given the important role of the epithelial cell as a physical barrier and in immune regulation, we studied potential underlying molecular mechanisms in an isolated epithelial cell culture model, using primary epithelial cells raised from CRS and control patients.

Objectives

The objectives of our study were as follows:

1. To identify molecular signatures of CRS via unsupervised analysis of gene expression profiling.

2. To verify molecular expression signature by characterization of the inflammatory cell profile in surgical biopsies from CRS patients and their demographic data

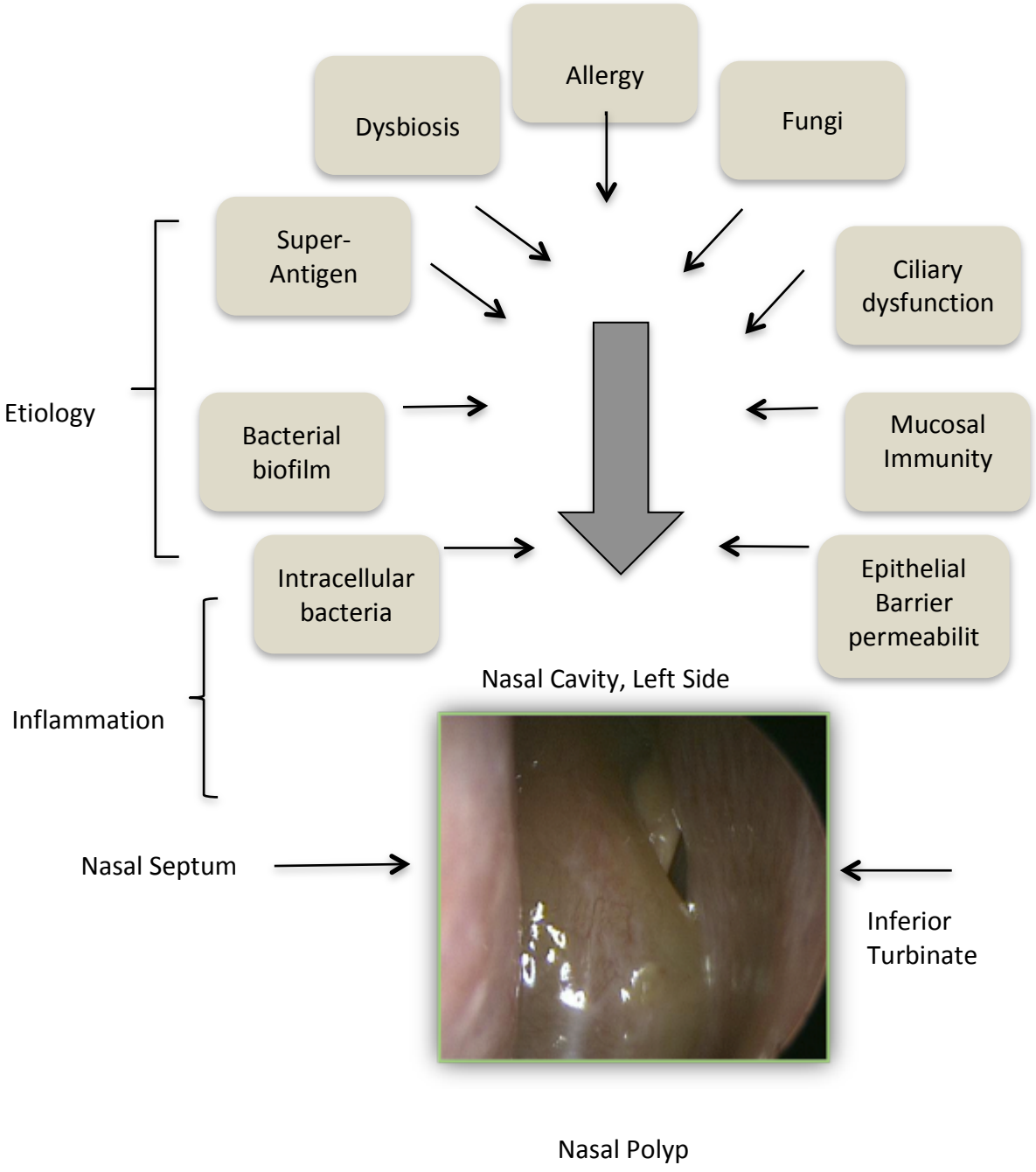


Figure 1.Multiple etiological factors cause the clinical phenotypes of CRS.

CHAPTER TWO: LITERATURE REVIEW

Chronic Rhinosinusitis (CRS)

CRS is defined as a persistent symptomatic inflammation of the nasal mucosa resulting from the interaction of multiple host and environmental factors. It is one of the most commonly reported diseases in the United States, with an estimated prevalence of greater than 10% of the general population (Bachert et al., 2009). The burden to society of CRS is considerable: Costs of medical and surgical care for sinusitis is estimated at over $6 billion yearly in the United State (Desrosiers et al., 2011). Beyond the limited scope of atopy, chronic inflammatory disorders appear to be increasing in incidence and occurring at earlier timepoints at interfaces between self and non-self, including the gut (Crohn's disease [CD] and ulcerative colitis [UC]) (Gaya, Russell, Nimmo, & Satsangi, 2006); lungs (asthma and chronic obstructive pulmonary disease) (Holgate, 2007; Q. Yang, Underwood, Hsin, Liu, & He, 2008); skin (atopic dermatitis AD and psoriasis) (Morar, Willis-Owen, Moffatt, & Cookson, 2006); and eosophagus (eosinophilic esophagitis) (Straumann, Bauer, Fischer, Blaser, & Simon, 2001).

Clinically, CRS may be suspected if two or more of the following symptoms are present: anterior or posterior mucopurulent discharge; facial pain/pressure or fullness; decreased sense of smell; or nasal obstruction, of a duration of greater than eight weeks. Nasal endoscopy and sinus computed tomographic (CT) scans are required for objective confirmation of the diagnosis because of the high false-positive rate using subjective criteria alone (Meltzer et al., 2004).

CRS is commonly subdivided into two subtypes based on the presence or absence of nasal polyps (hypertrophied sinus mucosa visible in the sinus cavity): CRS with nasal

polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) (Meltzer et al., 2004). Thus, these must be sought out on nasal examination.

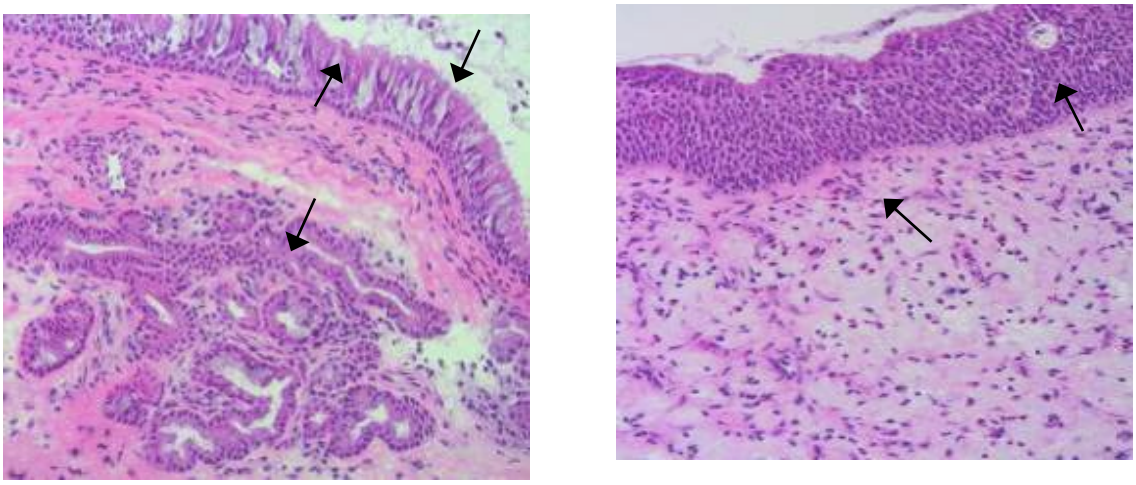
CRS has a considerable negative impact on patient quality of life. CRS patients have more bodily pain and worse social functioning than patients with chronic obstructive pulmonary disease (COPD), congestive heart failure, and back pain (Gliklich & Metson, 1995). The impact on quality of life is comparable in severity to that of other chronic conditions (Lanza & Kennedy, 1997).

Serious complications can occur with CRS because of the proximity of the sinuses to the orbit and cranial cavity. Approximately 75% of all orbital infections are directly related to sinusitis. Intracranial complications remain comparatively rare, with 3.7-10% of intracranial infections related to sinusitis (I. Brook, 2009). CRS may also coexist with other comorbidities such as asthma, cystic fibrosis, or of obstructive sleep apnea, further reducing the quality of life and the productivity of the affected person.

Medical therapy remains the cornerstone of management and relies on combinations of antibiotics and oral or topical corticosteroids. While these combinations are often effective in reliving symptoms at least temporarily, they are rarely curative. In individuals failing to respond to medical therapy, surgical management is required, in the form of endoscopic sinus surgery (ESS), to remove diseased tissue and clear obstructed sinus drainage passages. ESS restores sinus health with complete or moderate relief of symptoms in between 80-90% of patients with recurrent or medically unresponsive CRS (Desrosiers et al., 2011).

Anatomy

Knowledge of the anatomy of the paranasal sinuses is essential for understanding the pathophysiology and management of chronic sinusitis. The four pairs of paranasal sinuses are lined with ciliated, pseudostratified columnar epithelium (respiratory type epithelium). Goblet cells are interspersed among the columnar cells and seromucinous glands. The basement membrane of the epithelium is apposed directly to the bone. The CRSwNP histological structure of mucosa shows the hypertrophic epithelium without goblet cells and the inflamed mucosa with numerous inflammatory cells, mainly eosinophilic, and scarcity of glands at both the epithelium and subepithelium. (Figure 2)



(A) Normal histological structure of sinus mucosa.

(B) CRSwNP histological structure of sinus mucosa.

Figure 2. Normal and CRSwNP histological structure of sinus mucosa, H&E staining
(A) Normal histological structure of sinus mucosa, black arrows shows the ciliated, pseudostratified columnar epithelium Goblet cells are interspersed among the columnar cells and seromucinous glands.
(B) CRSwNP histological structure of mucosa, black arrows shows the hypertrophic epithelium with few goblet cells and the inflamed mucosa with numerous eosinophilic cells and a paucity of glandular structure in epithelium and sub epithelium area. (200X magnification).

The maxillary, frontal, and anterior ethmoid sinuses drain through their ostia located at the ostiomeatal complex lying laterally to the middle turbinate within the middle meatus. The posterior ethmoid and sphenoid sinuses open into the superior meatus and sphenoethmoid recess, respectively. The maxillary ostium is connected to the nasal cavity by a narrow passage called the infundibulum, located at the highest part of the sinus; hence, drainage from the maxillary sinus flows against gravity via mucociliary clearance.

Involvement of the surrounding bone and further extension of the infection into the orbital and intracranial compartments can result from inadequate treatment of sinusitis and specific types of sinusitis (e.g. fungal sinusitis). Because the floor of the maxillary sinus is the tooth-bearing part of the maxilla, dental infections can easily extend to the maxillary sinus (Figure 3).

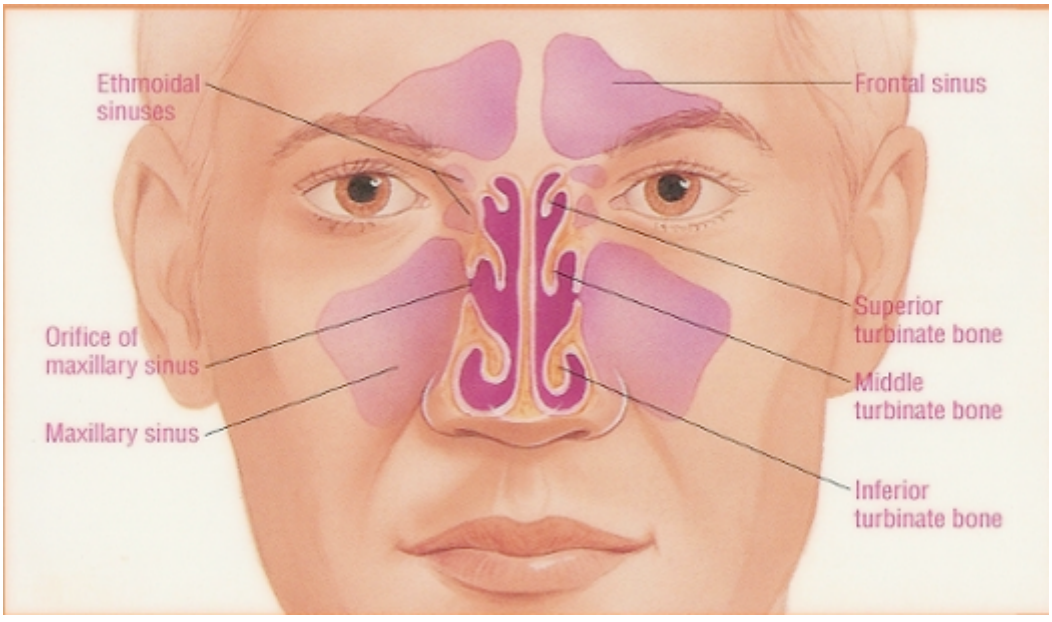


Figure 3. Anatomy of the sinuses. (Ghorayeb, updated 27 December. 2011)

Although the nasal cavity is usually colonized with bacteria, the sinuses are typically considered as sterile (Itzhak Brook, 2012; Van Crombruggen et al., 2011). However, these results from culture-independent bacterial detection methods are increasingly challenging this concept (Stephenson et al., 2010), showing aerobic and anaerobic bacteria are frequently identified with a wide variety of species present, and may play a role in pathogenesis of CRS.

Chronic Rhinosinusitis: Risk Factors

The following conditions and risk factors predispose patients to the development of chronic sinusitis by different mechanisms, such as alterations of the epithelial respiratory structure and remodelling, deposition of extracellular matrix proteins, damage to ciliated mechanical barrier, induction of inflammatory cytokines, impairment of the immunity system and obstruction the nasal drainage, and accumulation of secretions in the nose. These include (according to Meltzer et al., 2004):

- Allergic rhinitis and non-allergic rhinitis.
- Asthma
- Nasal polyps
- Hormonal disorders
- Tumoral obstruction
- Immunologic disorders
- Cystic fibrosis
- Primary ciliary dyskinesia, Kartagener syndrome.
- Repeated upper respiratory tract infections
- Smoking

- Environmental pollution
- Periodontitis and/or significant dental disease.

Etiology

Bacterial Involvement

The bacteria assumed to be involved in CRS differ from those involved in acute rhinosinusitis (ARS). The following bacteria have been reported in samples obtained through endoscopy or sinus puncture in patients with chronic sinusitis.

- Staphylococcus aureus (both methicillin-susceptible S aureus [MSSA] and methicillin-resistant S aureus [MRSA] strains; (I. Brook, Foote, & Hausfeld, 2008)
- Coagulase-negative staphylococci
- Haemophilus influenza
- Moraxella catarrhalis
- Streotococus pneumonia
- Streptococcus intermedius
- Pseudomonas aeruginosa
- Nocardia species
- Anaerobic bacteria
- Peptostreptococcus
- Prevotella
- Porphyromonas
- Bacteroides
- Fusobacterium species (I. Brook, 1989, 2007).

Microbiologic studies of chronic sinusitis often show that the infection is polymicrobial, with isolation of 1-6 isolates per specimen (I. Brook, 2007). In some cases, the baseline chronic sinusitis worsens suddenly or causes new symptoms. This acute exacerbation of chronic sinusitis is often polymicrobial as well, with anaerobic bacteria predominating. However, aerobic bacteria that are usually associated with acute sinusitis (e.g., *S pneumoniae, H influenza, M catarrhalis*) may arise (I. Brook, Foote, & Frazier, 2005). Gram-negative bacteria, including *P aeruginosa*, are more often isolated in patients with chronic sinusitis who have undergone endoscopic sinus surgery (Nadel, Lanza, & Kennedy, 1998; Parkins, Sibley, Surette, & Rabin, 2008).

