The role of bacterial biofilms in the pathogenesis of chronic rhinosinusitis

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TABLE OF CONTENTS	1
ACKNOWLEDGEMENTS	3
ABBREVIATIONS	4
1. AIMS OF THE THESIS	7
2. INTRODUCTION	8
3. BIOFILMS	9
3.1 Biofilm prevalence	10
3.1.1 in nature	10
3.1.2 in human disease	10
3.2 Biofilm properties	11
3.2.1 Cycle	11
3.2.2 Communication	13
3.3 Biofilm adaptability	14
3.3.1 Resistance to environmental stress	14
3.3.2 Resistance to antibiotic agents	14
3.4 Lab cultured biofilm vs natural in vivo biofilm	16
3.5 Biofilms in chronic rhinosinusitis	17
4. CHRONIC RHINOSINUSITIS	18
4.1 Epidemiology	19
4.2 Predisposing factors	20
4.3 Diagnosis	23
4.4 United airways	24
4.5 Treatment	25

5. MATERIALS AND METHODS	28
5.1 Study design	29
5.2 Inclusion and exclusion criteria	30
5.3 Parameters examined	31
5.4 Lund-Mackey CT score	32
5.5 Biopsies	33
5.5.1 Collection	33
5.5.2 Preparation	33
5.5.3 Storing	33
5.5.4 Incubation	33
5.6 Microscopy	35
5.6.1 Principles of confocal microscopy	39
5.6.2 Confocal microscopy and biofilms	41
5.7 Pilot study	42
5.8 Protocol	45
5.9 Statistics	46
6. SUMMARY OF RESULTS	48
6.1 Paper I	48
6.2 Paper II	50
6.3 Paper III	53
7. GENERAL DISCUSSION	57
8. CONCLUSIONS	60
9. FUTURE STUDIES	61
REFERENCES	62
PAPER I-III	77

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All this work started innocently as non-committal talks in the cafeteria at AHUS. Beware!

Abbreviations

AB-SA01 – A proprietary bacteriophage mix. Bacteria killing viruses.

AFRS - Allergic fungal rhinosinusitis

AHUS – Akershus University Hospital

AHL – Acyl homoserine lactones

AIDS – Acquired immune deficiency syndrome

CD4 – Cluster of Differentiation 4

CDC – Centers for disease control and prevention

CRS – Chronic rhinosinusitis

CRSsNP – Chronic rhinosinusitis without nasal polyps

CRSwNP – Chronic rhinosinusitis with nasal polyps

CT/CAT – Computed (axial) tomography

CT-PA - A proprietary bacteriophage mix. Bacteria killing viruses.

DNA – Deoxyribonucleic acid

EPOS – European position paper on rhinosinusitis and nasal polyps

EPS – Extracellular polymeric substance

ESS – Endoscopic sinus surgery

FESS – Functional endoscopic sinus surgery

ICAR - International Consensus Statement on Allergy and Rhinology

IL - Interleukins

MIC – Molecular imaging center

NP – Nasal polyps

PGE2 – Prostaglandin E2

PMT – Photo multiplier tube

PTC – Phenylthiocarbamide

SNOT-20 – Sinonasal outcome test (20 questions)

TH2 – T helper cell 2

VAS – Visual analogue scale

You can't depend on your eyes when your imagination is out of focus.

- Mark Twain

1. Aims of the thesis

In this thesis we wanted to explore the clinical significance of biofilms in the pathogenesis of chronic rhinosinusitis (CRS).

Specifically, we set the following aims for our papers.

Paper 1: Investigate the prevalence of biofilms in patients with CRS compared to controls.

Paper 2: Examine the differences between the prevalence of biofilms in CRSsNP versus CRSwNP, and biofilm distribution within the nasal cavity.

Paper 3: Explore the clinical and paraclinical data to more fully describe our population and determine if there are clinical differences between patients with biofilms compared to those without.

2. Introduction

Chronic rhinosinusitis is one of the most prevalent chronic infections in industrialized countries today (1-3). It is characterized by inflammation of the sinonasal mucosa, the mucosa of the nose and sinuses, but its pathogenesis is poorly understood (4-6). As a result, the treatment of CRS is still unspecific, with nasal corticosteroid treatment and saline solution the mainstay of medical treatment (7). Surgery, in the form of functional endoscopic sinus surgery (FESS), or endoscopic sinus surgery (ESS) is reserved for the cases where medical treatment fails. Up to 25% of patients submitted to surgery fail to achieve symptom control (8, 9). When we started this project, a few authors started to report the presence of bacterial biofilm in the sinonasal mucosa in a high percentage of patients with CRS as opposed to healthy controls, implicating biofilms in the development of CRS (10-13). Biofilms in humans are recognized as the culprit behind a diverse array of chronic infectious diseases, including infectious endocarditis, prosthetic joints and dental caries (14-17).

Biofilms consist of bacterial colonies, often several species, but may also consist of fungi. The colonies are protected by an extracellular polymeric substance (EPS) (18, 19), consisting mainly of carbohydrates and proteins, which in large colonies are responsible for its slimy appearance (13). Bacteria within the biofilm establish gradients for nutrients and oxygen, and communicate by secreting peptides, a process called quorum sensing (20-22). Bacteria also exchange DNA, notably resistance factors to different antibiotics. These factors contribute to the resilience of biofilms, making them hard to eradicate (23-25).

These characteristics made us decide to investigate biofilms and their association with CRS in this thesis.

3. Biofilms

The traditional understanding of bacteria is of free, planktonic bacteria. Free agents moving about in the biosphere. This is a grave oversimplification.

William J. Costerton “the father of biofilm”, amongst a few other colleagues realized that the “planktonic dogma” of bacteria was wrong (26). This discovery was made in the 1970s and made possible by the development of more powerful microscopes. Costerton spent much of his career to alert the medical community of the importance of biofilms and managed to put “the biofilm problem” on the agenda in most medical specialties.

Rapidly approaching the end of the (short) antibiotic era, our understanding of the bacterial life cycle has increased dramatically. Today we realize that bacteria have the capability to switch between the free, planktonic form, and the sessile, colony form (biofilm), but there is a lot we still don’t understand about this dynamism, and what actually takes place within a biofilm, especially in vivo (27).

Biofilms can consist of several species of bacteria, and often include fungi. A biofilm is characterized by surface bound microbes surrounded by a slimy coating, the extracellular polymeric substance (EPS). This is mainly composed of polysaccharides, proteins and water, and protects against external environmental factors. In vivo examples would be our immune system and antibiotics (18, 19).

The earliest fossil biofilms found to date, in the Dresser formation in Australia, are approximately 3,5 billion years old. Recently even more ancient fossils are reported in Greenland, dating approximately 3,7 billion years back (28). Ample time for evolution to fine-tune the bacteria’s survival mechanisms.

3.1 Biofilm prevalence

3.1.1 in nature

The predominant form of bacterial existence in nature is the biofilm form. Estimates suggest that at any given point in time, more than 99% of bacteria exist in biofilms (29-31).

3.1.2 in human disease

The Centers for Disease Control and Prevention (CDC), estimate that up to 65% of human bacterial infections involve biofilms (32). These include most chronic infections. E.g. infections on indwelling medical devices, dental plaque, urinary tract infections, chronic wound infections and infective endocarditis (14-16, 33-36). In otolaryngology, infections on ear ventilation tubes, cochlear implants and tracheal tubes, otitis media and chronic tonsillitis in addition to chronic rhinosinusitis are thought to be, at least partially, biofilm mediated (5, 6, 37-43). Important bacteria involved in human biofilms are Staphylococci, *Pseudomonas aeruginosa*, Streptococci and *Escherichia Coli* (17, 33, 44-51). The most important fungus involved in biofilm formation is *Candida Albicans* (52, 53).

3.2 Biofilm properties

A proper understanding of the features of biofilms is essential in order to develop effective treatments. The extreme adaptability of biofilms is a cause for concern.

3.2.1 Cycle

Figure 1. Biofilm life cycle. From Monroe (54), licensed under the Creative Commons Attribution 2.5 license.

Stage 1, initial attachment; stage 2, irreversible attachment; stage 3, maturation I; stage 4, maturation II; stage 5, dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to same scale (54).

Figure 1 shows the major steps in the cycle of a biofilm. This is based on an idealized pseudomonas model, and not all biofilms go through the same steps.

- 1) Bacteria settles reversibly on a host surface. If they are not displaced by mucociliary clearance, or attacked by the immune system they shift their phenotype by changing their gene expression (55-58) and
- 2) binds irreversibly to the host surface. The bacteria accomplish this by producing exopolysaccharides that binds with surface materials and/or receptor-specific ligands located on pili, fimbriae, and fibrillae, or both. At the conclusion of the second stage, adhesion becomes irreversible in the absence of physical or chemical intervention (59-65).
- 3) The colony starts to mature. Extracellular polymeric substance is produced to surround and protect the colony.
- 4) The colony start to mature fully. It often approximates a mushroom shape which increases the volume of the colony with proper oxygen tension. Canals are established in the EPS for communication between the bacteria and/or fungal cells. These canals also transport oxygen, nutrients and waste. The colony has assumed many of the characteristics of a superorganism (66-68).
- 5) The colony reaches "critical mass". It gets too large and some of the microbes receive insufficient oxygen and nutrition. That prompts some of the microbes to switch back to planktonic form and detach from the colony. They will be able to start new biofilm colonies elsewhere. Other microbes will enter a dormant phase. Due to physical shear forces, some bacteria, still in biofilm form, may also slough from the colony (69-72).

