INTRODUCTION

There is mounting evidence that bacterial and possibly fungal biofilms play an important role in the etiology and persistence of Chronic Rhinosinusitis (CRS). CRS affects nearly 16–25% of the US population each year, with billions of dollars of annual healthcare expenditures dedicated to its treatment **(Gliklich and Metson, 1995).**

Unfortunately, the recalcitrant nature of the disease, which often exhibits a chronic relapsing course, significantly contributes to these healthcare costs. The reasons for the persistent nature of the disease are likely secondary to a number of underlying pathophysiologic mechanisms. Asthma, allergic rhinitis, Gram-positive and Gram-negative infections, aspirin-sensitive asthma, fungus, osteitis, nasal polyposis, super antigens, and other factors have been implicated as etiologies contributing to the development of CRS. The chronic inflammation that develops as a fundamental hallmark of the disease can both cause and be a consequence of dysfunctional mucociliary clearance. Ultimately, stasis of sinonasal secretions will lead to subsequent infection and/or persistent inflammation. In some cases, persistent and recurrent infections occur despite multiple therapeutic interventions for CRS **(Leid et al., 2011).**

AIM OF THE WORK

This study aims to detect the role of biofilm in chronic rhinosinusitis through scanning by electron microscopy.

CHAPTER [1]

Chronic Rhinosinusitis

1.1 Definition, epidemiology and socio-economic implications:

Chronic sinusitis (CRS) can be considered as a group of disorders that is characterized by inflammation of the mucosal lining of nasal cavity and paranasal sinuses lasting for at least 12 weeks. Historically the disease was diagnosed clinically based on major and minor symptoms **(Jackson and Kountakis, 2005).**

Chronic sinusitis is a very common condition affecting up to 16% of US population. Its prevalence resembles that of hypertension and non-specific lower back pain and it remains the single most common self-reported chronic health condition affecting adults in the western world **(Anand, 2004).**

The financial burden of this condition is far reaching, with direct annual US health care expenditure in excess 5.8 billion US dollar **(Ray et al., 1999)**. Estimates of restricted activity days in the USA exceed 73 million days/year making financial costs of CRS to society significantly high **(Collins, 1997).**

1.2 Etiology & pathogenic factors:

Despite increasing research into pathophysiology of CRS over the last two decades, the exact pathogenic mechanisms still remain unclear. Without the identification of a single unifying cause for this condition, CRS is now considered a multi-factorial disease with varying levels of evidence for certain risk factors.

These factors have been categorized into extrinsic or non-host related factors and intrinsic or host related factors. Extrinsic factors are smoking, pollution and exposure to allergens as well as microbial infections (bacterial and fungal) and their associated pathogenicity (biofilms, superantigens, osteitis and non-IgE mediated eosinophilic inflammation). Intrinsic factors include; anatomical\structural abnormalities like cystic fibrosis, primary ciliary dyskinesia, disorders of innate and cell mediated immunity and adenoid hypertrophy in children **(Wolf, 2002).**

CRS is most likely the manifestation of interaction of multiple host and environmental factors suggesting that there may be genetic or epigenetic influences that predispose to disease. Environmental factors that have been proposed include viral, bacterial, and/or fungal colonization, as well as exposure to inhaled substances, such as cigarette smoke or allergens. The most reported CRS-associated bacteria in the literature are *S.*

aureus, *P. aeruginosa*, coagulase-negative Staphylococci, *S. pneumoniae*, and *Moraxella catarrhalis*. Using species-specific DNA probes, studies recently reported the presence of *H. influenzae in* ~80% of CRS patient lesions **(Sanderson et al., 2006).**This report was the first to demonstrate the prevalence of *H. influenzae* in CRS-associated nasal tissues. Within the last half decade, much attention has also been directed toward the contribution of fungi (in the sinonasal mucosa during development of polypoid disease. Subsequently, studies have demonstrated the presence of fungi and *H. influenzae* in CRS lesions associated with allergic rhinitis **(Hamilos and Lund, 2004; Gosepath and Mann, 2005).** Different techniques were used to demonstrate bacteria in sinonasal mucosa like fluorescent micrographs. (figure 1)