The exact roles all of these microbes play in the etiology of chronic sinusitis are unclear. Various researchers disagree on the role of anaerobe in chronic sinusitis. It has been suggested that much of the disagreement may be explained by methodology. According to Brook (1989), when proper techniques are used, anaerobic bacteria can be recovered in 50-70% of specimen. Additionally, the variable growth of microbes in samples may also be due to prior exposure of various broad-spectrum antibiotics in patients involved in the studies.

In support of a pathogenic role for anaerobes, Jyonouchi et al. successfully induced chronic sinusitis in rabbits via intrasinus inoculation of *Bacteroides fragilis*; the authors subsequently identified immunoglobulin G (IgG) antibodies against this organism in the infected animals (Incorvaia & Leo, 2010). In addition, IgG antibodies to anaerobic organisms have been observed in patients with chronic sinusitis (I. Brook & Yocum, 1999). These findings further support a role for anaerobes in chronic sinusitis.

Superantigen (SAg) Hypothesis

The SAg hypothesis proposes that *S. aureus*, perhaps protected by biofilms or sequestered within epithelial cells, secrete SAg toxins that result in a generalized stimulation of T cells, cytokine release, and a local polyclonal IgE response, all of which stimulate eosinophil recruitment and the clinical and histopathological changes associated with CRSwNP (Bachert, Gevaert, Holtappels, Johansson, & van Cauwenberge, 2001; Kern et al., 2008; Seiberling, Grammer, & Kern, 2005) (Figure 3.)

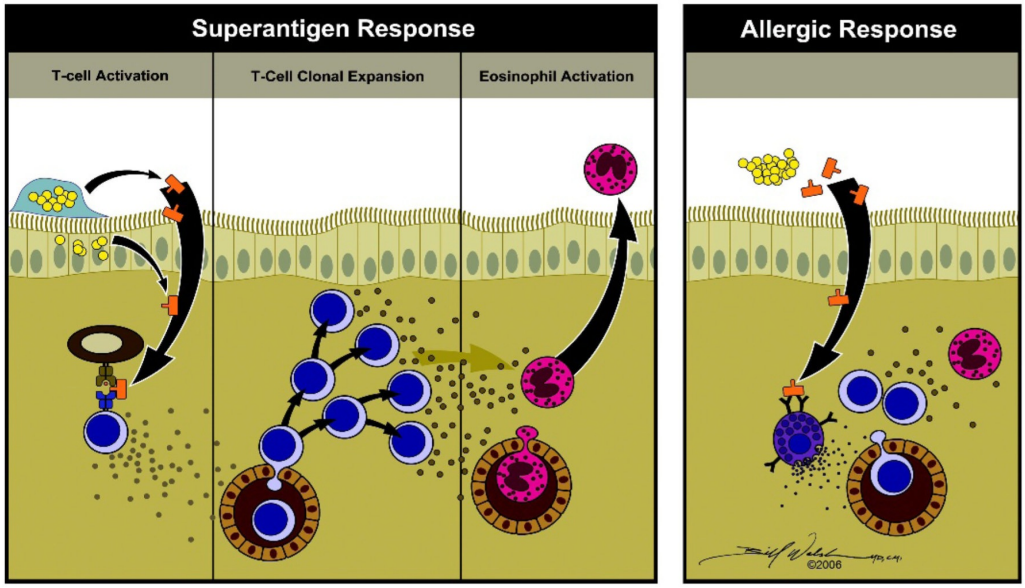


Figure 4. The superantigen (SAg) hypotheis.
(Used with permission from the author (Kern et al., 2008)

In support of the SAg hypothesis, studies have shown an association between the presence of staphylococcus by conventional bacteriology culture and nasal polyposis, Specific IgE directed against enterotoxins in polyp tissue has been established in ~50% of CRSwNP patients in Caucasian Belgian patients.

Evidence suggests that SAgs stimulate local immunoglobulin production in CRSwNP patients, possibly through direct effects on B cells in the nasal mucosa (Van

Zele, Gevaert, Holtappels, van Cauwenberge, & Bachert, 2007). These same studies also showed that another staphylococcal protein A (SpA) induces mast cell degranulation in nasal mucosa, further linking this organism with the pathogenesis of nasal polyposis. (Patou et al., 2008) In comparison, data supporting a SAg effect in CRSsNP is thus far lacking. Nasal tissue from CRSsNP and normal control patients had a comparatively low level of toxin-specific IgE (Zhang et al., 2005). This suggests that CRSwNP and CRSsNP are diseases with distinct etiologies (Seiberling, Conley, et al., 2005; Van Zele et al., 2004), but a mechanism for CRSsNP remains lacking.

In summary, multiple lines of evidence indicate that perhaps one-half of Caucasian CRSwNP patients show evidence of SAg exposure. Moreover, given the relatively universal nature of toxigenic staphylococci, it remains unclear why only a fraction of exposed individuals develop polyps, only one-half of the CRSwNP cases have no evidence of SAg responses, despite presenting with a similar phenotypic picture. Given the absence of a unique histological or molecular phenotype, it can be suggested SAgs are best considered to be disease modifiers in CRSwNP. The association of staphylococcal SAgs with other epithelial diseases such as atopic dermatitis (AD), asthma, and ulcerative colitis (UC) provides indirect support for this view (Hauk, Wenzel, Trumble, Szefler, & Leung, 1999; Kern et al., 2008; Shiobara et al., 2007).

Fungal Hypothesis

The fungal hypothesis proposes that patients with CRS mount an eosinophilic response to fungi, with initial evidence showing some degree of fungi and eosinophilic mucin in all patients with CRS (Braun, Buzina, Freudenschuss, Beham, & Stammberger, 2003; Davis & Kita, 2004; Ponikau, Sherris, & Kita, 2007). A 60-kDa component of the

Alternaria fungus was shown to trigger degranulation of eosinophils from CRS patients by acting on protease-activated receptors (PARs) (Inoue, Matsuwaki, Shin, Ponikau, & Kita, 2005). Collectively, these data were interpreted to be consistent with a T-cell–driven, non-IgE–mediated hypersensitivity response that culminated in the attraction and specific targeting of eosinophils against colonized fungi in the nasal lumen of CRS patients with subsequent degranulation and mucosal damage. In this hypothesis CRSwNP and CRSsNP are viewed as differing forms of one disease resulting from a single pathogenic mechanism of variable intensity.

The fungal hypothesis of CRS suggests that high levels of *Alternaria* can trigger effects on peripheral blood mononuclear cells (PBMCs) and eosinophils obtained from patients with CRS, although it is not clear that this is a disease-specific response. The clinical extrapolation of these findings suggests that intranasal fungi in a patient with CRS would possibly exacerbate the disease process through protease effects on nasal epithelial cells as well as activated eosinophils and lymphocytes present in the nose.

It is, however, unclear whether *Alternaria* has any relevance to the establishment of CRS in the first place. Furthermore, in contrast to initial promising results, subsequent trials using topical amphotericin failed to improve the clinical signs and symptoms in CRS patients (Ebbens et al., 2006). Given these issues, it is reasonable to conclude that the role of fungi in CRS etiology remains unclear, and is probably at least limited (Kern et al., 2008).

Mechanical and Immunologic Barrier of the Nasal Mucosa

Data confirming either fungi or staphylococci as the primary antigenic/etiologic agent triggering CRS are limited, however, and clinical success with either antifungals or

antibiotics have been unimpressive. Furthermore, these two classes of organisms can be identified in the nasal lumen of a high percentage of normal people without CRS, indicating that disease expression will manifest only in susceptible individuals. From this perspective, CRS may be viewed as analogous to inflammatory bowel disease, wherein the tolerance mechanisms toward commensal organisms are impaired. (Granucci & Ricciardi-Castagnoli, 2003). In this situation, it would appear worthwhile to search for defects in the immune response in CRS patients, in addition to attempting to identify unique environmental agents. The upper respiratory tract is not sterile, and the mechanical and immunologic barrier of the nasal mucosa is designed to expeditiously manage the constant load of foreign material with minimal collateral damage.

Mucosal Immunity in CRS

Mechanical barriers, effective mucociliary clearance, and optimal healing limit the degree of antigenic stimulation of immune cells residing in the mucosa.

Despite this impressive barrier function, animate and inanimate matter will stimulate the mucosal immune system, which must distinguish between commensal organisms and potential invading pathogens without excessive tissue damage. Two distinct but integrated immune responses to microbial entities and foreign proteins have been described: innate and acquired. The innate immune system refers to inborn resistance that is present before the first exposure to a pathogen. The innate immune responses are initiated by membrane-bound and cytoplasmic pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) found in microorganisms (Janeway & Medzhitov, 2002). PAMPs are conserved molecular patterns that are common among significant numbers of pathogens; recognition of PAMPs by

PRRs serves as a “danger” signal to the host immune system (Akira, Uematsu, & Takeuchi, 2006). PRRs also identify cellular damage through detection of debris from necrotic cells and the combined recognition of danger and damage signals sets in motion a response consisting of endogenous antimicrobial, antiviral, and anti-proteinase products designed to aid pathogen clearance and preserve the epithelial barrier (Meylan, Tschopp, & Karin, 2006). In addition to the release of innate protective agents, PRR activation triggers the release of chemokines and cytokines mediating the inflammatory response that attracts innate cellular defenses such as neutrophils (Figure 5).

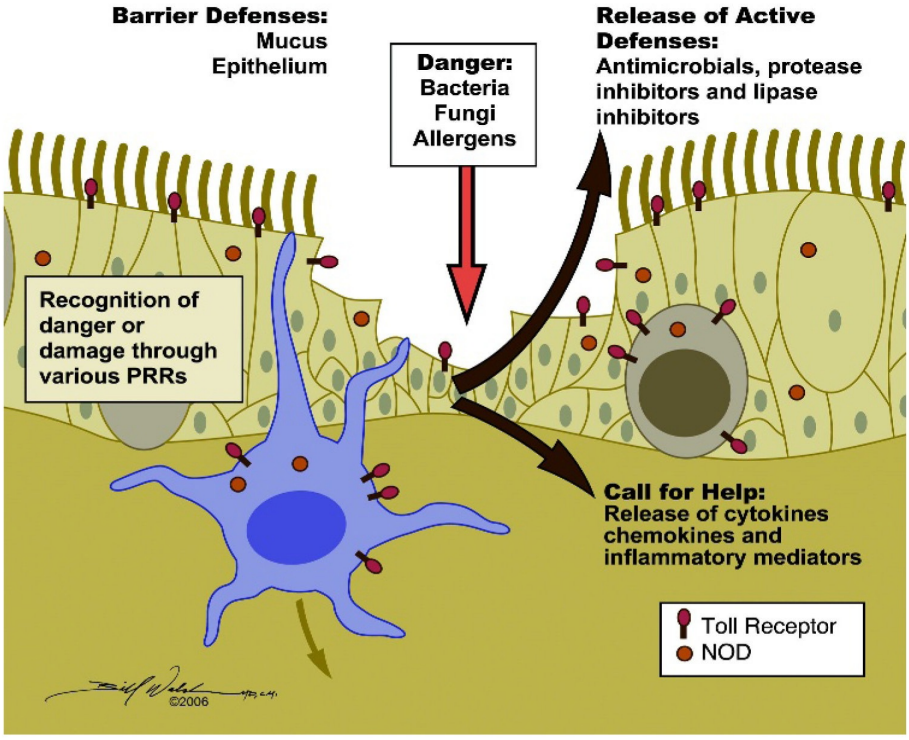


Figure 5. The mechanical and immunologic barrier hypothesis.
(Used with permission from the author [Kern et al., 2008]).

Epithelial defense in the nose first consists of mechanical barriers including mucociliary flow and tight junctions between respiratory epithelial cells that limit stimulation of (PRRs) through diminished exposure and access. (Kern et al., 2008).

The stimulation of PRR also sets in motion and ultimately determines the nature of the acquired immune response (Iwasaki & Medzhitov, 2004). The two best-characterized classes of PRRs are the toll-like receptor (TLR) family and the NOD-like receptor family. (Akira et al., 2006; Meylan et al., 2006). TLRs are transmembrane receptors expressed on multiple cell types, including respiratory epithelial cells. The innate immune response in the sinonasal tract includes antimicrobial factors that can directly interact with potential pathogens (Nochi & Kiyono, 2006; Ooi, Wormald, & Tan, 2008; R. P. Schleimer, Lane, & Kim, 2007). The integration of the innate and acquired immune responses in the sinonasal tract has not been extensively studied but likely begins with the recognition of PAMPs and cellular damage by multiple cell types that respond by secreting immune activating factors including cytokines that stimulate APCs and chemokines that attract the cellular components of the immune response. Damage to the epithelium likely exposes more PRRs to PAMPs, amplifying the immune response; if the PAMP stimulus is sufficiently strong, an acquired immune response will result.

Other studies indicate a significant decrease in expression of the S100 family of genes in CRS patients compared to normal subjects (Richer et al., 2008). These genes, part of the Epidermal Differentiation Complex, participate in epithelial defense and repair and are regulated by the T-cell cytokine IL-22 and its receptor (IL22R; (Wolk et al., 2006). Recent studies have suggested that IL22R may be deficient in nasal polyps, suggesting that this may be one mechanism for the observed deficit in S100 in CRS epithelial cells (Ramanathan, Spannhake, & Lane, 2007). In addition to S100, a significant decrease in expression was also observed for the gene SPINK5 in CRSwNP epithelial cells when compared with normal patients. SPINK5 is a secreted anti-protease

that likely protects gap junctions from the attack of proteases derived from host sources as well as microbes and allergens (Moffatt, 2004).

Both human and animal studies have indicated that defects in the epithelial barrier secondary to SPINK5 mutations are associated with chronic inflammation at epithelial surfaces (Moffatt, 2004). In the case of CRSsNP patients, results showed a strong trend for lower expression of SPINK5 mRNA compared with normal but the difference was not statistically significant (Marenholz, Heizmann, & Fritz, 2004). Richer et al.'s (2008) study demonstrates that protease inhibitor SPINK5 were found at decreased levels of expression in CRS with and without nasal polyps when compared with controls. Although preliminary, this report suggests that both forms of CRS may be associated with diminished expression of genes for epithelial repair and innate defense.

There is a hypothesis that abnormalities in PRR signalling may play a critical role in the development of idiopathic CRS, possibly TLR2, given its importance in recognition of both fungi and staphylococci. More significantly, however, epithelial cultures taken from CRS patients and normal controls indicated a decrease in some, but not all, functional responses to TLR2 ligands as assessed by release of cytokines after in vitro challenge (Grammer et al., 2007). These preliminary results show that epithelial cells from CRS patients have a poor spontaneous and TLR2-induced release of neutrophil attracting chemokine such as IL-8, extending previously reported observations, and suggest that there is an abnormality in TLR2 signalling in the nasal epithelium of CRS patients. (Damm, Quante, Rosenbohm, & Rieckmann, 2006). In support of this hypothesis, other epithelial cytokines associated with TLR2 responses such as IL-6 were preserved and possibly enhanced in CRS; therefore, a global decrease in nasal epithelial

TLR2 signalling was not observed. IL-6 is a key cytokine mediating the transition from innate to acquired immunity. Studies of tissue extracts indicate the presence of significantly higher levels of IL-6 protein and the soluble IL-6 receptor protein in CRSwNP when compared with CRSsNP and controls (Danielsen, Tynning, Brokstad, Olofsson, & Davidsson, 2006).

Tissue dendritic cells (DCs) are particularly important in the generation of the acquired immune response, acting as APCs. After stimulation by PRRs through PAMP recognition, DCs become activated, cease phagocytic activity, and acquire chemokine receptors that lead them to migrate to lymph nodes where they present antigen to TH cells. IL-6 has been proposed to be a key cytokine mediating the transition between the innate and acquired immune responses, helping to shut down many components of the innate response and promoting the acquired response (Jones, 2005). The subsequent TH responses have classically been divided into TH1 and TH2 based on cytokine profiles. TH1 responses (IL-12 and IFN-γ) facilitate defense against intracellular pathogens. TH2 responses (IL-4, IL-5, and IL-13) are of primary importance in parasitic immunity and are associated with allergy and asthma. The type, duration, and intensity of the PAMP stimulus shape the cytokine milieu and are believed to be critical in determining the TH profile. Additional TH subsets besides TH1 and TH2 have recently been recognized, including TH17 and Treg cells (Tato & O'Shea, 2006). TH17 responses are thought to play a role in defense against extracellular bacteria and Treg cells mediate immunosuppression and immune tolerance. Several cytokines, including IL-6, TGF-β1, and IL-23, appear to be key factors in fostering a TH17 response. TGF-β1 also promotes Treg differentiation, except in the presence of high IL-6, in which case this response is

suppressed. TH1 and TH2 responses reciprocally inhibit one another and both suppress TH17 responses (Tato & O'Shea, 2006). Treg cells appear to suppress TH1, TH2, and TH17 responses, acting to limit excessive immune responses (Romagnani, 2006). Treg responses are inactivated in situ by strong PRR stimulation, most prominently TLR2 (H. Liu, Komai-Koma, Xu, & Liew, 2006). These permit active protective responses to be mediated at the sites of strong PAMP stimulation while suppressing excessive or inappropriate immune responses.