3.2.2 Communication

The ability of the members of the biofilm colony to communicate with each other gives rise to some of the most troubling properties of a biofilm. They communicate with different signal molecules, e.g. oligopeptides and acyl homoserine lactones (AHL). This process can occur between bacteria within the same species but also between different species and regulates the gene expression of the bacteria. This process is known as quorum sensing and allows bacteria in the same area to coordinate their behavior. A differing proportion of the bacteria's genome is controlled by auto-inducers, the particles responsible for quorum sensing. When the molecules reach a threshold the specific gene or set of genes are activated or deactivated. This allows for relatively advanced control within the biofilm (20, 73-76).

Recently, a potentially very important discovery suggests that long range signaling between separate biofilm colonies are possible as well. This is mediated through potassium ion-channel electrical signaling (77-79).

3.3 Biofilm adaptability

Several of the factors mentioned above, in combination, contribute to the remarkable adaptability of biofilms.

3.3.1 Resistance to environmental stress

Biofilms have many specific mechanisms in dealing with different environmental stresses, but in the end their genetic diversity, incorporating different species, is the key factor to their survival. Being unable to reproduce sexually, they have specialized genes to cause random recombination in others (80-82).

3.3.2 Resistance to antibiotic agents

The emergence of completely resistant bacteria is a specter looming large. If no breakthroughs in pharmacology is forthcoming, the post-antibiotic era might be upon us in years.

Several attributes of biofilms contribute to their often-remarkable resistance to antibiotics.

A key point is that all current antibiotics are developed for bacteria in planktonic form. The phenotypic shift when they enter biofilm form, removes many of the targets the different antibiotics attack. The expressed genes between the two forms differs with up to 70% (24, 83, 84).

The EPS offers significant protection for bacteria in biofilm form. Antibiotic agents struggle to penetrate the matrix, reaching the cells. Studies has shown different penetration rates. Beta-lactam antibiotics has been shown to penetrate better through alginate matrix, and rifampicin, clindamycin and macrolides penetrated *Staphylococcus epidermidis* EPS better than vancomycin and teicoplanin (85-89).

In a biofilm, the microbes are sessile and generally less metabolically active than in planktonic form. This makes them less vulnerable to antibiotics, especially antibiotics targeting different metabolic pathways (90-95).

Other defensive mechanisms are the ability within a biofilm to exchange DNA carrying resistance factors, usually through the exchange of plasmids. Finally, we have the dormant bacteria, which where unsuitably placed in the original biofilm, receiving inadequate oxygen and nutrients. As bacteria above them dies and are shed off, they exit dormancy and becomes metabolically active

once more. If the course of antibiotics is not sufficiently prolonged, these bacteria will be able to rapidly rebuild the biofilm. This is an important mechanism for residual infections (67, 96-99). This also demonstrates the biofilms increased tolerance to antibiotics, e.g. not an irreversible resistance, but increased ability to tolerate antibiotics as pointed out by Snderholm et al. (100).

3.4 Lab cultured biofilm vs natural in vivo biofilm

Most of the experimental work so far on biofilms has been conducted on model biofilms in vitro. As mentioned above, especially *P. aeruginosa* in monoculture has been used. The reason for this is of course time, money and convenience.

These last years, unfortunately, experiments on animal models have shown that this is not always applicable to the conditions in vivo.

Again, the biofilms adaptability is thought to be the reason for the markedly different properties of biofilms in vitro and in vivo. For one, in vivo biofilms are exposed to a different environmental stressor than in vitro, prompting different gene expression. Another important reason is that the biofilm properties varies according to the specific bacteria inhabiting the colony. In vivo biofilms also often consist of several different species of bacteria and sometimes fungi, giving the biofilm unique properties.

As a result of this, more experiments are conducted on in vivo conditions in animal models. Strains of bacteria shown to be clinically relevant are also increasingly used (11, 101).

3.5 Biofilms in chronic rhinosinusitis

The pathogenesis of chronic rhinosinusitis is complex, and it is probably a multifactorial disease.

However, many studies over the last several years has documented the presence of biofilm in patients with CRS, indicating that biofilm likely are one of the causes of CRS (12, 13, 102-112).

Staphylococcus aureus biofilms seem to be the dominant type of biofilm in CRS. Interestingly, there are studies showing that uptake of *staphylococcus* intracellularly combined with the presence of biofilms indicate a poorer prognosis. Intracellular *Staphylococcus aureus* seems to be able to evade an immune system response. Biofilms in CRS also causes destruction of the ciliary clearance system (19, 91, 113-116).

Exciting new research have identified taste receptors, bitter and sweet, in the airways. Research indicates that deficiency in the bitter taste receptor T2R38, expressed in sinonasal ciliated cells, may disrupt the host's ability to detect quorum-sensing molecules, failing to launch a response from the innate immune system, leading to biofilm formation and refractory sinusitis. This receptor has been shown to have distinct genetic polymorphisms correlating with taste sensitivity to the bitter molecule phenylthiocarbamide (PTC), making it possible to identify patients at risk with a tasting test (107, 117).

While this research is new, it sheds light on vulnerability factors, explaining why some patients are more susceptible to biofilm infection than others.

4. Chronic rhinosinusitis

Chronic rhinosinusitis is a multifactorial disease, the hallmark of which is inflammation of the sinonasal mucosa. There exist several conditions with reduced ciliary function that greatly predisposes to CRS, as do aspirin sensitivity, immunodeficiency etc. (See below for predisposing factors). Among “regular” CRS, the subject of this thesis, two main groups are recognized. Patients with nasal polyps (CRSwNP) and without (CRSsNP) (5, 6). CRS is one of the most prevalent chronic infectious diseases in the industrialized world (1, 118-122). The pathogenesis is still poorly understood, and the mainstays of treatment are unspecific. As a result of this, a relatively large proportion of patients, up to 25 % of the patients submitted to surgery, do not achieve symptom control (8, 9). Their quality of life can be severely decreased (123-125), and CRS represent a major socioeconomic burden (126-130).

4.1 Epidemiology

The data on epidemiology is uncertain, as different articles employ different criteria. The estimates based on medical history range between 5-15% in Europe and the US (1, 118). CRS diagnosed by a physician is significantly lower, between 2-4% (3, 131, 132). A major problem is that most of these patients are diagnosed by GPs without the equipment or training to perform nasal endoscopy, leading to over diagnosis (133). Females are found to have a higher prevalence than men, up to a 6/4 ratio. Peak prevalence is in the age group 50-59 year (2).

The prevalence and incidence of CRSwNP are even more uncertain as this diagnosis requires nasal endoscopy, except in the patients with the largest polyps visible by inspection. Studies in the US have found a prevalence of CRSwNP of about 4% (134). In a prospective study on the incidence of symptomatic NP, Larsen and Tos found an estimated incidence of 0.86 and 0.39 patients per thousand per year for males and females, respectively (135). The incidence increases with age (136, 137). Several papers, including Eskeland et al. 2017 (138), find a higher symptom load in patients with nasal polyps.

4.2 Predisposing factors

There are a lot of different factors established or being investigated as possibly relevant in the pathogenesis of CRS.

Ciliary dysfunction: Primary ciliary dysfunction, e.g. Kartagener, has a high prevalence of CRS and airways diseases due to the inability to clear mucus. Cystic fibrosis, with its highly viscous mucus, also impairs mucociliary clearance. Cystic fibrosis is associated with a high prevalence of nasal polyps (139, 140).

Allergy: Clinically it is important to control any allergies to achieve satisfactory results when treating CRS (141). Epidemiologically it has not been proven that there is a causal relationship between allergy and CRS. At this stage, the association between CRS and allergy is unclear (142-144).

Interestingly several authors have found increased prevalence of nasal polyps in the presence of food allergy (136, 145-147).

Asthma: See chapter 4.5 (below).

Allergic fungal rhinosinusitis (AFRS). This is characterized by the presence of eosinophilic mucin and fungal hyphae in the paranasal sinuses without invasion into surrounding mucosa. The fungus is considered the inflammatory driver. This is a debated entity and is also known as eosinophilic fungal rhinosinusitis and eosinophilic mucin rhinosinusitis (148). In immunocompromised patients, acute invasive fungal sinusitis is a life-threatening disease with invasion of neural and vascular structures, causing thrombosis. The mortality is between 50-80% (149).

Aspirin-sensitivity (Samters triad): This condition is one of the rare sub conditions within CRS which was one of the exclusion criteria in our study. It is characterized by the presence of nasal polyps, asthma and aspirin- intolerance. Between roughly 40 and 90% of patients with aspirin sensitivity have CRSwNP (134, 150).

Immunodeficiency: CRS is more common in conditions such as common variable immunodeficiency and specific IgA deficiency. It is important to screen for these kinds of immunodeficiency in patients with recalcitrant CRS (151). In patients with AIDS, low counts of CD4 positive cells has been correlated with the development of CRS. Diseases more specifically linked to AIDS, such as Kaposi's sarcoma and atypical microorganisms such as *Aspergillus* spp. may explain these cases of CRS (152, 153).

Anatomical factors: While most clinicians intuitively feel that some anatomical features, e.g. concha media bullosa and septal deviation, must predispose patients to CRS, no conclusive epidemiological data supports this. One of the reasons for this might be that there is no unified system for categorizing anatomical deviations (154-159).