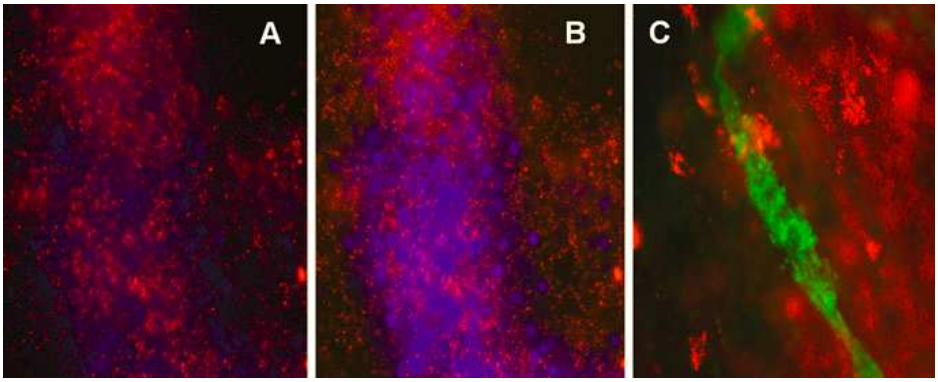

Figure (1): Fluorescent micrographs of explanted sinonasal tissue from human CRS patients undergoing functional endoscopic sinus surgery shows:

(A) The *red spheres* are *Haemophilus influenza (FISH stain).*

(B) The *blue* are eukaryotic nuclei.

(C) The *green* is a pan-fungal stain (**Leid et al., 2011).**

Is CRS a polymicrobial disease?

As medical technology has advanced, the idea of a single organism causing a disease has become outdated. Sophisticated techniques have identified new groups of microorganisms in every tissue of the human body. The same is certainly true for the human sinus cavity. The use of molecular tools has clearly shown that standard microbial culture techniques only identify 10–40% of the microorganisms present in many diseases. This is confounded by the inherent attachment of these communities making them hard targets for standard clinical diagnostics **(Veeh and Shirtliff, 2003).**

1.3 Treatment of CRS:

The treatment of CRS involves both medical and surgical interventions. Two recent surveys of US otolaryngologists found oral antibiotics and intranasal steroids are commonly employed first line agents in the management of CRS **(Sharp et al., 2007).**

Despite considerable disagreement surrounding the role of bacteria in CRS, two prospective randomized control trials (RCT) have demonstrated the efficacy of long term antibiotic treatment(>12 weeks) in management of CRS. Wallwork showed a statistically significant improvement of symptoms, endoscopic and rhinometric parameters in patients treated with

long term macrolide antibiotics compared to those in placebo arm **(Wallwork et al., 2006).**

A similar improvement in objective and subjective outcome measures was also found by Ragab et al, in patients medically treated with long term erythromycin, intranasal steroids and alkaline douches **(Ragab et al., 2004).**

A systematic review of literature by Cochrane Collaboration identified randomized controlled trials evaluating the use of nasal saline irrigation for the symptoms of CRS **(Harvey et al., 2007)**. Meta-analysis of three studies comparing the effect of saline versus no treatment revealed statistically significant improvement of symptoms and disease-specific quality of life scores in the saline arm **(Rabago et al., 2002).**

Functional endoscopic sinus surgery (FESS) has now become well established treatment of CRS refractory to medical treatment .A systematic review of literature by the Cochrane Collaboration in 2006,revealed that the vast majority of studies examining the effectiveness of FESS for CRS were either cohort or level 3 evidence at best **(Khalil and Nunez, 2006).** Although these studies generally demonstrated high efficacy of FESS, the 2 RCTs identified in the review **(Ragab et al., 2004), (Hartog et al., 1997)** did not demonstrate significant difference in outcomes between FESS and medical treatment of CRS.