The maturation of TH subsets has been studied extensively *in vitro* and in mice, but the conditions necessary for *in vivo* polarization of the acquired effector immune responses in health and disease in the human nose are unknown. TH1 and TH2 inflammatory patterns have been associated with CRSsNP and CRSwNP, respectively (Hamilos et al., 1995; Van Zele et al., 2006), but this is inconsistent. With regard to the TH17 subset, increased IL-17+ cells have been detected in CRSwNP by *in situ* hybridization (Molet, Hamid, & Hamilos, 2003). Immunohistochemistry has also suggested increased expression of IL-17 and its receptor in nasal polyp mucosa in comparison with inferior turbinate (Wang, Dong, Zhu, & Guan, 2006). On the other hand, ELISA studies done in on nasal tissue extracts from both CRSsNP and CRSwNP patients have failed to establish elevated expression of IL-17A, IL-17B, or IL-17E (Peters et al., 2010). With regard the Treg subset, recent evidence has emerged suggesting reduced numbers of Treg cells in allergic rhinitis and CRSwNP (Van Bruaene et al., 2008; Xu et al., 2007).

In addition to alteration in adaptive immunity, it is believed that dysregulated immunity play a role as well. The cell types of the innate and acquired nasal immune

responses, including epithelial cells, neutrophils, eosinophils, mast cells, and lymphocytes, all express protease-activated receptors (PARs) on their surface membranes (Hershenson, 2007). Although not classically considered host defense molecules, these receptors are activated by environmental proteases present in bacteria, fungi, and allergens (Ossovskaya & Bunnett, 2004). PAR receptors use many of the same intracellular signalling pathways (e.g., NFκB) triggered by PRR stimulation (Rudack et al., 2007). In consequence, at the nasal epithelial interface in vivo, PAR activation likely modulates both the innate and the acquired immune responses to animate and inanimate foreign material (Hershenson, 2007; R. P. Schleimer et al., 2007).

In summary, the mechanical and innate immune barriers across the nasal mucosa serve to appropriately repel the constant load of exogenous stimulation and limit activation of the acquired immune response. Genetic and/or acquired defects in this complex process may at least theoretically lead to the development of chronic inflammation seen in CRS (Holgate, 2007; Ramanathan & Lane, 2007; R. P. Schleimer et al., 2009).

Genetic Factors in the Development of CRS

Several studies support the existence of genetic factors, Cystic fibrosis (CF) is the prototypic example of genetic CRS, with 90% of CF patients demonstrating evidence of the disease (Sobol et al., 2002). Dysfunction of the mechanical and innate immune barrier presumably mediated through CFTR gene mutations has been shown (Claeys et al., 2005; Kern et al., 2008).

Genetics studies reported the associations between chronic rhinosinusitis (CRS) with nasal polyposis and single-nucleotide polymorphisms (SNPs) in the IL-1A genes

(Karjalainen et al., 2003; Mfuna Endam, Cormier, Bosse, Filali-Mouhim, & Desrosiers, 2010) and IL-33 gene (Buysschaert et al., 2010). Polymorphisms in the IRAK-4 gene showed a genotype-specific association with serum IgE level, which replicated in an asthmatic population (Tewfik et al., 2009).

A single pooling-based GWAS of CRS study has been performed by our group and has yielded additional insight into mechanisms of CRS (Mfuna-Endam et al., 2011). The top 10 reported associations suggest a potential role for interactions at the level of the basement membrane and extracellular matrix (laminin-α2 **[**LAMA2] and laminin-β)[LAMB1]), mitochondrial function (prolyl-tRNA synthetase 2, mitochondrial [PARS2]), and lipopolysaccharide degradation (acyloxyacyl hydrolase [neutrophil] [AOAH]). Additional genes identified by secondary analysis of our population according to severity also identified genes in SERPINA1 (Kilty, Bosse, Cormier, Endam, & Desrosiers, 2010) and TP73 (Tournas et al., 2010) as conferring an increased risk of severe, steroid-resistant CRS. Direct impact on function has not been demonstrated, but nevertheless, two tantalizing observations are associated with these genes. A knockout mice model of TP73 developed severe purulent rhinosinusitis, suggesting an important role for dysregulation of apoptosis in CRS1(13, 88] (A. Yang et al., 2000). Screening of a population with severe sinusitis identified low serum α-1-antitrypsin levels in an unexpectedly high percentage of CRS patients, again suggesting a role for a dysregulated α-1-antitrypsin metabolism (Desrosiers, 2010). However, homozygotes for the identified SNP did not show any genotype-specific alteration of level.

In a recent review of genetics of CRS (Mfuna-Endam et al., 2011), network analysis of published SNP's identifies nodal elements around NFkB, suggesting the

dysregulation in immune activation may play a key role in the genetic basis of this disorder.

Gene-Environment Interactions in the Development of CRS

The variation of clinical phenotype indicates that even in CF, the most straight - forward case of genetic CRS, multiple factors in an individual patient strongly determine disease expression and histologic findings. Th1/Th2 may differ (Hull & Thomson, 1998). Alterations in expression of genes other than CFTR, mediated via genetic variation or environmental effects, apparently combine to affect disease phenotype. Thus, much like asthma, CRS appears to be a disease of gene–environment interaction with complex immunobiology involving multiple genetic loci (Lilly, 2005; Thomsen, Ulrik, Kyvik, Ferreira, & Backer, 2006).

The relative importance of genetic versus environmental influences on disease expression in CRS is unknown, however, some insight may be gained from twin studies in asthma, the concordance rate for disease expression in identical twins is only 50% (Vercelli, 2004). From this study it is suspected that, alterations in a few genes dispersed within critical pathways create an inherited susceptibility to development of clinical disease that is heavily dependent on interaction with environmental exposures later in life (Farrall, 2004; Thomsen et al., 2006).

Although clear epidemiologic data are difficult to obtain, CRS is widely believed to be increasing in incidence and prevalence, similar to other chronic inflammatory diseases. In asthma and AD, the rate of increase is too rapid to be attributed to genetic mutation and is thus attributed to environmental effects, including changes in microbial exposure early in life (i.e., the “hygiene hypothesis”; (Kay, 2001; Rook & Stanford,

1998). The effects of changing environment on prevalence of CRS have not been directly studied but it is certainly reasonable to hypothesize that many of the same environmental factors that influence the prevalence of atopy also influence the prevalence of CRS (A. H. Liu & Murphy, 2003).

A mechanistic explanation of precisely how the hygiene hypothesis promotes clinical disease incidence remains elusive; however, it has been proposed that environmental factors may directly alter gene expression via epigenetic mechanisms such as DNA methylation and histone acetylation (Su, Becker, Kozyrskyj, & Hayglass, 2008; Vercelli, 2004). Epigenetic mechanisms produce transmissible modifications in gene function by altering gene expression without directly altering the DNA sequence. These concepts would suggest that the absence of appropriate microbial stimulation in childhood might result in epigenetic variations that mediate durable changes in gene expression that later manifest as disease on subsequent challenge.

In brief, the CRS phenotype most likely results from the combined effect of genetic variation interacts with toxic or acquired epigenetic effects across critical pathways that control the immunobiology of the nasal mucosa.

Chapter Summary

Current studies on the etiology of most chronic inflammatory mucosal disorders have emphasized abnormalities in the expression or function of genes that maintain the mechanical and innate immune barrier as it interfaces with the external environment, as well as the environmental changes that appear to be accelerating disease expression. In the study of CRS etiology, most interest still centers on identification of putative inciting microbial agents, likely reflecting an earlier era when sinonasal disease was primarily

infectious in nature.

Comparatively few studies on CRS etiology have focused on host defects; despite recent acceptance that CRS is best considered an inflammatory disease. Although the evidence for the hypothesis that mucosal immunity and barrier function is compromised in CRS is currently limited. It places the current controversies in rhinology in a framework consistent with modern concepts of complex genetic disorders and chronic mucosal inflammatory disease in general. Additional studies on host immune dysfunction in CRS will be necessary to generate a comprehensive understanding of the pathogenesis of this common disease and to make targeted therapies a reality.

CHAPTER THREE: MATERIAL AND METHODS

This chapter will go through the verification of our study objectives, using different techniques.

1. Microarray analysis method to identify the gene expression patterns and implicated canonical pathway(s) of the CRS disorder.
2. Molecular patterns suggested by gene expression signatures were validated with QRT-PCR and also by verifying the expression level of protein product of a putative epithelial marker, CCL2.
3. Infiltrating inflammatory cell profile in the surgical biopsies was identified using immunohistochemistry staining.

Study Subjects

The present study was approved and supervised by the institutional review board of the Centre de recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). All patients participated voluntarily and signed an informed consent.

Twenty subjects with chronic rhinosinusitis with nasal polyposis (CRSwNP, n=10) and without (CRSsNP, n=10) nasal polyposis, and ten controls aged 18 years and more were recruited. All subjects with CRS had a diagnosis of CRS with or without nasal polyps according to published AAO-HNS guidelines (Benninger et al., 2003), and had failed at least one course of maximal medical therapy. The control subjects were non-CRS patients undergoing endoscopic sinus surgery (ESS) for access to the structures of the orbit, the lachrymal apparatus or skull base. Patients with immune deficiencies, neoplasia, systemic disorders affecting immunity such as diabetes or renal insufficiency, on systemic immunosuppression, or with cystic fibrosis were excluded. No patient had

received oral corticosteroids or topical or systemic antibiotic therapy in the preceding 30 days. All subjects had ceased topical intranasal corticosteroids at least 14 days prior to surgery.

The following demographic parameters were assessed for all subjects: CRSwNP, CRSsNP and controls. Age, gender, asthmatic status, and antibiotic use were recorded and blood was drawn for complete blood count and differential, total serum IgE, and specific IgE test RAST for all subjects. Allergic status was determined according to self-reported history and presence of any one RAST greater than or equal Class 2. Endoscopic swabbing anterior ethmoid bulla was performed for bacteriology culture.

Biopsy Specimen Collections

In order to ensure maximal repeatability across disease conditions, surgical biopsy samples were all obtained at the level of the anterior ethmoid bulla. Two biopsies were collected; one used for cell culture and one for histopathology lab.

Biopsy Preparation for Air-Liquid Interface (Ali)

Primary nasal epithelial cells were isolated from surgical biopsy samples and cultured to differentiation in air-liquid interface (ALI). A biopsy sample was collected and immediately placed on a moist sterile compress in a sterile plastic container, and rapidly transported on ice to the cell culture facility situated within the same hospital. The tissue was incubated overnight in minimum essential medium (MEM) with 1% protease-DNAse at 4°C. The nasal epithelial cells were obtained by gently scraping the samples with a scalpel prior to incubation. The cells were recovered by centrifugation then cultured in T25 coated with Purecol in culture medium bronchial epithelial growth

medium (BEGM) supplemented with retinoic acid. The cells were maintained at 37°C, and 5% CO2 atmosphere.

The culture medium was changed every 2 days until the cells reached confluence. At this point cells could be frozen for differed culture, and resuspended at a later date. Then the primary human nasal epithelial cells were cultured on filters of 4 cm^2 in air-liquid atmosphere. The culture medium was changed every two days. Viability of the cells assessed by direct inspection daily.

Relevance of this in-vitro model to in-vivo behavior is supported by two previous studies assessing this. The morphologic features of normal sinus epithelial and chronic sinus epithelial cells in ALI cultures were quantitatively similar (Dejima, Randell, Stutts, Senior, & Boucher, 2006). Expression levels of cytokeratin, epithelial membrane antigen (EMA), vimentin, and CD45 remained similar in freshly dissociated primary epithelial cells and cells after first passage in culture, suggesting that fundamental gene expression were unaltered. In support of a relevant protein secretion, a second published assessments documented that growth-factor supplemented media retain their capacity to secrete pro-inflammatory cytokines (Fernandez-Bertolin et al., 2011).

RNA Isolation

In this study, total RNA from samples of cultured epithelial cells from the 20 CRS patients and 10 control subjects were used.

Total RNA was extracted from epithelial cells using RNeasy Mini Kit (Qiagen, Toronto, ON, Canada). The RNA was quantified and 1 µg was treated with DNase I Amp Grade (InvitroGen, Carlsbad, CA, USA) according to the manufacturer's protocols.

Microarray Gene Expression

RNA integrity values were assessed on an Agilent 2100 Bioanalyzer Nanochip (Agilent, Palo Alto, CA, USA). Total RNA preparations with RNA ratio [28s/18s] ratio between 1.51–2.53 and RNA integrity number ≥ 9.4 with enough quantity were used for the expression analysis. Labeled cRNA (50 ng) was hybridized 18 hours at 58°C to Illumina HumanHT-12 V3 Expression BeadChip Array (IIlumina, San Diego, CA, USA) according to the manufacturer's instructions. The Illumina Human HT-12 Expression BeadChip provides genome-wide transcriptional coverage of well-characterized genes, and splice variants, with significant portion targeting well established sequences supported by peer-reviewed literature. Each array targets 48,000 transcripts from 25,000 annotated genes with probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI), using the RefSeq (Build 36.2, Rel22) and the uniGene (Build 199) databases.

BeadChips were then washed and developed using fluorolink streptavidin-Cy3 (GE Healthcare). BeadChips were scanned using an Illumina Bead Array Reader. The microarray was performed at Génome Québec McGill University Inovation Centre (Montreal, QC, Canada).

Unsupervised Clustering Method

To explore the relationship among samples and underlying features of gene expression, we applied an unsupervised Hierarchical clustering method using the probe-set whose expression passed the filter (Figure 6). This unsupervised clustering divided samples into three major subgroups based on distinct patterns of gene expression: one

group formed by the control (CTL) samples and two different clusters, CRS1 and CRS2 from the CRS patients.

In order to identify a set of genes (markers) associated to each of the CRS1 or CRS2 groups identified from the unsupervised analysis step, we performed a class prediction analysis using the PAMR classifier (Tibshirani & Efron, 2002). PAMR is a Shrinkage method controlled by a threshold parameter obtained by cross validation. The choice of a threshold value is a tradeoff between a small number of genes and a good misclassification rate.

The result of unsupervised analysis of epithelial cell gene expression was interpreted by using fold change of difference between the (log^2) expression average of genes in the diseased and the control groups, and selected the gene which had significant expression difference with P: value less than 0.05.

Ingenuity Pathways Analysis

In order to identify implicated pathways from our list of genes with deferential expression pathway analysis was performed using Ingenuity Pathways Analysis (IPA; see http://www.ingenuity.com). IPA is a program and curated database of thousands of established relationships between proteins known in the current domain of literature (Henderson-Maclennan, Papp, Talbot, McCabe, & Presson, 2010). For the purpose of our study, it allowed us to input our dataset of differentially expressed genes and determine potential pathways, networks and transcriptions factors likely to be implicated from our expression data.