Genetic factors: Except in the setting of secondary causes like Kartagener and others, there has not been found genetic factors predisposing to CRS before the discovery of bitter taste receptors in the airways, and their possible role in the innate immune system (see chapter 3.5).

Gastroesophageal reflux/Helicobacter pylori: The possible link between gastroesophageal reflux and CRS has been investigated in recent years. No causality has been found (160).

Environmental factors: Smoking is the only environmental agent found to be linked to CRS so far. Lower socioeconomic status is also linked to higher prevalence of CRS (161).

The fungal theory (“precursor” of AFRS): In part this theory was motivated by the fact that bacteria didn’t grow routinely on cultures from CRS patients. It was speculated that the sinus was sterile. Several studies found evidence of fungi in patients with CRS. Some authors proposed that CRS was caused by fungi alone. This theory was dominant for several years. Today this is modified to fungi playing a part in some cases of CRS (AFRS). The emergence of biofilms, which do not routinely grow on regular culture media, has explained the absence of bacterial findings in earlier years (162, 163).

Deficiency in the eicosanoid pathway (linked to aspirin intolerance) explains all cases of nasal polyps: This theory postulates that a metabolic defect in the eicosanoid pathway triggers elevated levels of leukotrienes and lowered levels of certain prostaglandins (PGE2). The net result is inflammation, and presumably development of nasal polyps. However, there has been limited clinical efficacy of leukotriene pathway inhibitors, weakening the support for this theory (164, 165).

Staphylococcal super antigen: It has been reported that intracellular Staphylococcal Aureus only exists in patients with CRSwNP. It is believed that these bacteria evade the immune system by residing intracellularly, triggering excretions of super antigen exotoxins from the host epithelial cell. This leads to Th2- skewing, and tissue damage and remodeling due to increased activation of eosinophil and mast cells, explaining the formation of nasal polyps. Unfortunately, super antigens can only be demonstrated in approximately half of the patients with CRSwNP (164, 166, 167).

Dysfunctional immune barrier: This theory more broadly states that a dysfunction in the mechanical barrier and innate local immune system in the sinonasal mucosa leads to CRS by allowing colonization of microbes (168-170).

Biofilms (see also chapter 3.5): These last years, a lot of studies, including ours, have documented the presence of biofilms in patients with CRS, indicating that biofilm likely are one of the causes of CRS. Staphylococcus aureus biofilms seem to be the dominant type of biofilm in CRS. Biofilms in CRS also causes destruction of the ciliary clearance system. The microenvironment in the deeper parts of the nose, with lower shearing forces is conducive to biofilm formation as demonstrated by our own research, where there was a significant increase in biofilms in the ethmoid bulla compared to the front of concha media. The low oxygen tension that prevails, in part due to polymorphonuclear leukocytes in the biofilms periphery, forces the bacteria in the biofilm into slow metabolism, which also protects them from most antibiotics targeting different metabolic processes (10, 12, 13, 100, 102-106, 108).

4.3 Diagnosis

We used the following definition of CRS, proposed by EPOS 2007 (5), in this study:

“Chronic rhinosinusitis, with or without nasal polyps in adults is defined as:

- inflammation of the nose and the paranasal sinuses

characterized by two or more symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip):

- ± facial pain/pressure
- ± reduction or loss of smell for ≥12 weeks

This should be supported by demonstrable disease. Either endoscopic signs of:

- nasal polyps, and/or
- mucopurulent discharge primarily from middle meatus

and/or

- edema/mucosal obstruction primarily in middle meatus

and/or

- CT changes:
- mucosal changes within the ostiomeatal complex and/or sinuses”

4.4 United airways

The patients with both CRS and asthma have suffered from the arguably artificial separation of the airways into two medical disciplines, namely otolaryngology and pulmonology. Most research has been conducted within one or the other of these medical specialties. The relatively new focus on united airways, has yielded several exciting discoveries. The two parts of the airways are obviously anatomically linked. It is also lined with pseudo-stratified respiratory epithelium, and both CRS and asthma display similar inflammatory characteristics (171-173). Initially it was believed that the link between these two diseases was linked to the bypass of the functions of the nose, namely warming and humidifying the air, and micro aspirations of sinonasal secretions (174). This post-nasal drip theory is uncertain in all but neurological patients with impaired laryngeal function, and the effects of micro-aspiration of nasal content is unclear (175).

The most exciting new research has found probable neural reflexes between the sinonasal mucosa and the bronchi. Sinonasal inflammation has also been shown to exert systemic inflammatory effects through the blood stream and bone marrow (176, 177).

Asthma and CRS is strongly associated. Up to 50% of patients with CRS have been reported to have clinical asthma (171, 178). The exact etiological relationship between asthma and CRS are not completely understood (179).

4.5 Treatment

The treatment of CRS today is based on a thorough review of the available literature by the European Rhinologic Society (EPOS 2012) (6) resulting in treatment recommendations. The paper also highlighted the scarcity of high-quality studies in this field, making recommendations difficult. Since then the International Consensus Statement on Allergy and Rhinology (ICAR) (180) was agreed upon in 2016 by an international expert group, also lamenting the lack of rigorous treatment studies. Recently Walker et al. (181) published an updated review on the treatment options for CRS in the British Medical Journal (BMJ).

The mainstay of treatment comprises:

1) Intranasal saline solution.

This is a low-cost treatment modality. The effect compared to placebo is small, but thorough rinsing can remove mucous and reduce swelling. It is recommended to both CRSsNP and CRSwNP(182-185).

2) Intranasal corticosteroid spray.

Topical steroids are recommended to all patients with CRS. It is demonstrably better than placebo, but no one agent or method of delivery is proven to be superior (186-190). Second-generation agents minimize systemic bioavailability and are recommended over first-generation (191).

3) Bacterial lysates.

Bacterial lysates (e.g. OM-85 BV, Broncho-Vaxom), containing lysates of *Haemophilus Influenza*, *Streptococcus Pneumonia*, *Klebsiella Pneumonia* and *Ozaenae*, *Staphylococcus Aureus*, *Streptococcus Pyogenes* and *Viridans* and *Neisseria Catarrhalis* may have a beneficial effect. They work by stimulating the immune system and may prevent or reduce the frequency of airway infection (192-194).

4) Oral corticosteroids.

Systemic steroids have an unclear role in the treatment of CRSsNP due to insufficient data (195, 196). They are in mainstream use in the treatment of CRSwNP and proven to alleviate symptoms. This treatment is coined “non-surgical polypectomy” and lasts up to six months according to available data (195). Low dose of 30-40 mg for short spans of time is important to alleviate the risk of serious complications. Osteonecrosis is reported even in these low doses and Leung et al. concluded that the risks exceeds benefits if used more frequently than every two years (197).

5) Macrolides.

Antibiotics are best documented for acute exacerbations. The role of antibiotics outside of

this is debated. Several studies have shown the anti-inflammatory and immune-modulating effects of macrolides in airway inflammation. Some studies show short time effects of treatment with macrolides for three months for CRSsNP (198-202).

6) Surgery.

Patients who have undergone appropriate medical therapy, usually at least a course of nasal steroids and saline solution, are considered candidates for surgery (203). Surgical approaches are called endoscopic sinus surgery, ESS. This ranges from balloon-plasty to extensive surgery removing or opening all cells in the sinus system. The complications range from brain damage and blindness (both very rare) to CSF leaks and postoperative bleeding. There is evidence suggesting surgery in early stages of disease promises better results than late-stage (204). The principle guiding surgery is to open congested drainage paths from the sinuses. Regardless of the chosen surgical approach there is a high rate of revision surgery (205). Furthermore, there is a debate concerned with how aggressively the surgery should be performed. This is especially true in the case of CRSwNP where there is a high rate of relapse (6, 206, 207).

As apparent from the above the treatment options for CRS are limited. This reflects our incomplete understanding of the pathophysiology of the disease. A “revolutionary” treatment is still sorely missed, especially for the patients suffering from serious recalcitrant sinusitis.

Four exciting newer trials are:

Anti-IL-4Ra. IL-4 plays a role in Th2 cell-mediated inflammation, which is associated with CRSwNP, and possibly also potentiate the immune response in potentiate the immune response of fibroblasts in CRS (208-211). Recently dupilumab, an inhibitor of IL-4 and IL-13, was shown to significantly reduce polyp size and improve SNOT-22 compared to placebo (212).

Calcium channel blockers. A single study has tested the calcium blocker verapamil on patients with CRSwNP. Verapamil has been shown in other studies to possible have an immunomodulatory effect in addition to its established role in cardiology (213, 214). In the study with CRSwNP verapamil was shown to inhibit the secretion of IL-5, IL-6 and thymic stromal lymphopoietin comparable to dexamethasone (215).

Ivacaftor. This is a cystic fibrosis transmembrane conductance regulator potentiator. Cho et al. found a sinus stent covered with Ivacaftor and Ciprofloxacin to be effective against a *Pseudomonas* biofilm during in vitro studies. Ivacaftor has a synergistic bactericidal effect with Ciprofloxacin and also promotes sinus mucociliary clearance. The group is planning preclinical trials (216, 217).

Topical Bacteriophages. The Wormald group has tried topical bacteriophages in a mix they call CT-PA on a sheep CRS model with Pseudomonas biofilm. Bacteriophages are viruses that infect and kill bacteria. The group found that CT-PA was safe and effective against the Pseudomonas biofilm (218). They have also conducted a small experiment on patients with another bacteriophage mix, AB-SA01. These patients had recalcitrant CRS with a Staphylococcus Aureus biofilm. The treatment was deemed safe and showed improved and with 2 of 9 possible cured (219).