CHAPTER [2]

Bacteria and CRS

2.1 Controversy regarding the role of bacteria in CRS

The role of bacteria in acute rhinosinusitis is well defined; with streptococcus pneumoniae, Moraxella catarrhalis and Haemophilus influenza are the most common pathogenic organisms involved. The microbiology and importance of bacteria in the etiology of CRS remains debated. Summarizing the literature pertaining to the bacteriological evaluation of CRS patients is extremely difficult due to the multiple methodological differences existing between studies **(Brook, 2007)**.

Differences include; **1)** characteristics of patient (age, gender, immune state and presence of co-morbidities) **2)** duration, extent and severity of disease **3)** use of previous or concurrent treatments (antimicrobials and anti-inflammatory agents vs. surgical) **4)** site and sinus of sampling **5)** sampling methods used (irrigation, aspiration or blind vs. endoscopically guided biopsy/swab) **(Brook, 2007)**.

It has been postulated that in many cases bacteria may simply be present as non-pathogenic bystanders invading already inflamed tissue. This opinion has largely stemmed from the following observations: **1)** the finding that the paranasal

sinuses are not actually sterile as once thought, with more than half of all healthy sinuses culturing bacteria **2)** the relative absence of neutrophils and predominance of eosinophils and mixed mononuclear cells in the inflammatory infiltrate **3)** the poor correlation between clinical findings and microbiology and **4)** the often short lived or poor response to seemingly appropriate culture-directed antibiotic therapy **(Rontal et al., 1999**).

2.2 Bacterial Superantigens

The increasing evidence of superantigen mediated inflammation in chronic eosinophilic-lymphocytic inflammatory disorders such as atopic dermatitis, allergic rhinitis and asthma has led researchers to believe that they may also play a role in inflammation associated with CRS. Super-antigens are microbial derived toxins capable of triggering massive polyclonal T cell proliferation and activation. They do so by directly binding to and cross-linking the MHC (Major Histocompatibility Complex) class 2 molecules on antigen presenting to the region β chain of the T cell receptor. This bypasses the conventional MHC restrictions of the immune system, enabling them to activate up to 20-30% of the host T-cell population **(Herman et al., 1991)**.

CHAPTER [3]

Fungus and CRS

3.1 Role and terminology

Till one decade back bacteria implicated as pathogen in most form of CRS. Fungi may be responsible for few specific forms. Since 1999 Ponikau and associates claimed that fungi are responsible for nearly all cases of CRS. Study demonstrated the presence of fungi & eosinophils from nose & PNS from ~100% cases of CRS. Also study coined the term 'Eosinophilic fungal rhinosinusitis' (EFRS) **(Ponikau et al., 1999).**

More new terms coined depending on presence of fungal allergy or fungus:

1) NAFES: Non Allergic Fungal Eosinophilic Sinusitis
2) CFS: Chronic Fungal Sinusitis **(Ferguson and Stolz, 2005).**
3) AFS like Absence of fungi in mucin, but have fungal allergy **(Collins et al., 2003).**
4) NANFES (CES): Non Allergic, Non Fungal, Eosinophilic Sinusitis (Chronic eosinophilic sinusitis)
5) EMCRS: Eosinophilic Mucus Chronic Rhinosinusitis **(Pant et al., 2005).**

3.2 Innate immunity vs. fungi

- Proteases from fungi bind PAR (Protease activated receptor) on epithelial, airway cell, and blood vessels then release of cytokine and metalloproteinase occurs that leads to disruption of epithelial tight junction **(Guerra et al., 2002).**

- Fungi induce production of inflammatory cytokines IL-6, IL-8 from primary nasal epithelial cells **(Guerra et al., 2002).**

3.3 Allergic Fungal Sinusitis

Allergic fungal sinusitis occurs when a fungus colonizes a sinus cavity and then causes allergic mucosal inflammation through an IgE response to fungal protein. Patients present with nasal obstruction, rhinorrhea, facial pressure, sneezing, watery/itchy eyes, and periorbital edema. There are five major criteria used to make the diagnosis of AFS. These are the presence of eosinophilic mucin containing noninvasive fungal hyphae, nasal polyposis, allergy to the offending fungus, Immunocompetance of the patient, and the classic radiographic findings associated with AFS (**Manning et al., 1993).**