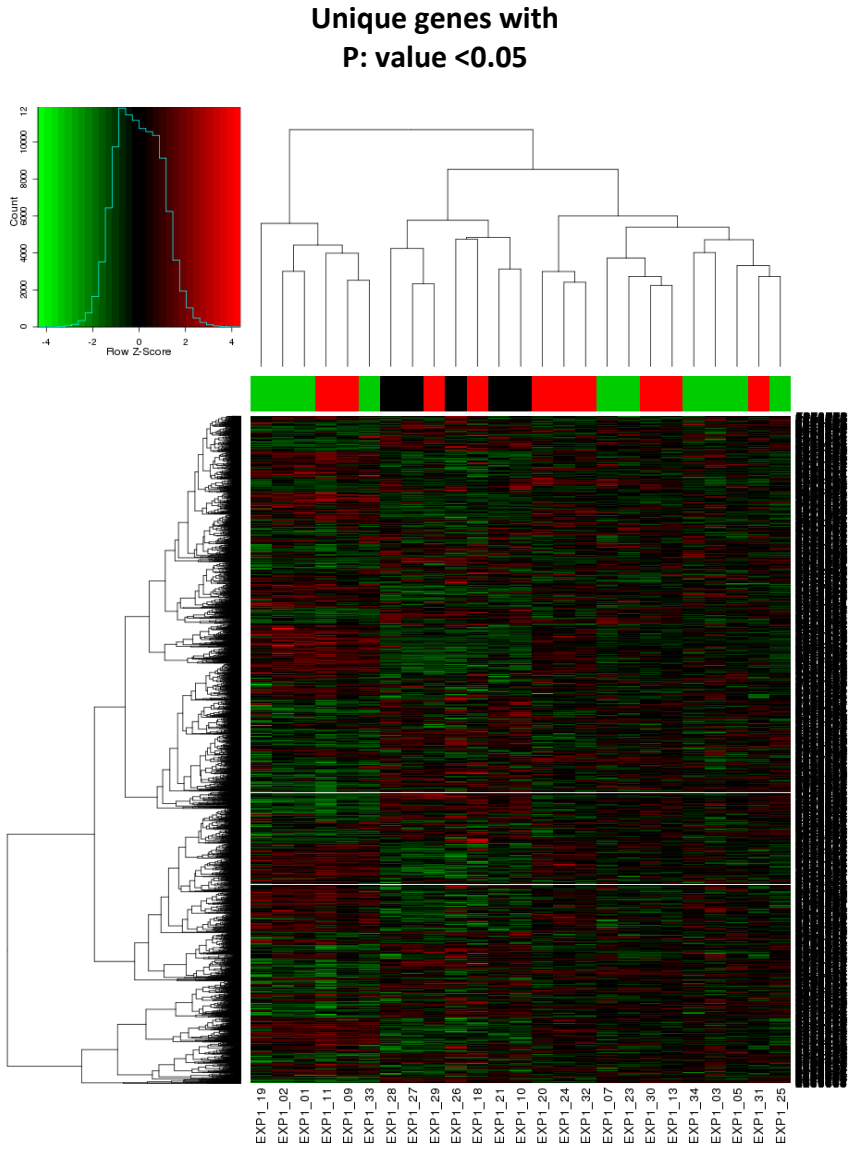


Figure 6. Unsupervised hierarchical clustering heatmap

Reverse Transcription-Polymerase Reaction (RT-PCR)/Quantitative-PCR (Q-PCR)

Following RNA purification, reverse transcription-polymerase chain reaction (RT-PCR) was performed to obtain complementary DNA (cDNA). Real-time PCR (q-PCR) was performed in 96 well plate format using SYBR Green based detection on a

Step-One-Plus machine (ABI) with each 10 µl reaction containing ~ 50 ng of cDNA, 0.3µL concentration 10µM sense and antisense primers (table 1), and 5µL iTAQ SYBR Green supermix with Rox (Bio-RAD). The plate was sealed and cycled under the following conditions: 95°C for 10 min, 50 cycles of 95°C for 10 s, and 60°C for 45 s, and 95°C for 10 min and 70°C for 45 sec. Each reaction was performed in duplicate, mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalization, and fold induction was determined from *Ct* values using the Δ CT method. PCR efficiency was determined from the slope of a standard curve generated using five-fold dilution series of the DNA template.

Biopsy Preparation for Immunohistochemistry

Surgical biopsies were taken from the anterior ethmoid bulla and immediately placed into sterile dishes and transported in dry ice directly from operating room to the pathology lab. In the pathology lab, biopsies were cut into small pieces roughly 5 mm^2. Optimal Cutting Temperature (OCT) compound was poured into a small plastic tray, the small tissue sample submerged in the compound, and the tray is placed in the vapor phase of liquid nitrogen for freezing. After hardening of the OCT, the tray was labeled with patient information and transferred to -80 ^{0}C freezer for storage until use.

Frozen biopsies were cut by cryostat microtome into five-micron sections and mounted on microscopic glass slides. Slides were fixed with ethanol/methanol 60/40% for seven minutes and air dried for 10 minutes, then stored at -80 ^{0} C until use.

One section from each biopsy samples was stained by Hematoxylin and Eosin (H&E) staining, and examined under light microscope to characterize the morphological structure.

Table 1

Primer Sequences Used in Q-RT-PCR

Gene	Primer sequences (5'-3')
TNF-a	Left primer: AGCCCATGTTGTAGCAAACC Right primer: TGAGGTACAGGCCCTCTGAT
IL-1b	Left primer: TCG TTA TCC CAT GTG TCG AA Right primer: GGA CAA GCT GAG GAA GAT GC
IL-8	Left primer: CTC TGC ACC CAG TTT TCC TT Right primer: GTG CAG TTT TGC CAA GGA GT
NFKBIZ	Left primer: CCGTTTCCCTGAACACAGTT Right primer: AATGGTTGGCATTTCTGAGG
KRT4	Left primer: CTGAGCTAAGACCATGCAG Right primer: TCCACCTTCAGGTAGGC
KRT13	Left primer: CCCGTAGCACCTCTGTTACC Right primer: CTCCTCTGGGTGAAGAC
ClCa4	Left primer: GCAACTTCCAAAATGGCCTA Right primer: GGAGGCACAGAAGAATTTGC

Immunohistochemistry (IHC) Staining

Immunohistochemistry (IHC) staining was performed on frozen sections obtained from CRS surgical biopsies and control groups. A modified immunoperoxidase method of immunohistochemistry was performed. Five micron frozen section were thawed and rinsed in PBS followed with 0.2% Triton X100 in PBS for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes at room temperature. The slides were washed in PBS and pretreated with universal blocking solution (Dako, Canada) for 30 minutes. Section slides were incubated overnight at 4°C with primary diluted mouse anti-human neutrophil elastase, mouse anti-human CD68 (Dako, Canada), mouse anti-human Major basic protein antibodies. (AbD biocompare, Canada) and rabbit anti-human CCL2 antibody (Abcam, Canada), The slides were rinsed and incubated with a biotinylated secondary rabbit anti-mouse antibody for 30 min at room temperature for the first three antibodies and with a biotinylated secondary goat anti-rabbit antibody for the CCL2 antibody. After washing in PBS, Streptavidin/Horse Radish Peroxidase complex (Vector, Canada) was applied for 30 min at room temperature. The reaction result was visualized with DAB/hydrogen peroxide (DAB Kit, Dako). Sections were finally rinsed in distilled water, lightly stained with hematoxylin, dehydrated, cleared, and cover slipped. Sample processed the same isotypes, but without primary antibody served as negative control.

Immunohistochemistry Scoring and Statistical Analysis

The positive cells of neutrophil, eosinophil and macrophage were detected by using light microscope and counted in the five randomly selected fields of the subepithelium mucosa at 20-x magnification. The mean value of the counting was

calculated and the result was analyzed by using a nonparametric test (Wilcoxon rank test) to assess the difference between the groups.

The area of CCl2 expression scale was generated (0-5) in the positive respiratory epithelium was scored on a level of 0 to 5, where is 0 = no expression, and 1 = 1-20% expression, 2 = 21-40%, 3 = 41-60%, 4 =61-80% and 5 =81-100% of all epithelial cells showing evidence of staining. Two different, blinded readers to avoid bias in the scoring did the scoring of the positive area. A Wilcoxon rank test assessed the difference in the expression of CCL2 marker in CRS and control. P value of ≤ 0.05 was considered statistically significant.

Immunohistochemistry Double Staining

The sections for double labeling with CD68 paired with CD206 respectively were incubated first with diluted mouse anti-human CD68 (Dako, Canada) antibody (1:800) for one hour, rinsed with PBS incubated with biotinylated secondary rabbit anti-mouse antibody for 30 minutes at room temperature, and completed by applying the Streptavidin /Horseradish Peroxidase complex (Vector, Canada) for 30 minutes at room temperature. The reaction result was visualized with DAB/hydrogen peroxide (DAB Kit, Dako). The sections were rinsed in PBS, blocked again with the universal blocking solution (Dako-Canada) for 30 min. and then incubated with diluted mouse anti-human CD206 (AbD biocompare, Canada) antibody (1:500) for overnight at 4°C. On the second day, the sections were rinsed with PBS then incubated with biotinylated secondary rabbit anti-mouse antibody for 30 minutes at room temperature, followed by application of the Streptavidin /HorseRadish Peroxidase complex (Vector, Canada) for 30 minutes at room temperature. After that the reaction result visualized with RED/hydrogen peroxide (DAB

Kit, Dako). The sections were finally rinsed in distilled water, lightly stained with hematoxylin, mounted with agues media and cover slipped.

Immunohistochemistry Double Staining Counting

The positive cells of CD68 classical macrophage and CD68+CD206 alternative macrophage were counted in the five fields of the subepithelium mucosa at 20-x magnification randomly; the mean value of the counting classical and alternative macrophages was calculated. The result was analyzed by using a two-tailed student T-test to assess the difference between the groups. The results of M1 and M2 macrophages were assessed in CRS1 versus CRS2 groups, and CRSwNP versus CRSsNP groups.

CHAPTER FOUR: RESULTS

Cell Culture Unsupervised Analysis

Microarray Analysis Results

Functional clustering using unsupervised hierarchical-clustering method successfully found genes that predict CRS1 and CRS2 groups. CRS1 group has 6 patients CRS2 group has 12 patients where both groups have patients from CRSwNP and CRswoNP. The Control group has 7 patients: 5 from clinical control and 2 from CRSwoNP. Figure 7 shows the gene expression heatmap of a set of 26 genes classifier obtained when the CTL samples were among the training group. This heatmap revealed that the majority of the 26 genes differentially expressed in CRS1 were involved in inflammation (Table 2), and were upregulated. In comparison to the same set of genes, CRS2 was downregulated (Table 3), and surprisingly similar to the control group in their genes expression pattern.

Ingenuity Pathways Analysis (IPA)

Using Ingenuity systems analysis software to explore the biological relevance of results and identify networks, we found most of the functional and canonical pathways identified by IPA were involved in inflammatory signaling events. The top networks in CRS1 showed a prominent high TNFR response and activity (Figure 8), suggesting a high inflammatory activity in CRS1 and lesser one in the CRS2 group.

We suggest this new molecular signature classification depends on NFkB-TNF-IL1 axis, with a greater activity in CRS1 (Figure 9). Marked discrepancy between CRSwNP/ CRSsNP clinical classification and the CRS1/2 molecular-signature based phenotype classification suggests differences between the two phenotypes.

Analysis of Demographic Data

When we analyzed our demographic data according to molecular classification, we found support for the existence of two differential phenotypes. The relevance of this new classification method is supported by differences in several demographic features associated with the disease. Differences in disease evolution are present, as assessed by an increased need for antibiotics and revision surgery in the CRS1 group. Associated inflammatory diseases differ as well, with presence of self-diagnosed aspirin sensitivity present in the CRS 2 group only. Interestingly, while Staphylococcus Aureus was recovered with equal frequency from both groups, Streptococcus Pneumonia was recovered in the low inflammation CRS2 group only. Additionally, total serum IgE differed between the two groups, with the low of level of IgE identified in the CRS1 group (Table 4). However, when we analyzed our demographic data according to clinical classification (CRSwNP or CRSsNP) we found there was no significant difference in this parameter between the groups.

Expression Level of Epithelial Cells Gene Results

Quantitative RT-PCR was performed for several of the putative genes identified by unsupervised analysis and confirmed our previous unsupervised analysis results. Analysis of the mRNA expression shows pro-inflammatory genes IL1-B, TNF-a, IL-8, NFKBIZ were strongly upregulated in CRS1 compared to the CRS2 and control (Figure 10), while others mRNA expression level of Cacl4, KRT4, and KRT13 genes were markedly downregulated in CRS1 compared to CRS2 and control (Figure 11).

We validated our results by verifying the correlation between the FC of qPCR Vers FC of microarray expression results for 13 genes (IL-1, IL-6, IL-8, KRT4, KRT13, ClCa4, poston, CEBPA, NFKB, TNF) in CRS1 vs. control, CRS2 vs. control, and CRS1

vs. CRS2. We found there is a strong correlation between them in CRS1 vs. control r2= 0.89, co CRS2 vs. control r2= 0.82. and CRS1 vs. CRS2 r2= 0.96, and it supports our results (Figure 12).

Tissue Biopsy Results

Analysis of Inflammatory Cells

Staining of obtained CRS biopsies by immunohistochemistry procedure detected positive staining in eosinophil, neutrophils, and macrophages mainly in the subepithelium (Figures 13, 14, and 15). All results are expressed as mean ± standard error of the mean. $P \leq 0.05$ was considered to be statistically significant as measured by Wilcoxon rank test.

Assessment of Inflammatory Cells in CRSwNP vs. CRSsNP:

When we assessed our results according to conventional phenotype, there was a significant increase in the number of eosinophil cells in CRSwNP compared to CRSsNP (740 ± 138.63 vs. 119.83 ± 72,14; p; 0.0016) and to control subjects (4.83 ±3.06; P<0.001). While there was an increase in the number of neutrophils in CRSwNP and CRSsNP compared to control subjects (220.2 ± 34.43, 231.2 ± 112.76 vs 85 ± 27.19; P< 0.01), there was no significant difference between CRSwNP and CRSsNP (P; 0.360). Macrophages were also significantly increased in the CRSwNP and CRSsNP compared to the control subject (CRSwNP; 40.8 ± 8.15, CRSsNP; 29.5 ± 6.78 vs. control; 2 ± 0.36; p<0.005); however, again there was no significant difference between CRSwNP and CRSsNP groups (P; 0.620) (Figure 17).

In summary, while measured inflammatory cells were increased in the CRS tissues in comparison to CTL, only differences in the level of eosinophil infiltration differentiated the two groups.

Assessment of Inflammatory Cells in CRS1 vs. CRS2

When we assessed our result according to molecular signature phenotype, we identified a different pattern. There was significant increase in the number of neutrophils in CRS1 compared to the CRS2 (353.5 ± 93.38 vs. 146.8 ± 27.23 P; 0.0159) and to control subjects (353.5 ± 9 3.38 vs. 84.66±; 27.19 P; 0.003). However, there was no significant difference in neutrophilia between the CRS2 and control groups (146.8 ± 27.23 vs 84.66±; 27.19 p; 0.130).

There was a similar pattern for macrophages, with a significant increase in the number of macrophages in CRS1 compared to the CRS2 (53.83 ± 10 vs 26.2 ± 4.65 P; 0.016) and between CRS1 and control subjects (53.83 ± 10 vs 2 ± 0.36; P; 0.0035). Again, while there was an increase in the number of eosinophil cells in CRS1 and CRS2 compared to the control, there was no significant difference in eosinophils between the CRS1 and CRS2 (CRS1; 449.17 ± 122.76, CRS2; 542.9 ± 177.85 P; 0.670 vs Control subjects 4.83 ± 3.06 P; 0.015) (Figure 18).

In summary, the CRS1 group is characterized by infiltration with neutrophils and macrophages, without any impact on eosinophilia, which is elevated in both the CRS1 and 2 groups.

Detection of CCL2 Expression

Immunohistochemistry Staining of CRS biopsies and control subject by CCL2 marker procedure was used to assess the intensity of CCL2 protein expression in epithelial cells in both groups (CRS, and control) (Figure 16). The expression of CCL2 markers was detected and analyzed in both groups (CRS and control). When we assessed our results according to the clinically classification, CCL2 was highly expressed in the

CRSwNP and CRSsNP groups compared to the control group, however there was no difference between the two CRS groups, (4.11 ± 0.31, 4.4 ± 0.43 P; 0.6; vs. 2.33 ±0.49; P; 0.009) (Figure 19). When we assessed it according to the molecular signature classification the expression of CCL2 was highly expressed in the CRS1 group compared to the CRS2 and control subject, with significant differences observed between the CRS1, CRS2 and control groups. (4.83 ± 0.17, vs CRS2 3.75 ± 0.31; P; 0.0296 and control 2.33 ± 0.49; P; 0.0028) (Figure 20).