5. Materials and methods

A cross-sectional study was conducted from 2010 to 2012 in Akershus University Hospital (AHUS), Norway. Eighty-six consecutive patients undergoing endonasal surgery were included. Sixty-one patients with CRS undergoing functional endoscopic sinus surgery (FESS) as the experimental group, and 25 patients undergoing septoplasty without CRS as controls. The study was approved by the hospital science board and the Regional Ethics Committee (reference number 2009/1720b).

5.1 Study design

We wanted to investigate the maximum number of patients within the time constraints of a PhD project, and the economic constraints of a hospital setting. We chose a cross-sectional design.

When we planned this study there were no other articles that had investigated biofilms in CRS vs septoplasty controls (Bezerra et. al (104) were published in 2011), and no papers investigating biofilm prevalence within different locations in the sinonasal cavity. This made it impossible to calculate statistical strength, and we had to set sample targets based on available time and resources.

5.2 Inclusion and exclusion criteria

The inclusion and exclusion criteria of this study are summarized in table 1 below. We sought to include the most common form of rhinosinusitis, excluding patients with immune deficiency, ciliary dysfunction etc.

CRS group included	CRS group excluded	Controls included	Controls excluded
Primary FESS	Pregnant	Septal deviation and/or concha media bullosa requiring surgery	Same as CRS group plus septal perforation
Bilateral disease	Immunodeficiency	Above 18 years old	
Above 18 years old	Reduced mucociliary clearance (e.g. Kartagener)	Lund-Mackay CT score of zero	
	Antibiotics within two weeks of surgery		
	Non-invasive fungal balls or invasive fungal disease		
	Systemic vasculitis		
	Granulomatous disease		
	Cocaine abuse		
	Neoplasm		
	Aspirin exacerbated respiratory disease		

Table 1. Inclusion and exclusion criteria

5.3 Parameters examined

Medical and surgical history, specifically regarding the use of nasal and systemic steroids, smoking, asthma and allergy were obtained. CT scan was performed on all subjects, including controls. The examinations were scored according to the Lund-Mackay CT- score system (220). Biopsies were obtained from all subjects, see below. They completed the SNOT-20 questionnaire and a VAS scale of symptom severity.

5.4 CT scan and Lund Mackay

We used the Lund Mackay CT score (220), seen below. This score has been shown to be relatively reproducible between researchers. However, the value of CT scans to evaluate the severity of CRS has been debated. Several studies have shown that healthy subjects have had significant opacification in their sinuses on CT scans. CT scans are however invaluable in the planning of surgery.

Lund-Mackay CT scan assessment
Paranasal sinuses
Maxillary (0, 1, 2)
Anterior ethmoid (0, 1, 2)
Posterior ethmoid (0, 1, 2)
Sphenoid (0, 1, 2)
Frontal (0, 1, 2)
Ostiomeatal complex (0, 2)*
Total
0 - With no abnormalities
1 - Partial opacification
2 - Total opacification

* 0: Without obstruction; 2: Obstructed.

Table 2. The Lund Mackay CT score form

5.5 Biopsies

Tissue samples were collected from every included patient perioperatively. This was done by, or under guidance from, experienced ENT surgeons. Small mucosa flaps were harvested, and the wound sites were covered with an antibiotic dressing (Terra-Cortril “Pfizer”, US). Every patient was in full anesthesia, and no post-operative infections related to the biopsies were reported. Apart from the biopsies the operations proceeded as normal.

5.5.1 Collection

In the CRS group, tissue samples were harvested from the anterior part of concha media, the uncinate process and the ethmoid bulla during primary sinus surgery. Mucosal samples in the control group were obtained from the anterior part of concha media. In the patients with nasal polyps, the polyp tissue was sent to routine check for atypia, and to our biobank. Tissue samples were immediately put on moist gauze and transported to the pathology department in clean plastic containers on ice separated by location.

5.5.2 Preparation

To obtain usable samples for microscopy, each biopsy was prepared at the pathology department. Bony tissue was removed, and the sample was washed three times in Millipore (MQ) water to remove any planktonic bacteria. This was deemed within the stress limits a biofilm should sustain.

5.5.3 Storing

After preparation, each sample was then snap frozen (isopentane cooled with dry ice) in a 1.8 ml cryotube and stored at -80 degrees Celsius in an ultra-freezer.

5.5.4 Incubation

At the day of laboratory analysis, samples were thawed at room temperature and washed three times in 25 ml MQ water (0.22 micrometer filter, EMD Millipore Corporation, Billerica, MA, USA) for 60 seconds. They were then transferred to Eppendorf tubes (Eppendorf AG, Hamburg, Germany) containing 1 ml MQ water. 1,5 µl component A (1.67 mM SYTO 9 nucleic acid stain, 1.67 mM propidium iodide solution in DMSO) and 1,5 µl component B (1.67 mM SYTO[®] 9 nucleic acid stain, 18.3 mM propidium iodide solution (Invitrogen's LIVE/DEAD[®] Backlight Bacterial Viability Kit,

Invitrogen, Burlington, Canada) followed by 10 seconds of stirring (IKA MS3 Digital, VWR International, Radnor, PA, USA). The reagents were incubated for 15 minutes in darkness on a nutating mixer (VWR International).

After incubation, the samples were washed three times in 25 ml MQ water. Prior to mounting, the tissue biopsies were squeezed gently between two microscope slides (ELKA, Karl Hecht GmbH & Co KG „Assistent“, Sondheim / Rhön, Germany). They were then mounted on a microscope slide with a drop of mounting oil (component C, Invitrogen`s LIVE/DEAD® Backlight Bacterial Viability Kit) and fitted with a cover slip (20x20 mm, 12 mm thick, VWR International).

After two to three days, visible decomposition had started and after one week the samples were rendered unusable. The fluorescent dyes were stable up to one week after staining, thus tissue decomposition was the limiting factor (findings from our pilot study, see below). As a result of this all our biopsies underwent same-day, or at the latest, next-day microscopy. We stored the samples in a dark, cold room. It is important to note that any bacterial growth occurring after preparation of the tissue will not be visible in the confocal microscopy, as only fluorescently marked tissue will light up during microscopy.

5.6 Microscopy

Confocal scanning laser microscopy was performed with an upright Leica TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany). Oil immersion lenses x40 and x63 were used. The examiner (this author) was blinded to patient identity and disease status. First the samples were scanned in the fluorescence mode, and then switched to confocal mode. Z-stacks were obtained in areas with possible biofilm. For the confocal part, the Argon laser 488 nm laser line was used. The strength was set to 15% and PMT1 was set to 554, PMT2 was set to 517. The strength and reception settings depend on the age of the laser and the thickness of the biopsy. Thus to different confocal labs may need slightly different values. Biofilms were scored when clusters of dots (presumed bacteria) of the right size, with intact membranes (green color), were present in both the x-y plane and in continuous exposures along the z-axis. Typical sizes of a single bacterium are 0,2 to 2 microns in diameter for spherical species, and 1 to 10 microns in length for nonspherical species. Z-stacks were useful to visualize the three-dimensional properties of the biofilms. With samples that this author found ambiguous regarding the presence of biofilm, the whole group was consulted. During these discussions the whole group were of course blinded to biopsy site and disease status of the patient.

One of the challenges was separating artefacts from cellular and bacterial structures. Tilting artefacts, autofluorescence and superfluous dye were among the most prominent. Most of these were relatively easily resolved by scanning along the z-axis and excluding areas that were homogenously dyed from investigation. Tilting artefacts had the potential to ruin the biopsy, necessitating painstakingly preparation of the slides, to make them flat, avoiding this pitfall. Most samples were subjected to microscopy the same day as they were prepared in the lab. Two sets of samples were subjected to microscopy the day after preparation. We determined in the pilot that this was acceptable.

Figure 2. A biofilm positive tissue sample. The epithelial cells in red, with larger nuclei. The bacteria as green dots with significantly smaller nuclei. Scanning in the z-axis, the observer can get an impression of the three-dimensional structure of a biofilm.

Figure 3. A biofilm negative tissue sample. The epithelial cells in red. This view contains just a few bacteria in the lower left corner. Not enough to be scored as a biofilm. Here we get a clearer impression of three-dimensionality, when looking at the nuclei of the epithelial cells.

During each session of incubation and microscopy we used a control sample not related to the study as an indication of the quality of fluorescence. In addition, we scored each sample on the quality of incubation as follows:

++++ = Very good quality, almost no background color, easy to distinguish cells and tissue structures.

+++ = Good quality, some background color. Good result.

++ = Mediocre quality, some background color, harder to distinguish tissue and cells.

+ = Poor quality, not suitable for examination

Scores such as these are inherently prone to subjectivity. The most important distinction was, however, between + (unusable sample) and ++ (usable, but suboptimal). It was easier to score these two consistently than the difference between +++ and +++, but this difference is not as critical as between the two lowest scores.

5.6.1 Principles of confocal microscopy

A confocal microscope can be thought of a specialized version of a fluorescence microscope.

Figure 4. The author and the confocal laser scanning microscope at Akershus University Hospital (AHUS, Lørenskog, Norway).

The basic principle of a confocal optical system is to restrict the light beams reaching the image sensor to only detect light at the focused position. This is done by having a pinhole opening at the position that corresponds with the focal position of the objective lens (see figure 1 below).