Eosinophilic mucin is pathognomonic for AFS, and is described as a thick, tenacious and highly viscous material that is tan to brown or dark green in appearance. Under microscopic

examination, the mucin contains branching fungal hyphae, sheets of eosinophils, and charcot-leyden crystals. Charcot Leyden crystals are slender and pointed crystals consisting of a pair of hexagonal pyramids joined at their bases. They result from the breakdown of cells by enzymes that are released by eosinophils (**Manning et al., 1993).**

Chronic invasive fungal sinusitis (CIFS) is occurring over months to years. Patients with this disease tend to be immunocompetent. The most common pathogen is Aspergillus, seen in about 80% of cases. Other fungi include Mucor, Rhizopus, Bipolaris, and Candida **(Gourley et al., 1990).**

Retrospective analysis of the results of FESS by Xavier Dufour and associates performed in 175 patients suffering from paranasal sinus fungus ball with all maxillary (n=150), sphenoidal (n= 20), and ethmoidal (n = 4) locations have been treated exclusively by FESS to obtain a wide opening of the affected sinuses, allowing a careful extraction of all fungal material without removal of the inflamed mucous membrane. No major complication occurred. Postoperative care was reduced to nasal lavage with topical steroids for 3 to 6 weeks. Only 1 case of local failure have been observed (maxillary sinus = 1), and 6 cases of persisting of fungus ball (maxillary sinus, n = 4; frontal sinus, n = 2) with a mean follow-up of 5 years. No medical treatment (antibiotic, antifungal) was required. This study revealed that surgical treatment of a fungus ball consists

in opening the infected sinus cavity at the level of its ostium and removing fungal concretions while sparing the normal mucosa. No antifungal therapy is required. Finally, through this 175 patients study, FESS appears a reliable and safe surgical treatment with a low morbidity **(Dufour et al., 2005).**

CHAPTER [4]

Biofilm

4.1 Historical perspectives

The first description of biofilms was made by Anton Van Leewenhoek in 1683. Scientists became focused with the free floating form that became popularized by Robert Koch in his doctrine of bacterial causation of acute diseases. It was not well known that that the concept of bacteria existing in biofilms until the emergence of chronic diseases **(Costerton et al., 1978).**

The interest in the biofilm world is now overwhelming, with more than 6500 biofilm related articles published since 1990 **(Morris and Hagr, 2005).**

4.2 Definition

The definition of a biofilm is constantly evolving one, reflecting advances in scientific research and technology. The early definitions focused entirely on the structure of the biofilm, namely the bacterial clusters and their encasing matrix. Soon after, it became evident that biofilms were not static, homogenous structures but rather exhibited spatial and temporal heterogeneity as well as many differences to their planktonic counterparts in terms of growth, metabolic rate and genetic expression. As a consequence the most recent definition put

forward by Donlan and Costerton encompasses both the readily observable structural features of a biofilm as well as the specific physiological features of the organisms existing within these structures **(Davies et al., 1998**).

They now define a biofilm as a microbially derived sessile community, characterized by cells that are irreversibly attached to a substratum or interface or both, are embedded in a matrix of self-produced extracellular polymeric substances, and exhibit an altered phenotype in terms of growth rate and genotype **(Donlan, 2002).**

4.3 Biofilm ultrastructure

The conceptual understanding of biofilm ultrastructure has evolved with the advent of new imaging modalities. From early light and transmission electron microscopy studies biofilms were viewed as:

Homogenous, unstructured, planar accretions of bacterial cell embedded within the cells' exopolysaccharide matrices **(Nyvad and Fejerskov, 1997)** by using the differential interference contrast (DIC) microscope to study water-system biofilms **(Keevil and Walker, 1992).**

Laser Microscopy to biofilm research probably represents the most significant advancement in our understanding of biofilms. Laser Microscopy circumvents many