Assessment of Classical and Alternative Macrophages

When we assessed our result according to conventional phenotype, there was no significant difference in the number of classical macrophages M1 in CRSwNP compared to CRSsNP subjects (22.3 vs 22.2 P; 0.9969), while there was a significant increase in the number of alternative macrophages M2 in CRSwNP compared to CRSsNP (18.5 vs. 7.2; p; 0.0306) (Figure 21).

When we assessed our result according to molecular signature phenotype, we identified a different pattern. There was significant increase in the number of classical macrophages M1 in CRS1 compared to the CRS2 (35.3 vs. 14.5 P; 0.0197) and to control subjects (35.3 vs. 2 P; 0.00349). However, while there was trend towards an increase in alternative macrophages M2 in CRS2 compared to CRS1 group, this did not attain staistically significant difference. (18.5 vs. 11.7; p; 0.3131) (Figure 22).

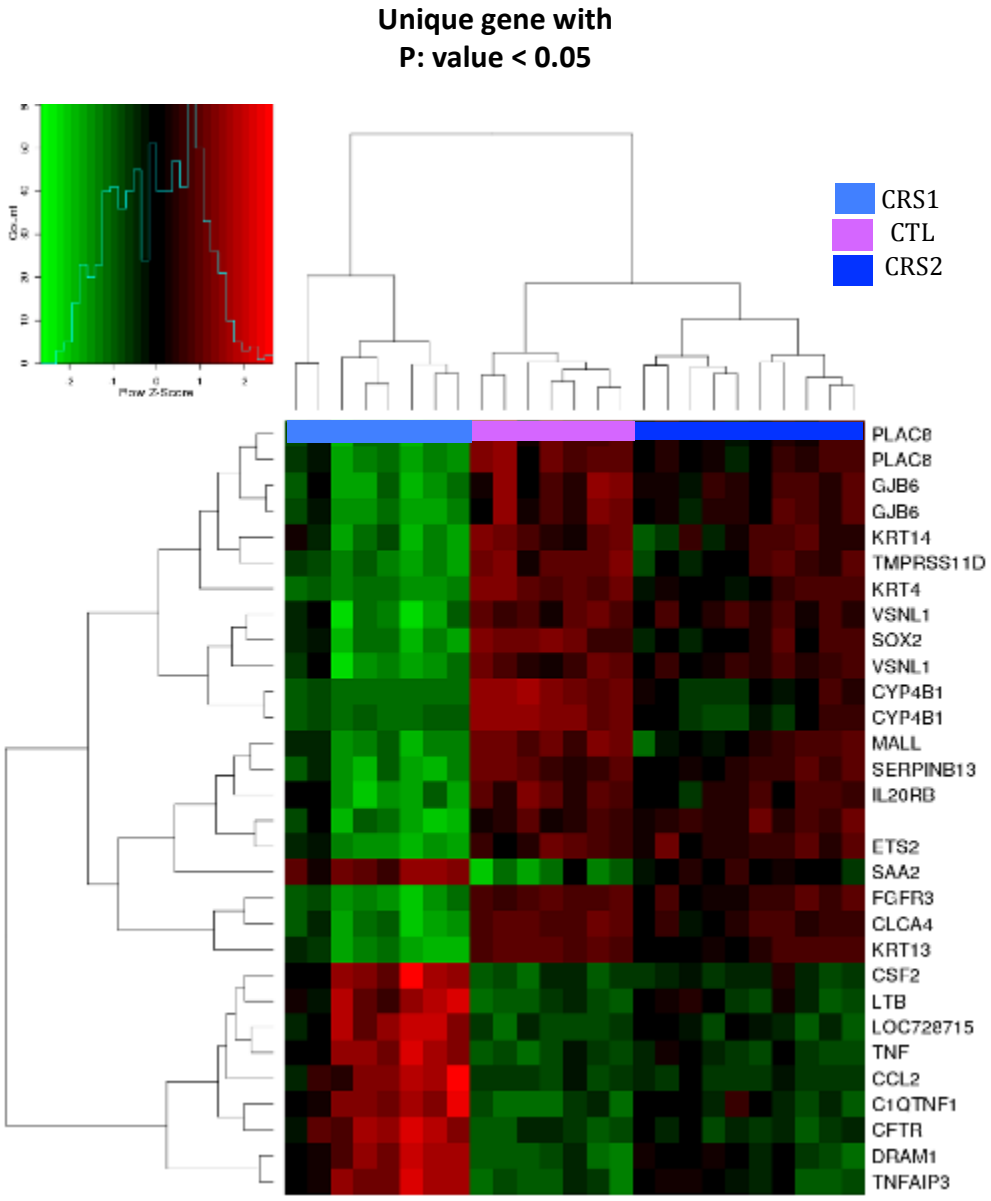


Figure 7. Gene expression heatmap of the set of 26 genes

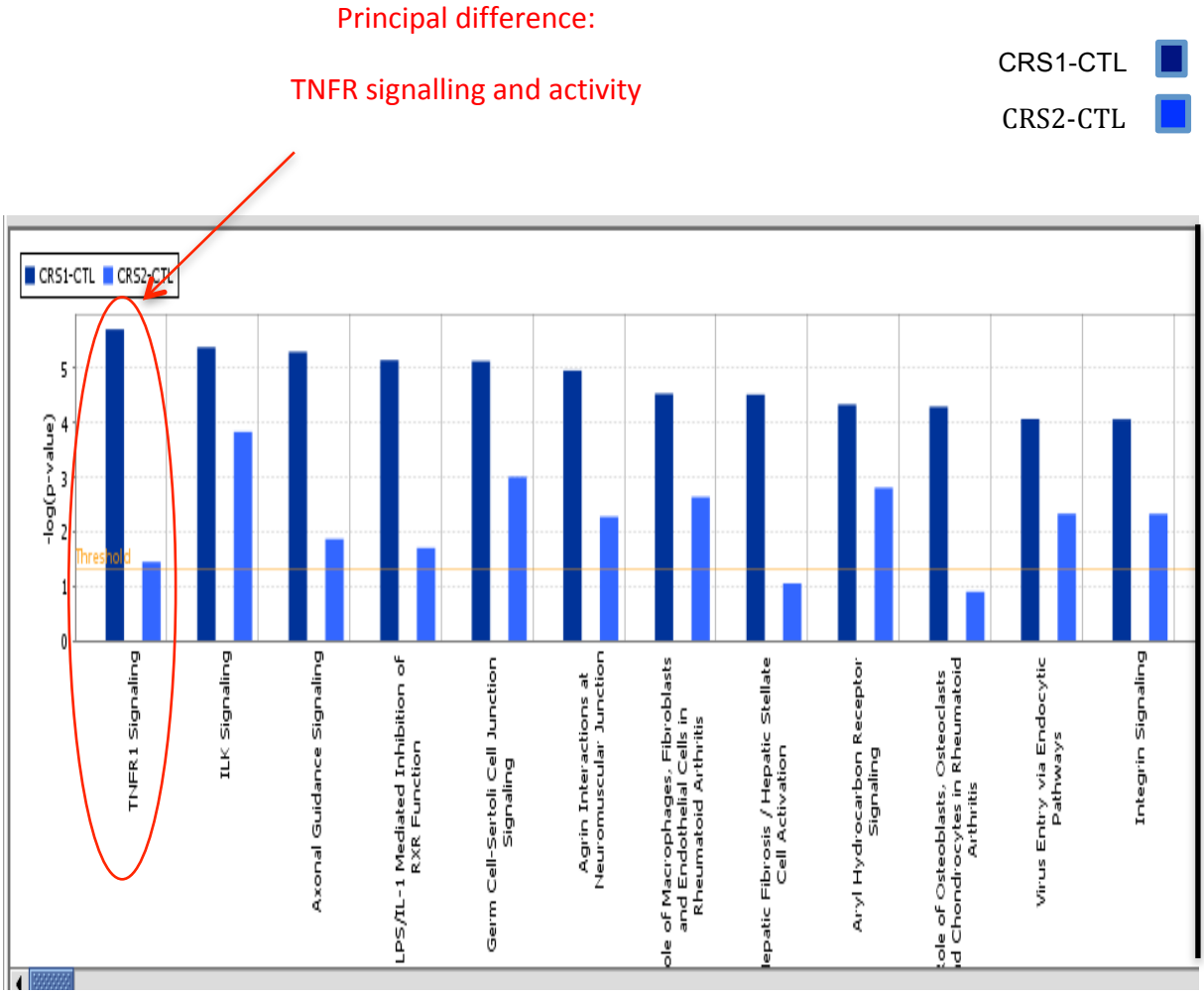


Figure 8. Canonical pathways of gene expression in CRS1 vs. CRS2

Shows higher inflammatory activity in CRS1 compared to the CRS2.

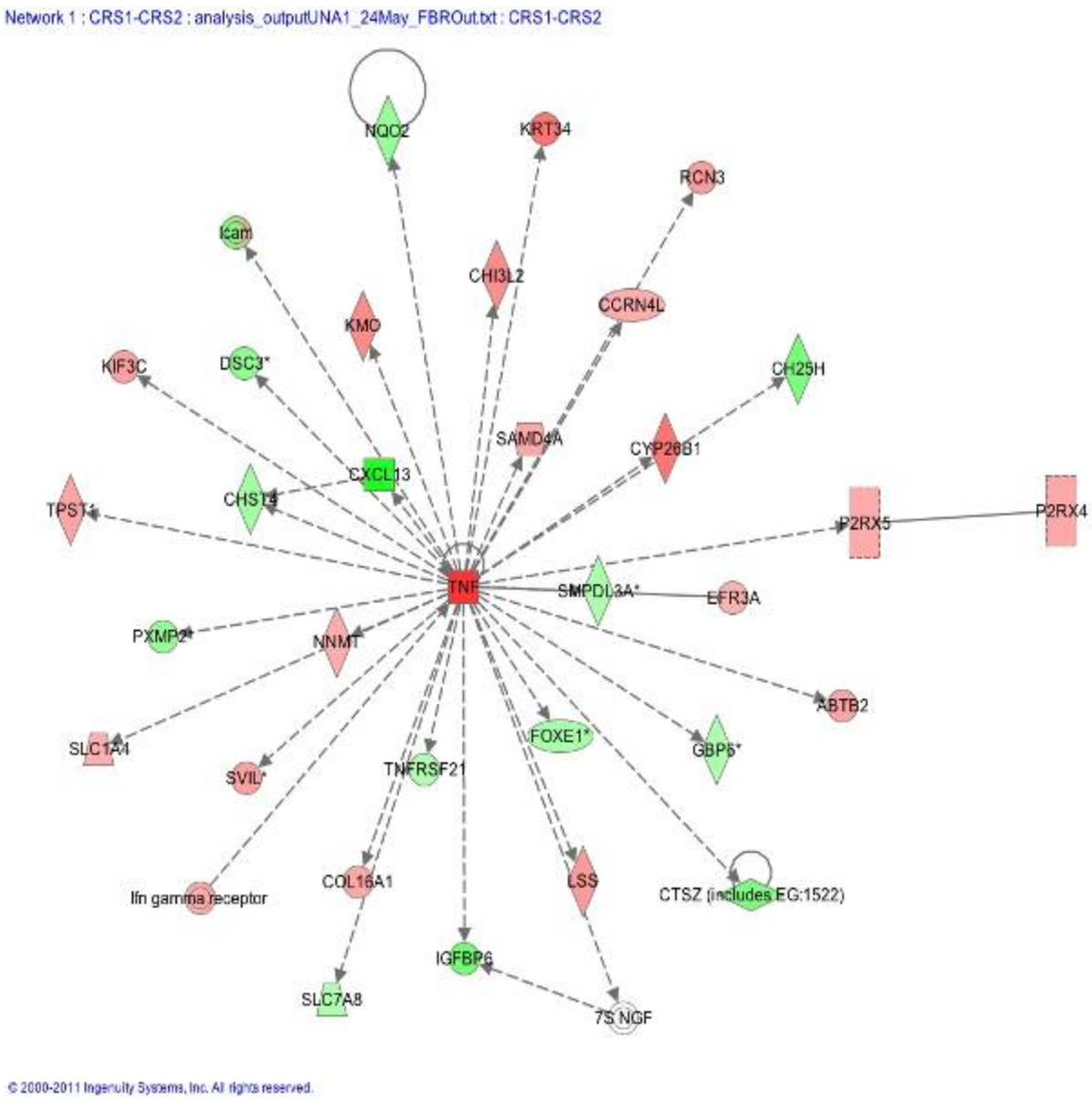


Figure 9. Ingenuity pathway analysis (IPA) networks.

IPA identified TNF as a central mediator in the inflammatory process in CRS1 compared to CRS2. Colored boxes represent genes differentially expressed in our dataset with red representing upregulation and green representing downregulation (different shades of the color signify the degree of expression). Direct relationships are shown as solid arrows and indirect relationships are shown as dashed arrows.

Table 2

Microarray Results of CRS1 vs. CTL Gene Expression

Up-regulated symbol	Enter gene name	FC	P-value
UBD	Ubiquitin D	34,702	$3{,}16 \times 10^{-7}$
CCL2	Chemokine (C-C motif) ligand 2	22,239	$8{,}07 \times 10^{-8}$
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	19,777	$8{,}07X10^{-8}$
CXCL10	Chemokine (C-X-C motif) ligand 10	19,723	$9{,}70X10^{-5}$
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	18,698	$4{,}39X10^{-9}$
TAGLN	Transgelin	16,335	$1{,}37X10^{-6}$
IL32	Interleukin 32	14,563	$2{,}70X10^{-7}$
ADAM19	ADAM metallopeptidase domain 19	14,243	$7{,}37X10^{-7}$
Down-regulated			
KRT13	Keratin 13	-189,864	$1{,}54 \times 10^{-9}$
KRT4	Keratin 4	-114,356	$8{,}94X10^{-8}$
SPRR3	Small proline-rich protein 3	-90,009	$6{,}75X10^{-7}$
CLCA4	Chloride channel accessory 4	-40,994	$1{,}71X10^{-8}$
CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	-38,568	$1{,}90X10^{-7}$
KRT14	Keratin 14	-26,137	$2{,}15X10^{-7}$
PLAC8	Placenta-specific 8	-25,801	$8{,}15X10^{-9}$
CYP2F1	Cytochrome P450, family 2, subfamily F, polypeptide 1	-25,644	$1{,}49X10^{-5}$

Table 3

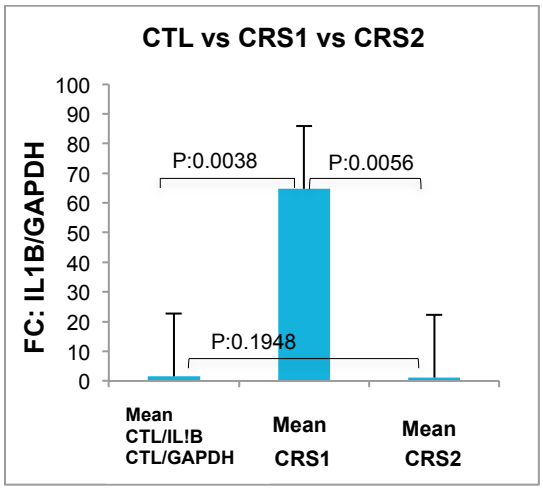
Microarray Results of CRS2 vs. CTL Gene Expression

Up-regulated symbol	Enter gene name	FC	P-value
UBD	Ubiquitin D	5,551	$2{,}29X10^{-3}$
VCAN	Versican	4,526	$1{,}41X10^{-3}$
CXCL10	Chemokine (C-X-C motif) ligand 10	4,351	$3.26X10^{-3}$
VIM	Vimentin	4,179	$1.21X10^{-3}$
ADAM19	ADAM metallopeptidase domain 19	4,015	$2.02X10^{-3}$
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	3,74	$1{,}80X10^{-3}$
COL4A1	Collagen, type IV, alpha 1	3,648	$1{,}09X10^{-3}$
IL32	Interleukin 32	3,612	$5{,}71X10^{-3}$
Down-regulated			
SPRR3	Small proline-rich protein 3	-10,168	$2{,}13X10^{-3}$
CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	-8,577	$2{,}96X10^{-4}$
KRT4	Keratin 4	-6,887	$3{,}85X10^{-3}$
C20orf114	Chromosome 20 open reading frame 114	-5,313	$7{,}85X10^{-3}$
CYP2F1	Cytochrome P450, family 2, subfamily F, polypeptide 1	-5,082	$1{,}31X10^{-2}$
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	-4,012	$3{,}41X10^{-2}$
HOPX	HOP homeobox	-3,892	$1{,}80X10^{-3}$
KRT13	Keratin 13	-3,884	$9{,}92X10^{-3}$