The light source is a point light (usually laser light, as in our application) that is focused on a specific point in the sample, as opposed to an ordinary light microscope, where the whole sample is more evenly illuminated. This reduces background scatter and improves contrast.

Figure 5. Image from the Olympus website (221) illustrating the principle of confocal microscopy.

Even more importantly, it allows for scanning in three planes. Because the focus is so precise, and there is no superimposition along the depth axis, z-axis, the examiner can scan a sample in three dimensions.

This allows for the acquisition of z-stacks, a series including the same x-y square, but from different depths in the sample. The only spatial variable is the depth value. These frames can then be viewed as short films, to get a better understanding of the three-dimensional attributes of a sample.

With a laser scanning confocal microscope, as in our application, it is necessary to incubate the sample with fluorescent molecules to see anything (excluding the materials which autofluoresces, e.g. oil). Finally, the laser light, which has distinct wave lines, as our chosen Argon laser line at 488 nm, must be tailored to the excitation spectra of the chosen fluorescent probes. The emission spectra of the probes must not have too much of an overlap (more on this in the section on our pilot) (221).

Finally, it is important to fine tune the strength of the laser light and the strength of the “receivers”, photomultiplier tubes (PMTs) to optimize the image capture. If you have too high laser strength you induce photo bleaching, rendering the sample unusable as you get no additional fluorescence on repeat scans of the same area. Too low you get no excitation and no image. The PMTs, if set too low, will give no signal, and therefore no image, if set too high, you get too much noise.

5.6.2 Confocal microscopy and biofilms

For the specific investigation of biofilms and CRS, Confocal laser scanning microscopy has been shown to be the gold standard compared to electron microscopy and ordinary light microscopy (11)

The reason for this is the ability to scan in three dimensions, and the ability to incubate the samples with probes, like in our study, which separate live from dead cells, thus making use of the extraordinary resilience bacteria have in biofilm form. You cannot scan into the sample with a scanning electron microscope, it just shows the surface. Ordinary light microscopy has the same shortcomings in addition to lower resolution (11).

Another very useful application of the confocal microscope in the setting of biofilms are the scanning disc confocal laser microscope with an incubation chamber. This type of microscope can obtain scans on distinct times, allowing time-lapse series. This allows the researcher to perform experiments with biofilm models in vitro. One application is to examine the effect of different antibiotics on model biofilms.

5.7 Pilot study

This study was the first clinical study performed at the ENT department, Akershus University Hospital. We wanted to investigate the early claims that biofilms were part of the pathogenesis of CRS. To accomplish this, we had to decide on a protocol to detect biofilms, and test this. We also had to establish a protocol for collection of clinical and paraclinical data, and the whole department became involved in this. We also depended on help from the pathology department to handle the biopsies and established a very productive cooperation with them.

The most challenging part was going to be the detection of biofilm. After researching the relevant publications, we decided that a protocol developed by the Wormald group in Adelaide, Australia showed most promise. They had developed a protocol where the samples were collected and frozen for later microscopy and had shown that this gave satisfactory results compared to immediate microscopy. They used confocal microscopy with samples incubated with the LIVE/DEAD kit to detect biofilms (12). The possibility to delay microscopy was essential for us to make the study feasible (10). We corresponded with the Wormald group, and got several useful tips, amongst others to be very particular about the preparation of the biopsies, as any bone left would lead to an uneven surface for microscopy. This would lead to major artefacts and render the sample useless.

We had access to great facilities at EpiGen, the research block at AHUS, which also contained a laser scanning confocal microscope. This author was tasked with performing the microscopy. There was not sufficient expertise to help us with our application in-house, so I made use of the expertise at MIC, Molecular imaging center, which is the core facility for confocal microscopy in Norway. They are situated at Haukeland University Hospital in Bergen. They helped with the necessary training for our application. I also depended on them later. We started to test out the staining procedure in conjunction with microscopy to establish a firm protocol for our study. One of the first issues was the auto fluorescence in our set up.

Figure 6. Autofluorescence from our mounting oil, component C of the Baclight Kit, which fluoresces in the green spectrum. Picture from the pilot. The other components gave no autofluorescence. This was done to ensure there was no autofluorescence artefacts that could be interpreted as bacteria. For biofilm positive scan, see above. This autofluorescence was only visible in the fluorescence application and did not light up under confocal microscopy.

There are a host of commercial incubation agents which are geared towards investigating different parts of the cell. Our kit, Invitrogen's LIVE/DEAD® Baclight Bacterial Viability Kit (Invitrogen, Burlington, Canada) consist two nucleus stains, SYTO9, which colors the nucleus green, and Propidium iodide, which colors the nucleus red. SYTO9 easily penetrates the cell membrane, always staining the nucleus green, while the propidium iodide only does this if the cell membrane is compromised. If both enters the nucleus, propidium iodide will dominate, i.e. it will be red. Thus, it colors the nucleus green if the membrane is intact and red if it is compromised.

To achieve an image, we also had to decide on a suitable laser line which suited the excitation

spectra of our kit. Using the Fluorescence Spectra viewer tool available online (<https://www.thermofisher.com/no/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html?ICID=svtool&UID=1304dna>) we decided on the following setup:

Figure 7. Screen export from the Spectra viewer with our setup. Propriumiodid is the orange line while SYTO9 is the blue line. The dotted lines are the excitation spectra's, and the solid lines are the emission specters. The solid vertical line is our excitation laser.

With the 488-nm laser line of the Argon laser, we would be close to peak excitation for SYTO9, which were the weakest by far. The relative low excitation intensity of propriumiodide we achieved with the 488 wavelengths was more than enough to balance the SYTO9.

Finally, we determined the optimal strength of the laser and the values of the photomultiplier tubes (PMTs) to achieve the best image quality possible without excessive photo bleaching.

The lab work with the incubation was fairly straightforward. The biggest challenge was optimal preparation of the biopsies in order to mount them flat.

Another essential element to investigate was how long the biopsies could be stored after incubation. The mounted slides were stored in a dark storage room at -20 degrees C. The fluorescent dyes were stable at least a week after incubation. The limiting factor proved to be tissue decomposition. One day of storage showed no visible changes. Two days showed minor changes, and after three days the samples were unusable. We therefore decided that the samples had to be subjected to microscopy the same day or the day after.

5.8 Protocol

Based on our experiences from the pilot study, we developed the following protocols.

Lab:

The samples were thawed, and the vials marked with tissue ID. The samples were checked for bone residue, which were removed. They were rinsed and 1,5 ul component A and B from the Live-Dead kit were added with 1 ml distilled water. The contents of the vial were mixed on a shaker for 10 seconds, and then incubated in darkness on a rocker. The sample was then rinsed again and mounted with two drops of mounting oil on a microscopy slide.

Confocal microscopy:

The microscope with fans and argon laser was turned on. The strength was set to nine, the 488-nm laser line was set to 15%, gain on PMT1 to 554 and PMT2 to 517. The fluorescence mode with 40x oil lens was used for preliminary investigation. In areas suspect for biofilm, the confocal mode was used. Single scan was chosen, and for interesting images, a format of 2048x2048 with averaging set to two was chosen. It was often necessary to increase the laser strength to 11-12 and adjust the PMTs to optimize the image. For Z-stacks, a format of 512x512 with averaging set to one was chosen. Mode was set to continuous. The area was scanned along the z axis and a start and end area was set. The number of images was chosen, usually around 30. Format for capture was set to 1024x1024 and series was chosen. The capture was exported as AVI. The experiment was named after the current date, saved and the equipment turned off.

5.9 Statistics

The data presented in the first two papers of this study were analyzed with SPSS 19 (IBM Corp. SPSS, Armonk, NY, USA). We used Chi-square with continuity correction and odds ratio, when comparing dichotomous data, and student's t-test for continuous data. For all results a two-sided significance level of 5% and 95% confidence interval were used.

In the third paper we used SPSS 23. One-way analysis of variance, ANOVA, was used to compare biofilm formation, Lund-Mackay CT score, SNOT-20 and its sub score between the CRSsNP, CRSwNP and the septoplasty patients. Student's t-test was used to assess the significance of the mean differences for time to referral, VAS, Lund-Mackay CT score, SNOT-20 and its sub score between CRSsNP and CRSwNP. Pearson correlation was used to evaluate the correlation between Lund Mackey CT score and SNOT-20/VAS, and correlation between SNOT-20 and VAS.

In the following I want to present the different statistical methods and terms, and why they are used.

Significance: Statistical significance is a more precise term. In statistical hypothesis testing we often want to find out if a given experimental result is meaningful or not. This is done by formulating a conservative null hypothesis and testing it. In this work one of those questions was if x percent of the subjects in the control group biofilm were positive versus y percent in the experimental group reflected a probable difference. The null hypothesis is then that the differing results is just chance; it does not reflect an actual difference between the two groups. A significance level is then decided (beforehand) where the null hypothesis is rejected, e.g. the observed difference is likely to reflect a real difference between the groups. If you use two-sided significance you accept that the difference can go either way and to get significant results, they must be on half the significance level in either extreme before we reject the null hypothesis. In medical research a 5% significance level is often chosen, and we followed that convention in this work (222).

Confidence interval: This is a given interval where one, based on observed data, expects to find a new, unknown observation. If we set the confidence interval to 95%, we expect to have a 95% chance of any new observations to fall within this interval. As with significance level, the value of 95% chosen is based on convention (223).

Student's t-test: A test that can be used to determine if two sets of data are significantly different from each other. It was introduced in 1908 by William Sealy Gosset who found it to be a convenient way to monitor the quality of Stout in the Guinness Brewery, Dublin (224). He used the pen name Student, hence the name of the test. The application in these papers were as a two-sample location

test where the conservative hypothesis states that the means of the groups compared are equal. The main assumptions when using this test is that the data are continuous and that they follow a normal distribution. The test is not suitable if data are dichotomous, e.g. yes or no. When sample size increases and two-sample location test is used the test is relative robust regarding deviation from the assumption of normal distribution (225).

Chi-square: This test is non-parametric and can be used to analyze group differences in dichotomous (yes-no, up-down) data. The full name of the test is Pearson Chi-square test. It can test categorical variables like gender and is a significance test. It measures variances between two groups (226). We used 2 X 2 tables when comparing groups and used Yates continuity correction. This is a correction based on sample size divided by two, and in 2 X 2 tables are thought to give more exact results (227).

Odds-ratio: Odds is related to probability in statistics. Odds deals with binary outcomes. The difference is that odds is ratio between studied/wanted outcome divided by other outcome while probability is the studied/wanted outcome divided by all other outcomes. Probability will therefore always be lower than its related odds. In this study we used odds-ratio to compare two groups with binary outcomes (asthma yes-no). This is simply dividing the odds of asthma in one group by asthma in the other group. This was then compared with the 95% confidence interval, see above, for the odds ratios (228).

ANOVA: This is a one-way analysis of variance. It is based on the same assumptions as the t-tests, see above, but is utilized when there are several groups to compare. It uses variance instead of means to reduce error when comparing multiple groups (229).

6. Summary of results

6.1 Paper I

The aim of this study was to establish a reliable protocol for detection of biofilm using confocal microscopy. An extensive pilot phase led up to this study. We wanted to investigate any differences in occurrence of biofilm in patients with CRS and controls.

The total number of patients in the CRS group was 61, 23 females and 38 males, and median age was 40 years. There were 25 individuals in the control group, 8 females and 17 males with a median age of 40 years. Patients within the CRS group had significantly higher occurrences of allergy (50,9% vs. 17,4%, $p = 0.006$) and asthma (34.0% vs. 8.7%, $p = 0.022$) than the controls. In our material, there was a higher rate of current smokers in the control group (34,0% vs. 8.7%, $p = 0.014$).

Bacterial biofilms were detected in 90% of patients with CRS, significantly more prevalent than controls, in which 56% were biofilm positive ($p < 0.001$, chi-square score 13.1) (see table 3).

	Controls	CRS Group	p-value	Chi-square and odds ratio
No. of patients	25	61		
Age; Median	40	40		
Male	17	38		
Female	8	23		
Smokers	34.8 %	11.1 %	($p = 0.014$)	Chi Square 6.1, OR 0.23 (0.07-0.78)
Allergy	17.4%	50.9 %	($p = 0.006$)	Chi Square 7.5, OR 4.9 (1.5-16.5)
Asthma	8.7%	34.0%	($p = 0.022$)	Chi Square 5.3, OR 5.4 (1.1-25.6)
Biofilm positive	56.0%	90.2%	($p < 0.001$)	Chi Square 13.1, OR 7.2 (2.3-22.9)

Table 3. The demographic data and results in paper 1

Figure 8. A montage showing the different images generated in a z-stack (depth). This gives a feeling of the dynamism along the depth axis. Top left – most superficial. Depth denoted in the bottom right of each image. Red areas are the nuclei of epithelial cells with compromised membranes, and the green small dots are bacteria with intact membranes.

6.2 Paper II

This study is based on the same patients as in article one, see above for demographic data, but here we examined additional biopsies to stratify the findings of biofilm according to localization within the nasal cavity. We also split the experimental group according to the presence or absence of nasal polyps.

Twenty-seven of the patients in the experimental group had CRSsNP, and the rest, 34, had CRSwNP.

Bacterial biofilms were detected in 97.1% of patients with CRSwNP, 81.5% of patients with CRSsNP, and 56% of controls. Patients with CRSwNP had highly significantly increased prevalence of biofilms compared to controls ($p=0.001$, Chi-Square=15.0). The increased prevalence of biofilms in patients with CRSsNP compared to controls was also significant ($p=0.047$, Chi-Square=3.96). Patients with CRSwNP also had significantly higher prevalence of biofilms compared to CRSsNP ($p=0.042$, Chi-Square=4.12). The prevalence of biofilms in different anatomical locations within the nasal cavity differed. In total, 159 samples from patients with CRSsNP and CRSwNP were collected from the middle turbinate, uncinate process, and ethmoid bulla.

Biofilms were detected in 79.6% of the samples from the ethmoid bulla, 70.9% of the samples from the uncinate process, and 62.0% of the samples from the middle turbinate. Biofilms were significantly more prevalent on the ethmoid bulla compared to the middle turbinate ($p=0.047$, Chi-Square=3.93). The difference in biofilm prevalence between the other locations was non-significant.

Figure 9. The point prevalence of biofilm in the three groups

Figure 10. The point prevalence of biofilms in the three localizations tested.

6.3 Paper III

This paper is also based on the same patients included in article one and two. The focus here was the clinical parameters in our study.

Patients with CRSsNP and CRSwNP had significantly higher degree of symptoms compared to the septoplasty group (SNOT-20: 39.8, 43.6 and 29.9, respectively, ANOVA $p=0.03$). There were no differences in the total SNOT-20 or VAS scores between the CRSsNP and CRSwNP groups (39.8 vs 43.6, T-Test $p=0.46$, and 63 vs 68.6, T-Test $p=0.27$, respectively). Regarding the SNOT questions related to nasal discomfort, patients with nasal polyps showed significantly higher scores as compared to patients without nasal polyps with regards to cough, runny nose and need to blow nose (T-Test $p=0.01$, $p=0.05$ and $p=0.001$, respectively).

The mean duration of CRS symptoms prior to evaluation at our clinic was 79 months. The CRSsNP had a mean duration of CRS symptoms of 99 months prior to evaluation, while the CRSwNP had an average duration of 59 months. This difference did not reach statistical significance (T-Test $p=0.325$).

The CRSwNP patients had a higher score compared to the CRSsNP patients (12.06 versus 8.00, T-Test $p=0.001$). There was a significant correlation between the VAS- and SNOT-20 scores ($p=0.001$ $r=0.53$).

	Control group	CRSsNP	CRSwNP	ANOVA	T-test
Number of patients	22	23	30		
Time to referral (months)		99	59		0.33
Biofilm positive (%)	56	81.5	97.15	<0.001	
VAS score		63	68.6		0.27
SNOT-20 (total score)	29.9	39.8	43.6	0.034	0.46
Need to blow nose	2.23	2.35	3.5	<0.001	0.001
Sneezing	1.27	1.57	1.87	0.29	0.43
Runny nose	1.86	2.17	2.93	0.02	0.05
Cough	1.27	1.09	2.10	0.02	0.01
Post-nasal discharge	1.73	1.48	2.3	0.19	0.08
Thick nasal discharge	1.73	2.3	3.07	0.02	0.11
Facial pain / pressure	1.27	2.43	2.03	0.04	0.36
Lund Mackey CT score (mean)	0	8.00	12.06		0.001

Table 4. Demographic data and results from paper 3. The ANOVA test was applied to calculate the significance of differences between the three groups and the t-test for comparison of CRSsNP with CRSwNP

Figure 11. The difference between key items in SNOT-20 between CRSsNP and CRSwNP

Figure 12. CT picture of patient illustrating an individual variability between CT score and SNOT-20 score. The patient had SNOT-20 score of 16 and LM core of 20.

7. General discussion

It was our hypothesis going into this project that biofilms play an important role in the pathogenesis of most patients with CRS. Specifically, CRS not accounted for by rare subgroups within the CRS spectrum, motivating our exclusion criteria.

A cross-sectional design allowed us to state correlations between the occurrence of biofilm and chronic rhinosinusitis. This design allowed us to complete the study within a reasonable timeframe. It also gave us the opportunity to include many more patients than a prospective design would have allowed. In a prospective design, we would have to have included healthy subjects, identify a cohort with biofilm and followed them over a considerable timespan to identify the proportion that developed chronic rhinosinusitis compared to biofilm-negative controls.

The drawback to our design is, of course, that we are not able to infer causality between the presence of biofilms and the development of sinusitis.

In our first paper we found a significantly higher prevalence of biofilms in patients with CRS compared to controls (103, 112). Most other papers investigating CRS and biofilms report comparable results. Notably, Mladina et. al. found biofilms on almost all subjects, controls and patients with CRS both, leading that group to conclude that biofilms were of no significance in the pathogenesis of CRS (230, 231). However, the methodology of the Mladina group, utilizing scanning electron microscopy to detect biofilms, has been questioned. Ha et. al.(11) found that confocal microscopy is the gold standard for detecting biofilms in patients with CRS. Major challenges with scanning electron microscopy being its inability to scan into the biopsy, and to identify bacteria that were intact at the time of fixation, indicating biofilms. See also chapter 5.5.2.