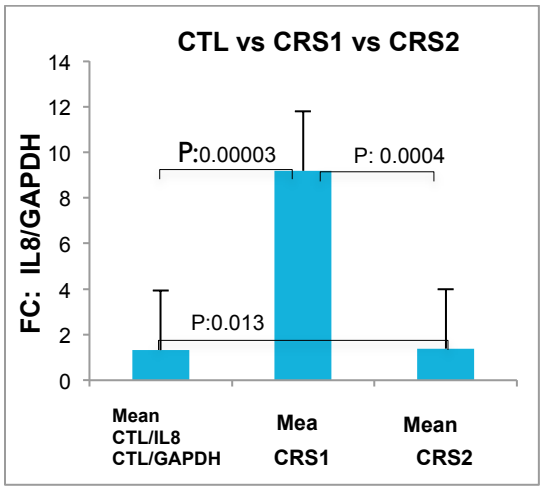
Table 4

Demographic Data of Recruited Patients for Surgical Biopsies

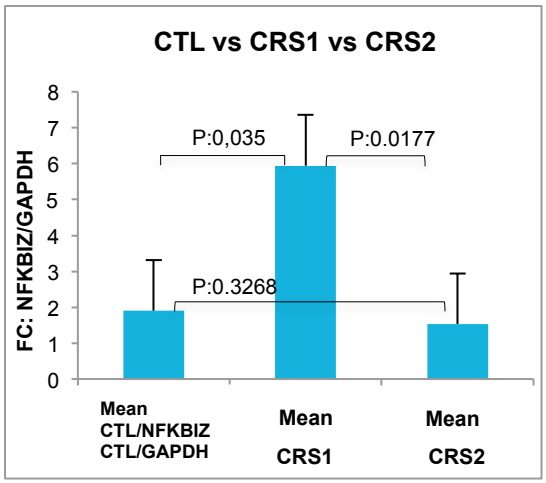
Datum	CRS1	CRS2	CTL	P value CRS1 vs. CRS2	CRSsNP	CRSwNP	CTL	P value CRSwNP vs. CRSsNP
Number of subjects	6	12	7		10	10	11	
Gender (% Men)	50%	75%	55%		70%	70%	55%	
Age, mean (SD)	49(14)	48.9(14)	46.4(16)	0.889	48.4(16)	48.5(10)	46.4(16)	0.99
Allergies	33%	75%	31%	0.071	60%	60%	27 %	
ASA	0%	33%	0%	0.032	20%	20%	0	
Number ABX last year	5	0.45	n/a	0.021	3.5	0.8	n/a	
Family CRS	33%	33%	9%					
Asthma	67%	42%	0	0.023	30%	70%	0	
Age Asthma	45.3	21.2						
IgE (Mean)	45.5	160.4	66.73	0.049	101.3	123.1	66.73	0,5629331
RAST + ?	17%	25%	17%		12.5	30%	17%	
IgE>150 ?	0%	33%	33%		25%	40%	27%	
% EOS	5%	5%	1%	0.096	2.5%	7.5%	1%	
E-Coli	0%	14%	18%		14%	0%	18%	
H Influenza	17%	0%	0%		10%	0	0	
SAE	16.7%	25%	46,1%		30%	20%	36.4%	
STREP	0%	38%	0%	0.026	0	30%	0	0,0406



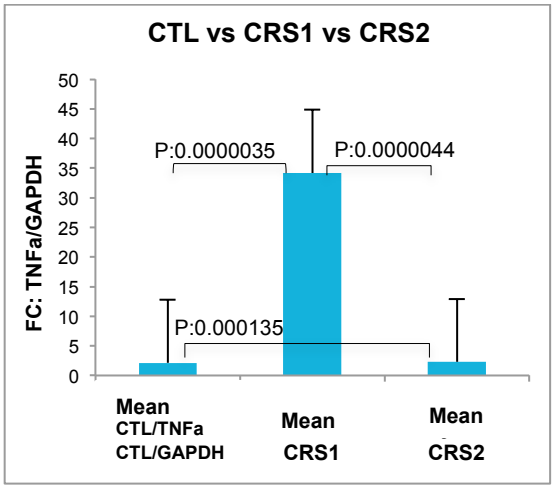
A) IL-1B mRNA expression



B) IL-8 mRNA expression

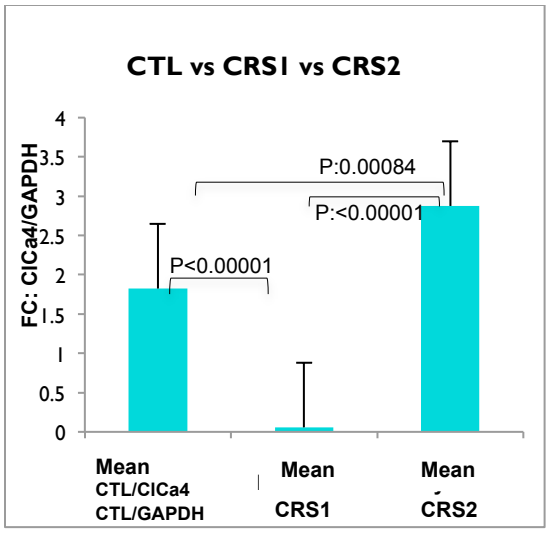


C) NFKBIZ mRNA expression

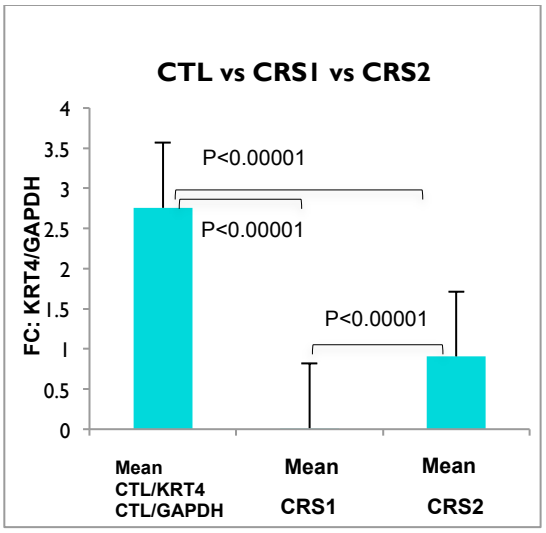


D) TNF-a mRNA expression

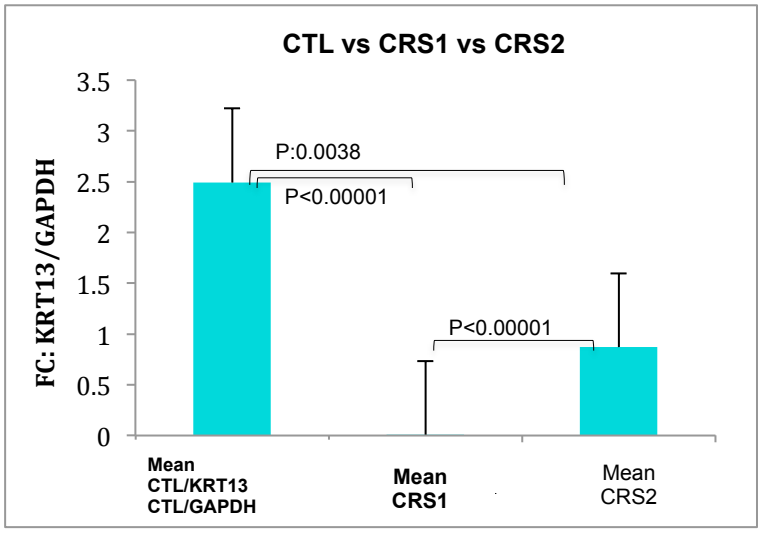
Figure 10. Q-RT-PCR gene expression graphs I



A) Clca4 mRNA expression



B) KRT4 mRNA expression



C) KRT13 mRNA expression

Figure 11. Q-RT-PCR gene expression graphs II

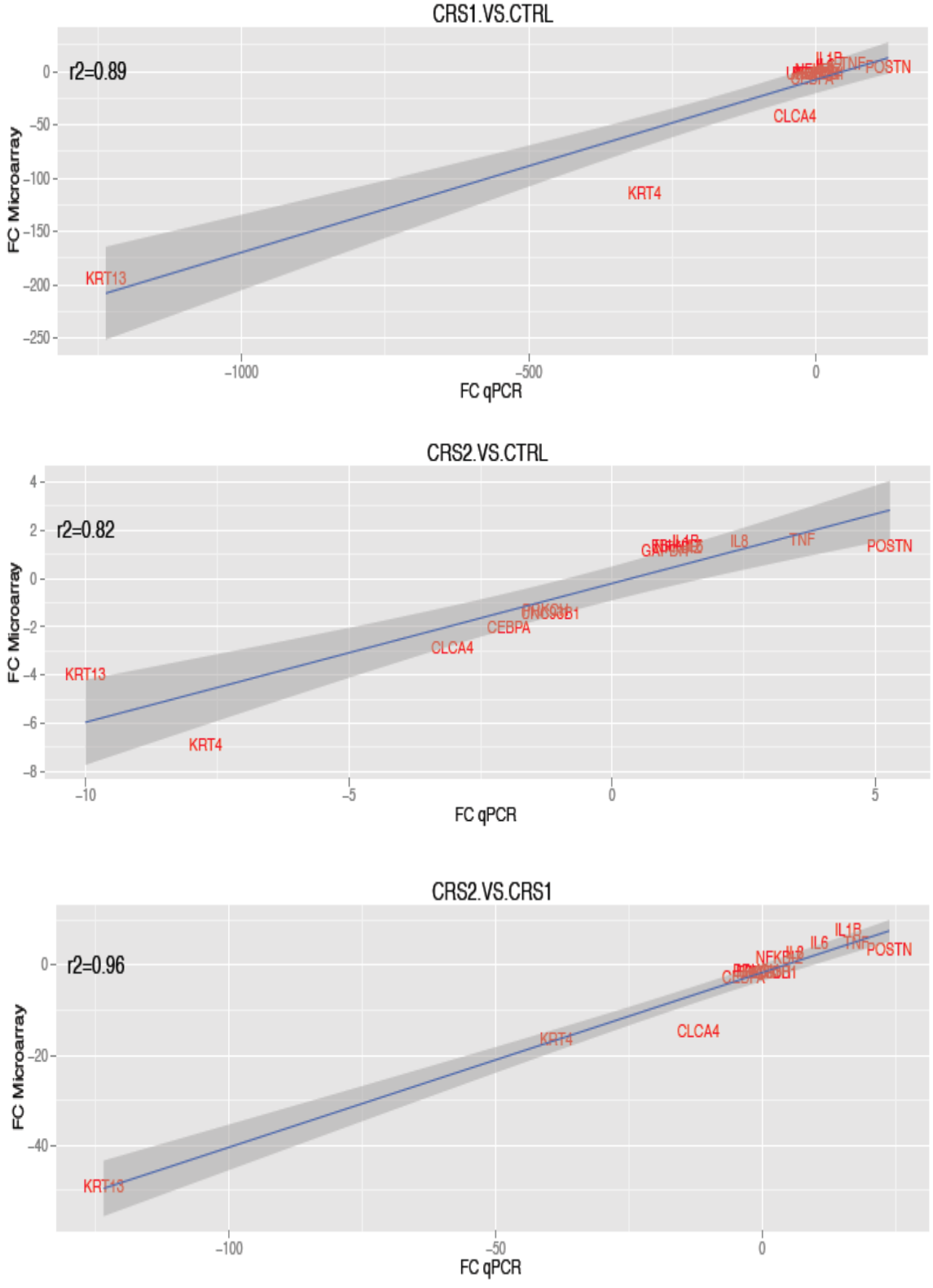
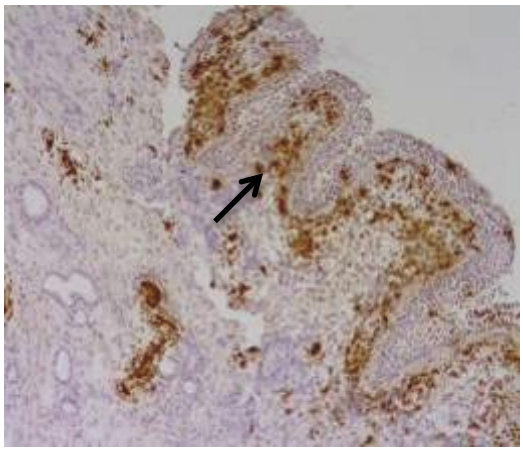
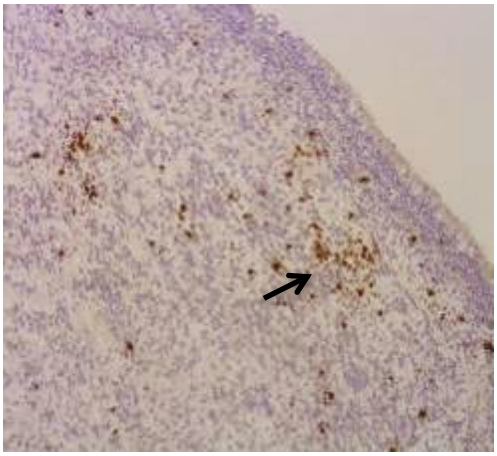


Figure 12. Validation: over-all correlation qPCR. FC vs. FC microarray.



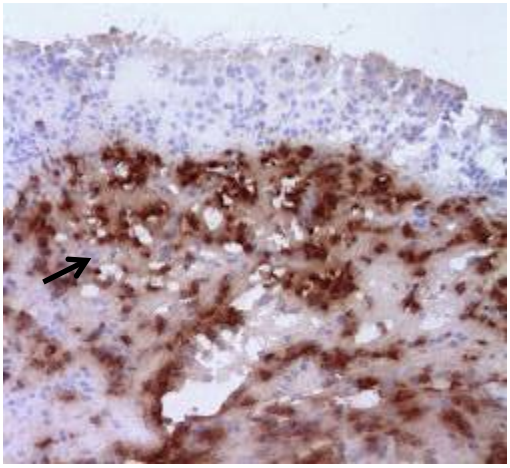
A) CRS1- 20X



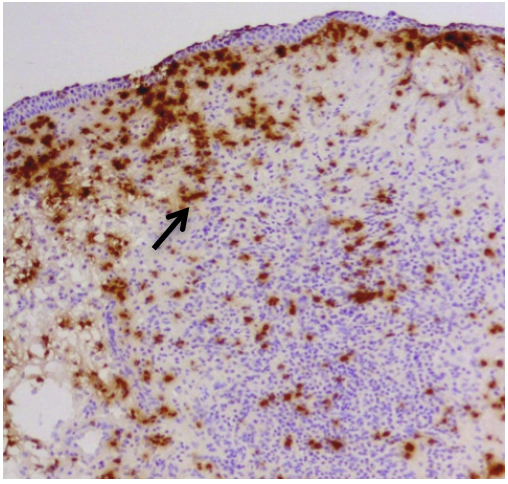
B) CRS2- 20X

Figure 13. Immunohistochemistry staining of Neutrophil elastase in CRS.

IHC staining was used to detect the Neutrophil Elastase. The positive cells were stained with dark brown (black arrow), A) CRS1, B) CRS2.