The multitude of theories concerning the pathogenesis of CRS presented in chapter 4.2 suggests a multifactorial nature of CRS. However, some of the theories can be combined. Especially the biofilm and immune barrier theory. As mentioned in chapter 3.5 the discovery of bitter taste receptors and their possible role in the innate immune system fits snugly with the immune barrier hypothesis, predisposing individuals with defects in these receptors to biofilm formation and CRS (107, 117). Furthermore, *Staphylococcus Aureus* has been shown to be the most common microbe in sinonasal biofilms. Finally, *Staphylococcus Aureus* has been shown to alter the eicosanoid metabolism (10, 116).

Thus, a synthesis of the different existing theories is emerging. It seems that biofilms play a role in the pathogenesis of CRS. Increased susceptibility to infection, and the properties of the different biofilms, determined by the different species of bacteria and fungi, and their relative number, are

important to determine who develops CRS, and why some patients develop nasal polyps. It is important in the coming years to conduct larger, prospective studies to determine if there exists a causal relationship between biofilms and CRS. The last two years after our third paper was published there has been a paucity of new studies investigating biofilms and their role in the pathogenesis of CRS (232). Cho et al. (216, 217) and the Wormald group (218, 219) has published some interesting articles on new treatment options for biofilms, as mentioned in the treatment chapter (4.6). In my opinion, we still need a better understanding of biofilms and their potentially causative role in CRS.

In our second paper the CRS patients were investigated with three biopsies each, allowing us to stratify on location. The findings here were interesting. The highest prevalence of biofilms was on the ethmoid bulla, significantly higher than on the middle turbinate. Both the finding that the prevalence varies within the sinonasal cavity, and that it increases deeper in the middle meatus is interesting. This means that comparable biopsies must be harvested on the same anatomical site to make meaningful comparisons, having implications for the design of future studies. The finding that biofilms are more prevalent deeper within the middle meatus strengthens the likelihood that they are significant in the pathogenesis of CRS. Most nasal polyps originate in the ethmoids, of which the ethmoid bulla is the start. Biofilms in this area might be responsible for the inflammation that triggers polyps. *Staphylococcus Aureus*, the most prevalent bacteria found in biofilms, have been found intracellularly only in patients with nasal polyps, and might have a role in their genesis (116).

In our third paper, we did not find significant differences between the overall SNOT-20 sum and VAS between patients with CRSwNP and CRSsNP. Three items of the inventory scored significantly higher in CRSwNP versus CRSsNP, namely need to blow nose, runny nose and cough. Some studies have reported better quality of life scores in patients with nasal polyps compared to patients with CRSsNP, but patients with recurrent nasal polyps after initial surgery represent the patients with the most recalcitrant CRS. Up to 30% of people with NP are asymptomatic or does not contact a physician. This illustrates the large differences within the group with NP (145, 233).

We also found previously diagnosed asthma to be significantly more prevalent in the CRS group compared to the control group, but no significant difference between the patients with CRSsNP versus CRSwNP. This association is well established in the literature and supporting the united airways hypothesis. Several papers report asthma to be more prevalent in the group with nasal polyps. We did not run diagnostics for asthma in this study, leaving the possibility for undiagnosed asthma to skew the data (171, 178).

Finally, we found the VAS scale introduced by the EPOS group to correlate well with SNOT-20. This supports the use of the simple VAS in clinical practice.

Strengths and challenges of the study

The main challenge in this thesis was to decide on and establish a method for the detection of biofilms. We followed the advice of Ha et. al. (11) that concluded that confocal microscopy is best suited for detection of biofilms in the sinonasal mucosa. However, the practical application was not easy, as no member in the group had used a confocal microscope. The help from the Molecular Imaging Center, MIC, the core facility for confocal microscopy in Haukeland, Bergen, was invaluable. Foreman's article (10) which found that delayed microscopy was feasible, made this study possible. The rest of the method is based on Psaltis et. al. (12). Both authors are a member of the Wormald group, Adelaide Australia. This group is at the forefront of biofilm research in the setting of CRS. The pilot, which lasted several months, was necessary to get sufficient practice. Still we have not found established criteria for what constitutes a biofilm in the confocal microscope. This made it necessary to establish our own criteria, based on published images and descriptions from available papers. MIC was also helpful here. We decided that there had to be a prominent, coherent group of bacteria, visible in several images along a z-stack. As an additional safety mechanism, the whole group was consulted on difficult z-stacks (blinded).

A very interesting result in this work is the high percentage of biofilm occurrence in septoplasty patients.

When we found that 56% of our controls had biofilms, we were unsure of our scoring. Doing additional searches in the available literature we found that Bezerra et. al. had comparable scores, at 48,1%, also using septoplasty patients as controls (104). Why are biofilms so prevalent in septoplasty patients? Most of the groups having used skull-base patients as controls reported biofilm presence in controls between zero to ten percent.

Is septal deviation one of the risk factors for the later development of CRS because of biofilm formation? Closed, poorly aerated areas are more prone to biofilm formation.

Despite ultimately having too few patients to stratify the CRS group on biofilm, a strength in this work was the number of patients examined. Confocal microscopy is labor intensive, and a total of 86 patients (including controls) is a respectable amount. Prior to starting this study, most studies performing confocal microscopy reported featured fewer patients, many only 10-20 patients and 0-10 controls. There was also a scarcity of studies, making power analysis meaningless. Instead we decided to investigate the maximum number of patients and controls feasible within our time limit.

Another strength in this study was our pilot phase, establishing rigorous protocols for the lab work and microscopy. This was a prerequisite for the studies to follow.

8. Conclusions

We showed that biofilms were present both in controls, which was septoplasty patients, and patients with CRSsNP and CRSwNP. They were significantly more prevalent in patients with CRSwNP compared to both controls and CRSsNP. The location of biofilm within the nose also varied. There was higher prevalence of biofilms on the ethmoid bullae compared to the uncinate process and the middle turbinate. Only the difference between the middle turbinate and the ethmoid bullae was significant.

Because of our high number of biofilm positive subjects, we could not do meaningful statistical analyses on our CRS groups, as there were too few biofilm negative CRS patients.

We found that our CRS groups had a higher symptom load on VAS and SNOT-20 than the septoplasty controls. The patients with CRSwNP had higher scores on VAS, SNOT-20 and shorter time for symptom debut to evaluation compared to CRSsNP, but none of the differences were significant.

There was a significantly higher score for CRSwNP vs CRSsNP on the questions in SNOT-20 regarding cough, runny nose and need to blow nose.

In summary, we established a method for detecting biofilms in patients with CRS and found a significant correlation between the presence of biofilms and CRS.

9. Future studies

There is still some debate as to the precise role biofilms play in the pathogenesis of CRS. Is it the main cause? One of several factors? There is a need of larger, prospective studies examining if the presence of biofilm causes CRS. Most of the studies so far only shows correlations.

It is also important to work more on the inflammatory profile of biofilms. Do they trigger local inflammation? We plan to address this question in a future paper.

There is a host of studies investigating treatment of biofilms on the sinonasal mucosa, where some are showing promising results.

Further treatment modalities need to be investigated. Today the mainstay in treatment of CRS is symptomatic relief, not causative treatment.

There is a need to perform biofilm studies on septoplasty patients. Does septal deviation predispose to biofilm formation? If so, does this put these patients at increased risk of developing CRS? Does biofilm formation exacerbate the feeling of nasal congestion?

Regardless of the final verdict on biofilms and CRS, biofilms are implicated in most chronic infectious diseases. There is an urgent need to develop antibiotics effective against biofilms in the near future.

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Bacterial biofilms in patients with chronic rhinosinusitis: a confocal scanning laser microscopy study*

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Abstract

Background: Recent research into the pathophysiology of chronic rhinosinusitis suggests an important role for biofilms. They can be detected in both healthy and diseased nasal mucosa. Several different methods of detecting biofilms have been described. This study investigates the presence of biofilm in a larger group of patients with chronic rhinosinusitis undergoing primary functional endoscopic surgery.

Methods: Sixty-one patients with chronic rhinosinusitis and 25 controls, with septal deviation, were included from 2010 to 2012. Endonasal biopsies were harvested during surgery, snap frozen in isopentane, cooled on dry ice and stored at -80°C. The samples were prepared with Invitrogens' BacLight LiveDead kit, and investigated with confocal scanning laser microscopy for the presence of biofilm.

Results: In the chronic rhinosinusitis group 55/61 were biofilm positive as opposed to 14/25 in the control group. The difference was highly significant. The odds ratio was 7.2.

Conclusion: Patients with chronic rhinosinusitis have a highly significant increased point prevalence of biofilms compared to controls.

Key words: biofilm, chronic rhinosinusitis, confocal microscopy, FESS

Introduction

Chronic rhinosinusitis (CRS) is increasingly viewed as an entity composed of several different subgroups of diseases. However, the pathogenesis of CRS is still poorly understood, resulting in a lack of targeted therapy for these patients⁽¹⁻³⁾.

Recently, several authors reported the presence of bacterial biofilm in the sinonasal mucosa in a high percentage of patients with CRS as opposed to healthy controls, implicating biofilms in the development of CRS⁽⁴⁻⁶⁾. Biofilms in humans are recognized as the culprit behind a diverse array of chronic infectious diseases, including infectious endocarditis, osteomyelitis and dental caries⁽⁷⁻¹¹⁾.

Biofilms consists of bacterial colonies, often several species, but may also consist of fungi⁽¹²⁾. The colonies are protected by an extracellular polymeric substance (EPS), consisting mainly of carbohydrates and proteins, which in large colonies are responsible for its slimy appearance⁽¹³⁾.