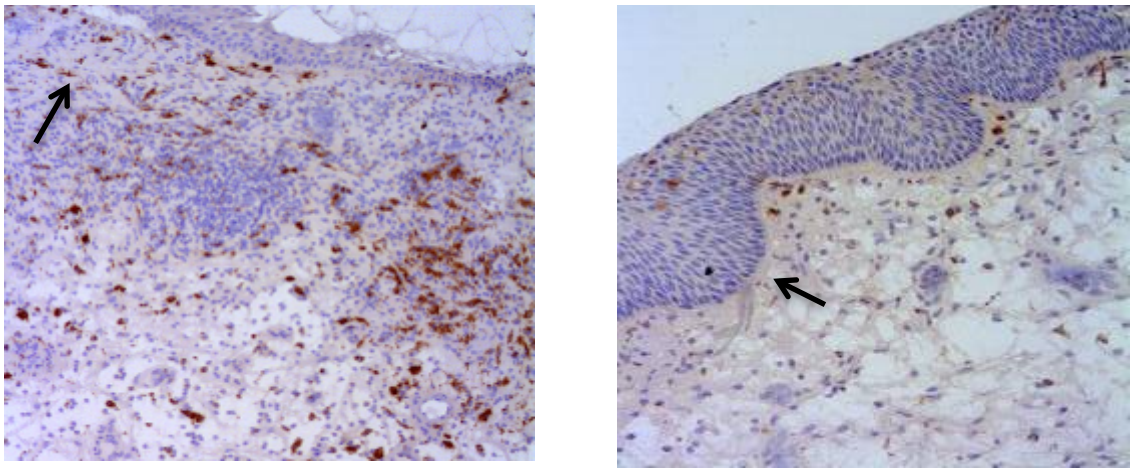
A) CRS1- 20X



B) CRS2- 20X

Figure 14. Immunohistochemistry staining of MBP in CRS

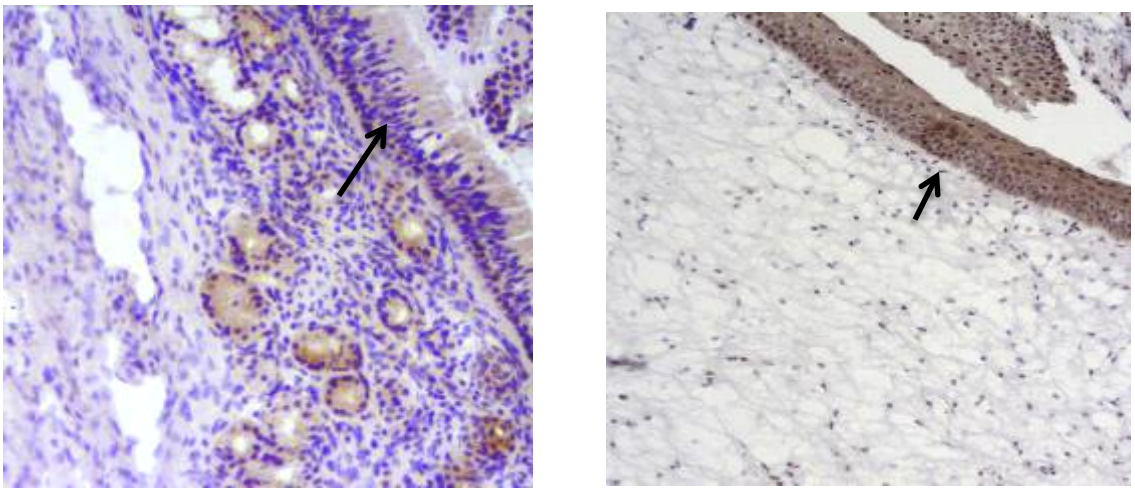
IHC staining was used to detect the MBP in eosinophil. The positive cells were stained with dark brown (black arrow). A) CRS1, and B) CRS2



A) CD68 in CRS1, 20X B) CD68 in CRS2, 20X

Figure 15. Immunohistochemistry staining of CD68 in CRS.

IHC staining was used to detect the CD68 macrophage. The positive cells were stained with dark brown (black arrow), A) CRS1, B) CRS2.



A) Control, 20X B) CRS, 20X

Figure 16. Immunohistochemistry staining of CCL2 in CRS and CTL.

IHC staining was used to detect the CCL2 expression in epithelial cells. The positive cells were stained with dark brown (black arrow). A) Control subject, and B) CRS patient.

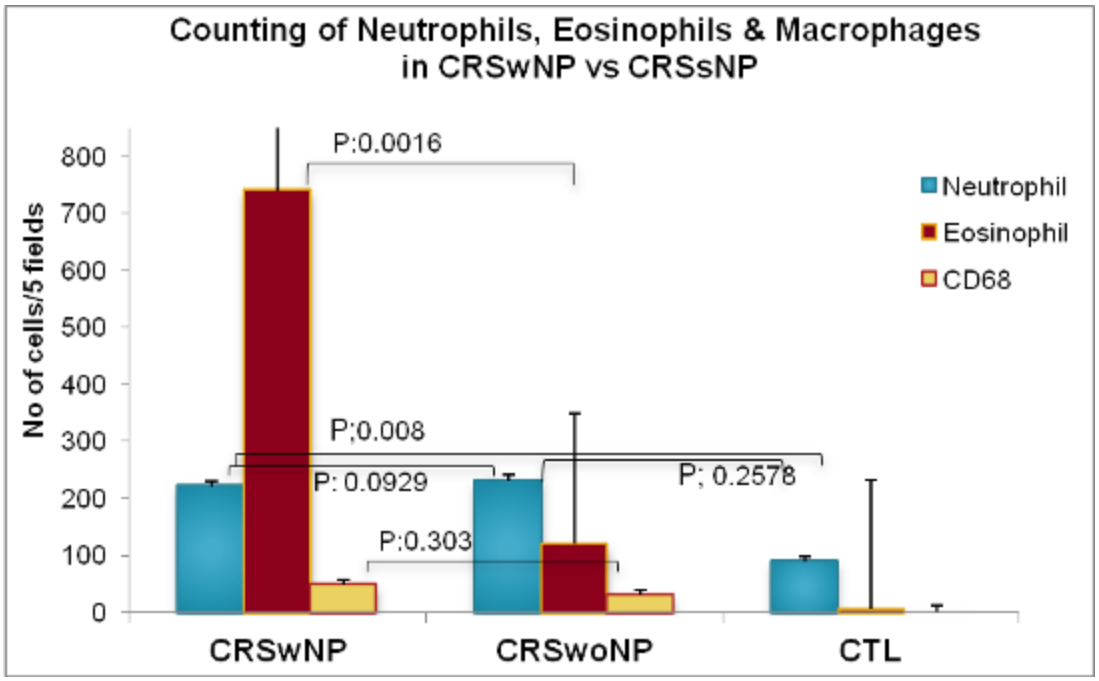


Figure 17. Graph of inflammatory cells results in CRSwNP vs. CRSsNP

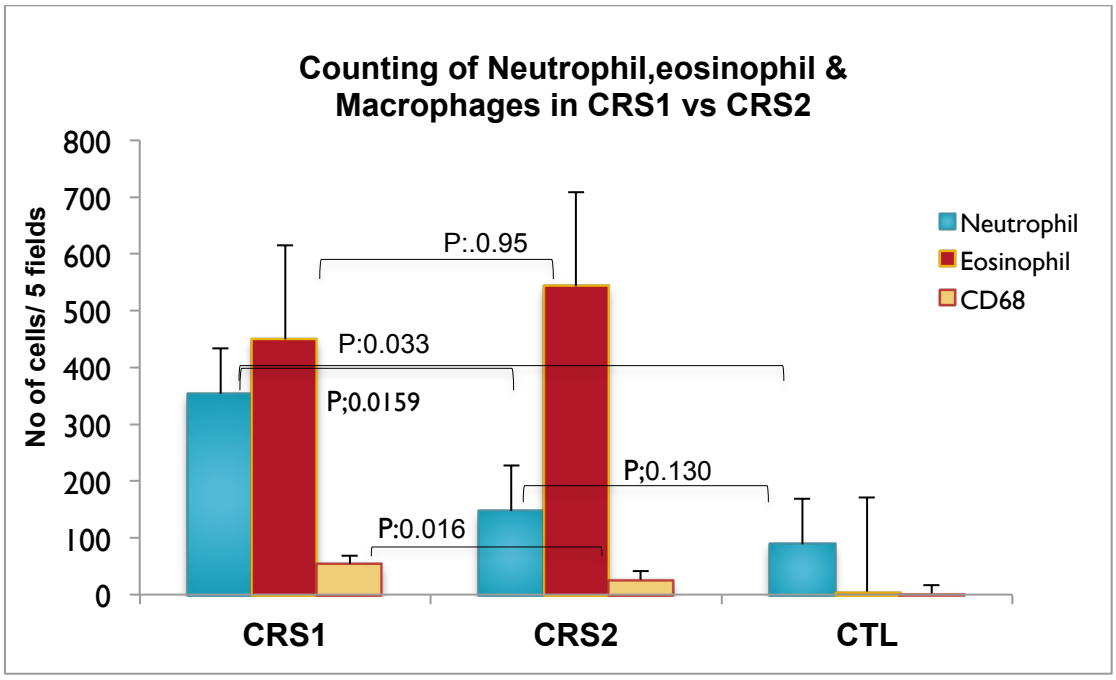


Figure 18. Graph of inflammatory cells results in CRS1 vs. CRS2

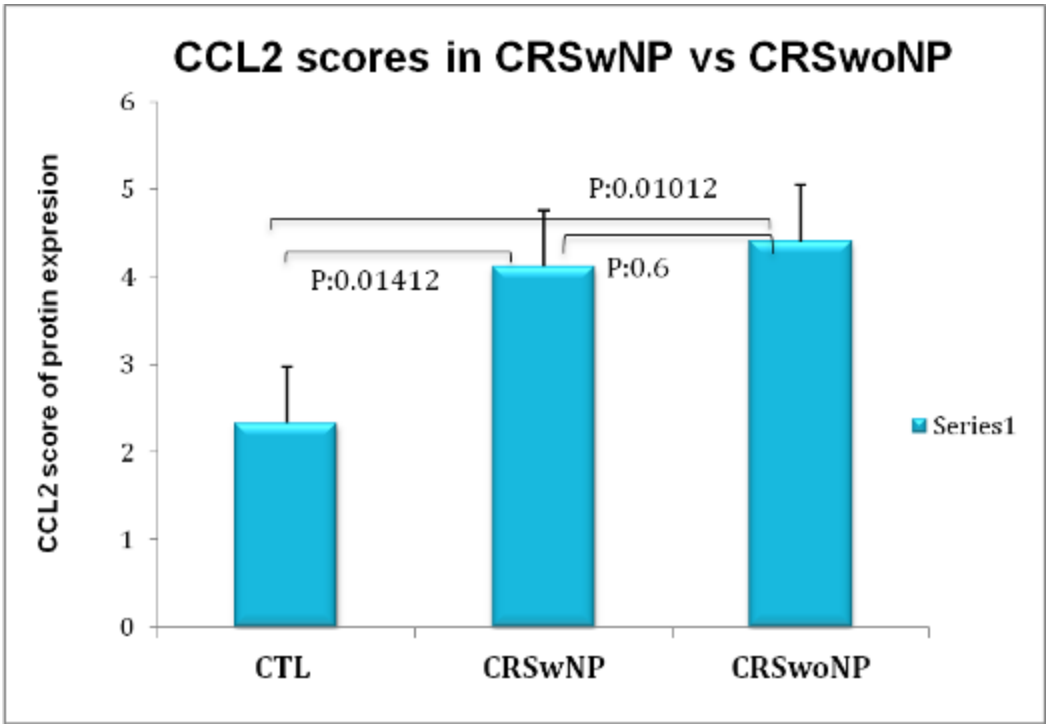


Figure 19. Graph of CCL2 expression in CRSwNP, CRSsNP and control.

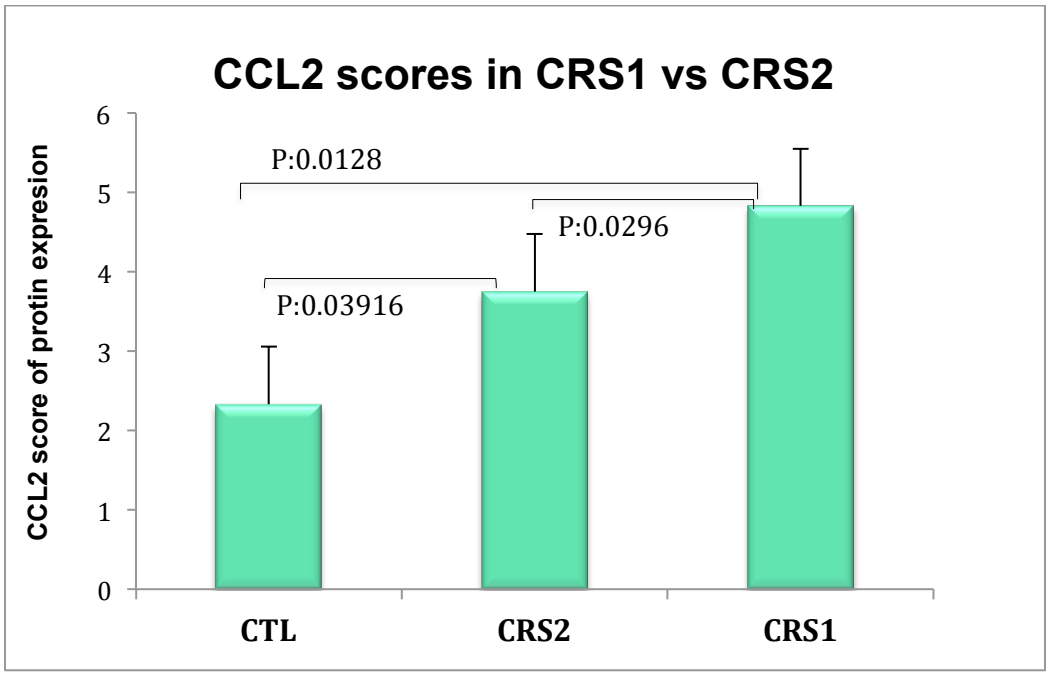
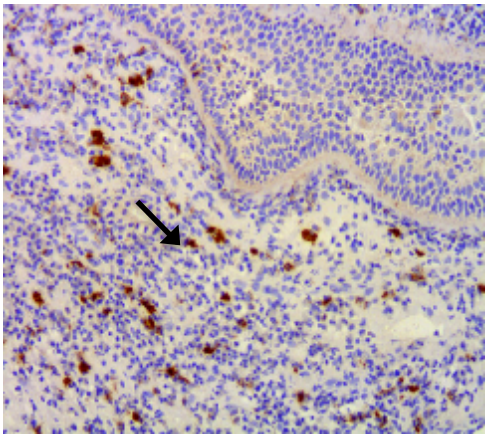


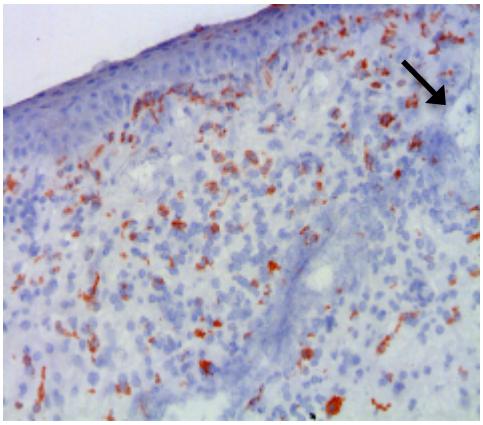
Figure 20. Graph of CCL2 expression in CRS1, CRS2 and control.

CD68 in CRS, 20X



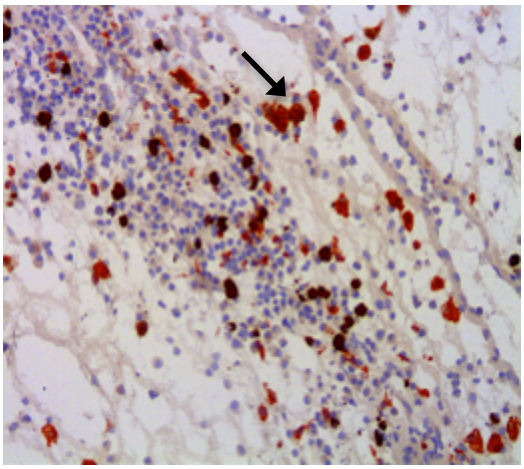
A) IHC staining was used to detect the CD68 macrophage. The positive cells were stained with dark brown (black arrow)

CD206 in CRS, 20X



B) IHC staining was used to detect the expression of CD206.The positive cells were stained with red (black arrow)

CD68+CD206 in CRS, 20X



C) Double IHC staining was used to determine the M2 macrophage CD68+CD206. The positive cells were stained with dark reddish brown (black arrows). All of the pictures are taken under 200_ light microscope.

Figure 21. Immunohistochemistry double staining of CD68 and CD206

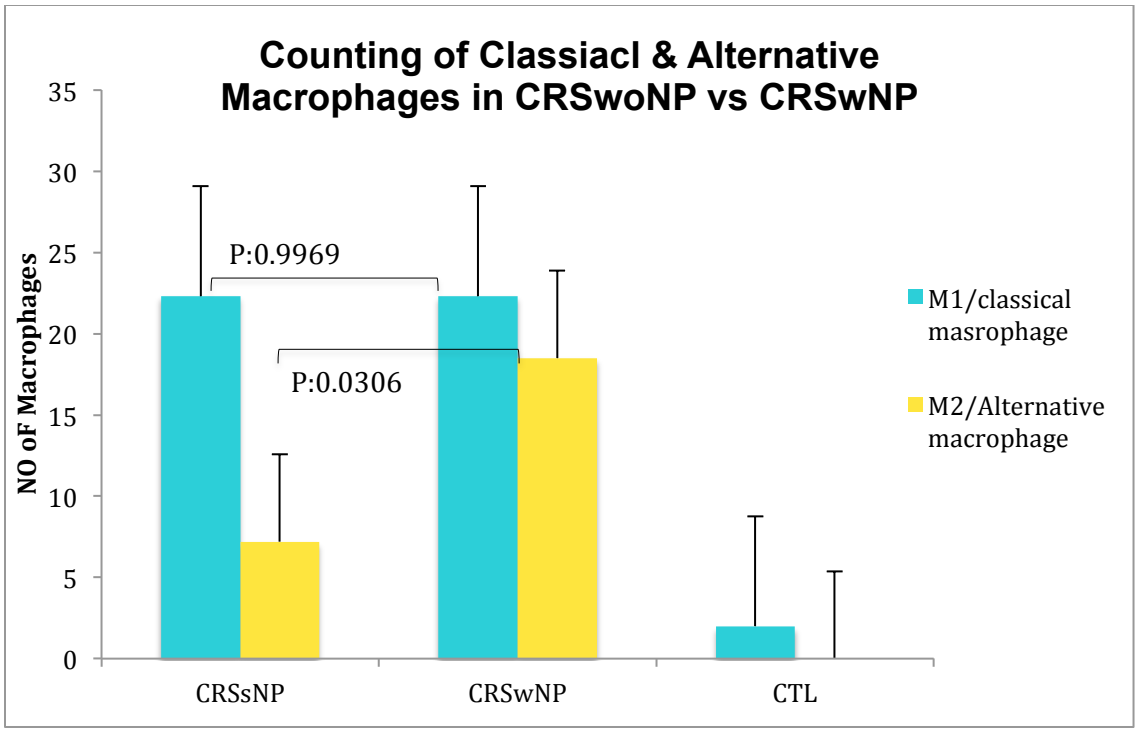


Figure 22. Graph of classiacal / alternative Macrophages in CRSwoNP, CRSwNP

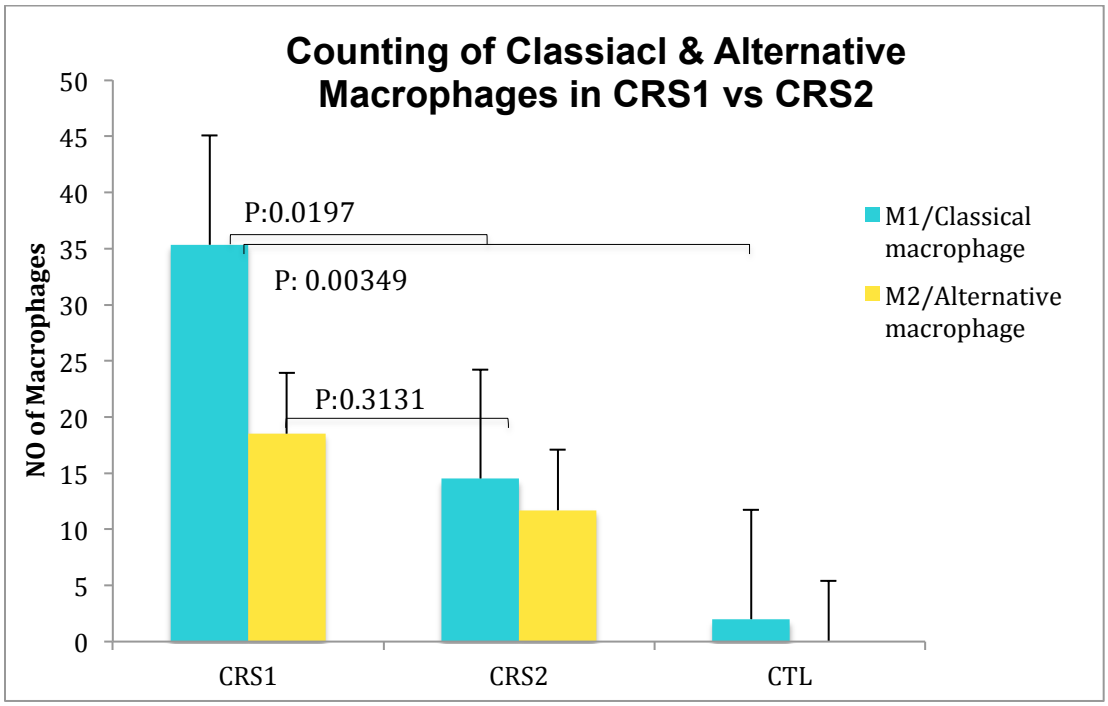


Figure 23. Graph of classiacal / alternative Macrophages in subepithelium of CRS1and CRS2.

CHAPTER FIVE: DISCUSSION

Based on this series of studies, we suggest that the clinical classification of chronic rhinosinusitis, which is based on presence or absence of nasal polyps, does not appropriately differentiate or predict mechanism. We also suggest a new means of classification based on the molecular signature of CRS, using several techniques to support our hypothesis.

Unsupervised Analysis of Microarray of Epithelial Cell Gene Expression Identifies Possible Underlying Mechanism for Development of CRS

We present a novel molecular signature for characterizing subjects with chronic rhinosinusitis based on constituent characteristics of nasal epithelial cells isolated from CRS patients, rather than from whole surgical biopsy samples. This suggests the existence of two distinct, pathogenic mechanisms in CRS characterized by the existence of low- and high-spontaneous inflammation groups. Based initially upon unsupervised clustering analysis of microarray results, these were confirmed via individual PCR for several of the implicated genes, and by demonstration of varied levels of gene products and infiltrating inflammatory cells.

The expression of IL-1B, IL-8, NFKB, TNF-α and CCL2 is markedly increased in the epithelial cell cultured of CRS1 as compared to the CRS2 and control subjects. All these genes code for pro-inflammatory cytokines, which induce inflammation. This supports presence of higher baseline inflammation in CRS1 compared to CRS2 and control.

The expression of KRT4, KRT13 and ClCa4 is decreased in CRS1 compared to CRS2 and control subjects. The implications of this are less immediately obvious, yet some hypotheses may nevertheless be generated. KRT4 and KRT13 are members of type II

cytokeratin, which play an important role in differentiation of simple and stratified epithelial tissues and tumorigenesis. While the impact of reduced KRT4 levels has not been described for CRS, suppression of KRT4 promoter activity increases inflammation in a model of vascular inflammation. Thus the KRT4 reduction observed in our CRS1 group may be contributing to the disease, either through deficient actin cytoskeleton rearrangement, or else deficient formation of the epithelial barrier.

ClCa4, Calcium-activated chloride channel regulator 4 (CaCC family) is involved in mediating calcium-activated chloride conductance. Lee et al. (2005) reported that in biopsy samples of normal tissue (inferior turbinate), ClCa2 is highly expressed in the epithelial layer and submucosal glands, while in nasal polyps; lower levels of ClCa 1,2,3 expression are seen in the epithelial layer. This suggests that solute disorders in solute transport and ionic equilibrium are contributing to the inflammation in this group. Support for the concept of disordered ionic equilibrium is afforded by cystic fibrosis, where defects in the Cl^- channel contribute to the development of chronic sinusitis (Sang Hag Lee, 2005).

These results contrast to the previous literature based on clinical phenotypes, which indicate that TNF, TNFR (Karosi, Csomor, & Sziklai, 2012) and CCL2 (R.P. Schleimer, 2012) genes are upregulated in CRSwNP but not in CRSsNP (Bernstein, 2001; R.P. Schleimer, 2012). However, this may be at least partially explained by the diversity observed in our clinical phenotypes, where CRS1 and CRS2 groups included elements of both the CRSwNP and CRSsNP groups within these categories, highlighting the heterogeneity of results reported on the basis of current classification schemes.

Our results, taken from cultured epithelial cells, nevertheless reflect cellular inflammatory changes identified in simultaneously obtained surgical biopsy samples,

offering additional support for the validity of this approach, as does the existence of two clinically and biologically different phenotypes. Experience from other groups supports this. Mechanism-based phenotypes have been described as influencing disease behavior. In a first example, evolution of patients after endoscopic sinus surgery varied according to NFKB expression in tissue biopsy samples obtained at operation, with poorest evolution noted in the high-activation group. In the second, tissue neutrophilia at the time of surgery was associated with lesser response to a course of oral steroid therapy (Wen et al., 2012).

Support for this classification is also afforded by significant variations in the clinical profiles of patients, where patients with the CRS2 phenotype present a more seemingly indolent clinical course than those with a CRS1 profile. Based on clinical data, CRS2 patients had a lower consumption of antibiotics, and a bacterial flora, which differs from CRS1, with a higher recovery rate of Streptococcus Pneumonia. Limited serum biomarkers demonstrate lower levels of WBC, and a higher total serum IgE level in the CRS2, as compared to CRS1 group.

Inflammatory Cell Populations

In our study we used immunohistochemistry staining to characterize and assess the inflammation in the biopsies obtained from the same patients recruited for the microarray analysis, choosing four inflammatory markers, neutrophil elastase, Major basic protein (MBP), CD68, and CCL2. While tissue eosinophilia was greater in the CRS1 and CRS2 groups as compared to controls, there is no significant difference in increase of eosinophil between CRS1 and CRS2. We however detected a significantly increased neutrophilic infiltrate in submucosa of biopsy samples from CRS1 patients compared to CRS2 and control subjects, along with a corresponding increase in macrophage infiltrate, again

greater in CRS1 than CRS2 and control.

Neutrophilic infiltration is a characteristic feature of proinfllamatory TNF-a, NFkB, and IL-8 gene activation and can be considered as a surrogate marker of the activity of this pathway. The increased neutrophilic infiltrate seen in CRS1 corresponds to the in-vitro observations from the same patients demonstrating increased pro-inflammatory gene activity and offers additional support to our concept.

The expression of CCL2 (MCP-1), which acts as a potent chemoattractant and activator of monocytes/ macrophages, was also detected in the epithelial tissue of surgical biopsies, with a significantly higher level of CCL2 expression protein in CRS1 compared to the CRS2 and control but with no significant differences in expression between CRSwNP and CRSsNP. These support results of unsupervised analysis showing upregulated CCL2 expression in CRS1 compared to CRS2 and control. CCL2 expression may be induced by inflammatory cytokines and again offers support for a greater inflammation in CRS1. In addition, these observations are supported by results reported by another group (Wen et al., 2012), which indicted the increase expression of CCL2 m-RNA and other inflammatory cytokines in neutrophilic nasal polyp than non-neutrophilic phenotype.

The mechanism of CRS2 remains elusive, however, assessment of macrophage activation may offer some insights. We detected two different patterns to macrophage activation in chronic sinusitis using double staining to identify classical M1 and alternative activated M2 macrophages. M1 macrophages express high levels of pro-inflammatory cytokines, and participate in the induction of a Th1 response and prevent pathogen persistence (Gordon, 2003). M2 macrophages are producing anti-inflammatory cytokines,

immunosuppressive and may support intracellular survival of bacteria and viruses (Benoit et al., 2008).

In biopsy samples, M1 macrophages were dominant in CRS1 compared to the CRS2 while M2 macrophages were more dominant in CRS2. This observation supports our concept that CRS1 is a high inflammatory profile subgroup of CRS patients, and suggesting that a more immunotolerant pattern is present in CRS2 (Gordon, 2003). Increased presence of M2 macrophages in CRSwNP as compared CRSsNP has previously been described (Krysko et al., 2011), and supports our results. Interestingly, in those studies, the increase in M2 macrophages is highest in CRSwNP subjects who have high levels of IL5 in biopsy samples (IL5+) and is associated with a defect in phagocytosis of S Aureus, suggesting that the M2 activation phenotype could potentially contribute to persistence of chronic CRS by reducing clearance of pathogenic bacteria thus facilitating their persistence.

While the concept of phenotyping chronic sinusitis according to cellular infiltrate is novel, this is an increasingly studied topic in the lower airways. Asthma, as a clinical syndrome affecting the lower respiratory tract, characterized by variability in disease expression and severity, presents several similarities to chronic sinusitis and may serve as a basis for comparison. In effect, when we compare our results to asthma, we found there is support for the concepts presented here. Gibson, et al demonstrate (Gibson, Simpson, & Saltos, 2001) that there is heterogeneity of airway inflammation in persistent asthma, with the typical eosinophilic pattern occurring in only a minority of subjects. The majority of subjects have non-eosinophilic asthma with neutrophil degranulation and a neutrophil influx that may be mediated by IL-8. Moore et al (Moore et al., 2010) used an unsupervised

hierarchical cluster analysis of demographics of severe asthma cases and identified five distinct clinical phenotypes of asthma that differed in clinical, physiological, and inflammatory parameters. In results surprisingly similar to those from our study, they found severe asthma classified to five groups according to disease severity, clinical symptoms, treatment response, bacterial infections and hospitalization need and inflammatory cells in expectorated sputum samples. While eosinophilic infiltrate in expectorated sputum samples was common to all groups, two of these five clusters (clusters 3 and 5) had high neutrophilia, In an interesting parallel with our results, these two groups reported the highest prevalence of sinus disease, with nearly half of those in cluster 5 reporting prior sinus surgery and more episodes of pneumonia, which is similar to the CRS1 pattern.

Taken as a whole, this body of work suggests that in chronic rhinosinusitis, two distinct mechanisms of pathogenesis exist, with overlapping clinical features of nasal polyposis, which appear to be independent of these two mechanisms.

Host factors or initiating events are currently unknown, but speculation as to their origin is in order. Inherited modifications of the genetic code for key genes may lead to mucosal dysregulation with increased inflammation or to increased susceptibility to bacterial infection. Examples in humans include cystic fibrosis, where a primarily neutrophilic form of nasal polyposis is observed, and MHC-I immune deficiencies, where rhinosinusitis is a frequent feature of the clinical presentation. In addition, pathogens such as viruses or bacteria producing exotoxins may also colonize sinuses and contribute to disease development by behaving as biological modifiers. This supports the concept advanced by other groups that factors released by resident bacteria such as Staphylococcus aureus and Streptococcus pneumonia may be the key of inflammation (Huvenne et al.,

2010; Thurlow et al., 2011).

In reality, CRS development probably combines features of both genetic susceptibility and interaction with bacterial and viral agents.

CHAPTER SIX: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Using unsupervised analysis of gene expression analysis of epithelial cultures from control and CRS patients, we have identified a novel, gene expression-based molecular signature that allows differentiation of chronic rhinosinusitis patients according to pathogenic mechanism, which is independent of the presence or absence of nasal polyposis. Constitutive difference in gene expression pattern in epithelial cells from CRS1 and CRS2 patients with high pro-inflammatory gene expression at baseline are accompanied by a corresponding pronounced neutrophilic infiltration in CRS1 in surgical biopsy samples,

We also identified two different patterns to macrophage activation: A predominantly classical microbicidal M1 macrophage infiltrate in CRS1, and an increased percentage of alternative macrophages M2 immunosuppressive inflammation in CRS2, suggesting an immunosuppressive or immunotloreant environment, which could potentially contribute to persistence of chronic inflammation in CRS2.

We hope our results may help to widen current understanding of the pathogenesis and assessments of disease mechanism and response to therapy according to molecular, as opposed to clinical phenotypes which may hopefully allow us to “personalize” our management of individuals with this disease.

Future Directions

Our future directions are:

1. To replicate our findings in a second CRS population
2. To identify novel biomarkers for CRS1 and CRS2 patterns applicable in outpatient clinic which will hopefully help improve the assessment of the CRS patient by phenotyping the patient as to underlying disease mechanism rather than clinical phenotype.

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