Bacteria within the biofilm establish gradients for nutrients and oxygen, and communicate by secreting peptides, a process called quorum sensing⁽¹⁴⁾. Bacteria also exchange DNA, notably resistance factors to different antibiotics. These factors contribute to the resilience of biofilms, making them hard to eradicate⁽¹⁵⁾.

Despite the possible role of biofilms in CRS, some authors do not find significant differences between patients with CRS and controls. Indeed one group, Mladina et al. ^(16,17), concludes that the reported biofilms represent a mucociliary blanket.

The question arises whether these reported differences could be caused by methodological differences amongst the groups investigating biofilms and CRS? Currently used methods to investigate biofilms in the sinonasal mucosa range from traditional light microscopy, electron microscopy to confocal laser microscopy ^(6,16-18).

Ha et al. ⁽¹⁹⁾ showed that confocal microscopy is better suited for biofilm detection than the other available modalities, and is therefore the method we have chosen in this study.

The primary goal of the present study was to determine the point prevalence of bacterial biofilms in our patients with CRS, after establishing a suitable methodology in a pilot study.

Materials and methods

A cross-sectional study from 2010 to 2012 was performed in Akershus University Hospital, Norway. Eighty-six consecutive patients undergoing endonasal surgery were included. Sixty-one patients with CRS undergoing functional endoscopic sinus surgery (FESS) as the experimental group, and 25 patients undergoing septoplasty without CRS as controls. The study was approved by the hospital science board and the Regional Ethics Committee (reference number 2009/1720b).

Inclusion and exclusion criteria are detailed in Table 1. Patients were diagnosed with CRS as defined by the EPOS group in 2007

⁽²⁰⁾. Medical and surgical history, specifically regarding the use of nasal and systemic steroids, smoking, asthma and allergy were obtained. Written consent was obtained from all patients. The study was performed according to the Helsinki declaration.

Tissue collection and preparation

In the CRS group, tissue samples were harvested from the anterior part of concha media, the uncinat process or the ethmoid bulla during primary sinus surgery. Mucosal samples in the control group were obtained from the anterior part of concha media. Tissue samples were immediately put on moist gauze and transported to the pathology department in clean plastic containers on ice separated by location. Bony tissue was removed and the sample was washed three times in millipore (MQ) water to remove any planktonic bacteria. Each sample was then snap frozen (isopentane cooled with dry ice) in a 1.8 ml cryotube and stored at -80°C).

At the day of laboratory analysis, samples were thawed at room temperature and washed three times in 25 ml MQ water (0.22 micrometre filter, EMD Millipore Corporation, Billerica, MA, USA) for 60 seconds. They were then transferred to Eppendorf tubes (Eppendorf AG, Hamburg, Germany) containing 1 ml MQ water. 1,5 µl component A (1.67 mM SYTO 9 nucleic acid stain, 1.67 mM propidium iodide solution in DMSO) and 1,5 µl component B (1.67 mM SYTO® 9 nucleic acid stain, 18.3 mM propidium iodide solution (Invitrogen's LIVE/DEAD® BacLight™ Bacterial Viability Kit, Invitrogen, Burlington, Canada) followed by 10 seconds of stirring (IKA MS3 Digital, VWR International, Radnor, PA, USA). The reagents were incubated for 15 minutes in darkness on a

Table 1. Inclusion and exclusion criteria.

CRS group included	CRS Group excluded	Controls included	Controls excluded
Primary FESS	Pregnant	Septal deviation and/or concha media bullosa requiring surgery	Same as CRS group plus septal perforation
Bilateral disease	Immunodeficiency	Above 18 years old	
Above 18 years old	Reduced mucociliary clearance (eg Kartagener)	Lund-Mackay CT score of zero	
	Antibiotics within two weeks of operation		
	Non-invasive fungal balls or invasive fungal disease		
	Systemic vasculitis		
	Granulomatous disease		
	Cocaine abuse		
	Neoplasm		
	Aspirin exacerbated respiratory disease

Figure 1. Biofilm positive (x40 objective). Epithelial cells are the large red structures, and the bacteria are shown as the small green dots dominating the view.

Figure 2. Biofilm negative (x40 objective). Only epithelial cells are seen with no signs of bacteria.

nutating mixer (VWR International).

After incubation the samples were washed three times in 25 ml MQ water. Prior to mounting the tissue biopsies were squeezed gently between two microscope slides (ELKA, Karl Hecht GmbH & Co KG, „Assistent“, Sondheim / Rhön, Germany). They were then mounted on a microscope slide with a drop of mounting oil (component C, Invitrogen's LIVE/DEAD® BacLight™ Bacterial Viability Kit) and fitted with a cover slip (20x20 mm, 12 mm thick, VWR International).

After two to three days, visible decomposition had started and after one week the samples were rendered unusable. The fluorescent dyes were stable up to one week after staining, thus tissue decomposition was the limiting factor (unpublished data). We stored the samples in a dark room at -20°C. Microscopy was therefore performed on the same day, or the day after, the tissue samples were prepared. We would like to point out that any bacterial growth occurring after preparation of the tissue will not be visible in the confocal microscopy, as only fluorescently marked tissue will light up.

Confocal Laser Microscopy

Confocal scanning laser microscopy was performed with an upright Leica TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany). Oil immersion lenses x40 and x63 were used. The examiner was blinded to patient identity and disease status. First the samples were scanned in the fluorescence mode, and then

switched to confocal mode. Z-stacks were obtained in areas with possible biofilm. For the confocal part, the Argon laser 488 nm laser line was used. The strength was set to 15% and PMT1 was set to 554, PMT2 was set to 517. The strength and reception settings depend on the age of the laser and the thickness of the biopsy. Biofilms were scored when clusters of bacteria with intact membranes, green colour, were present in both the x-y and x-z axes. Typical size of a single bacterium is 0,2 to 2 microns in diameter for spherical species, and 1 to 10 microns in length for nonspherical species. Figure 1 shows a biofilm positive sample, while Figure 2 shows a biofilm negative sample. Z-stacks were useful to form an idea of the three dimensional properties of the biofilms (Figure 3). With samples that the first author found ambiguous regarding the presence of biofilm, the whole group was consulted.

Statistical analyses

The data obtained in this study were analysed with SPSS 19 (IBM Corp. SPSS, Armonk, NY, USA). Chi-square with continuity correction and odds ratio, when comparing dichotomous data, and student t-test for continuous data were used. For all results a two-sided significance level of 5% and 95% confidence interval were used.

Results

The total number of patients in the CRS group was 61, 23 females and 38 males, and median age was 40 years. There were 25 individuals in the control group, 8 females and 17 males with a

Figure 3. Individual slides in a z-stack, providing a 3-dimensional overview of the sample.

median age of 40 years.

Bacterial biofilms were detected in 90% of patients with CRS, significantly more prevalent than controls, in which 56% were biofilm positive ($p < 0.001$, chi-square score 13.1) (Table 2).

Patients within the CRS group had significantly higher occurrences of allergy (50,9% vs. 17,4%, $p = 0.006$) and asthma (34,0% vs. 8,7%, $p = 0.022$) than the controls. In our material, there was a higher rate of current smokers in the control group (34,0% vs. 8,7%, $p = 0.014$) (Table 2).

Discussion

In this study a high point prevalence of biofilms in patients with CRS (90.2%) was found. This is higher than previously reported, but in line with other studies^(4,18,21). A relatively high point prevalence of biofilms was also found in controls (56.0%), which is higher than most other studies but on level with Bezerra et al.⁽²¹⁾. A possible explanation is that patients undergoing septoplasty were used as controls in both this and Bezerra's study. Several other studies reporting low prevalence of biofilms in controls used subjects completely free of nasal complaints undergoing

Table 2. Results.

	Controls	CRS Group	p-value	Chi-square and odds ratio
No. of patients	25	61		
Age; Median	40	40		
Male	17	38		
Female	8	23		
Smokers	34.8 %	11.1 %	(p = 0.014)	Chi Square 6.1, OR 0.23 (0.07-0.78)
Allergy	17.4%	50.9 %	(p = 0.006)	Chi Square 7.5, OR 4.9 (1.5-16.5)
Asthma	8.7%	34.0%	(p = 0.022)	Chi Square 5.3, OR 5.4 (1.1-25.6)
Biofilm positive	56.0%	90.2%	(p < 0.001)	Chi Square 13.1, OR 7.2 (2.3-22.9)

skull base surgery ^(4,6). This may point to a role for biofilms in the pathogenesis of patients with nasal obstruction without CRS. Further studies are needed.

In the current study, we have harvested biopsies from the structures surrounding the middle meatus as not all patients underwent surgery of the frontal recess, posterior ethmoid cells or sphenoid sinus. In a later study, we plan to investigate whether there is an unequal distribution of biofilms in the different compartments of the sinonasal cavity.

We chose to follow the protocol for biofilm detection with confocal microscopy outlined by Psaltis et al. ⁽⁶⁾ with cryopreservation instead of direct microscopy for convenience. In a later article, Foreman et al. ⁽⁴⁾ reported that cryopreservation gave results similar to direct microscopy.

Research into the pathophysiology of CRS increasingly points out the need to differentiate several distinct subgroups; e.g. Samters triad, ciliary dysfunction, eosinophilia and immunodeficiency ⁽¹⁾. In this study, we wanted to examine the association with biofilm infection in patients with CRS without these underlying conditions at the time of primary rhinological surgery. In our opinion, a biofilm point prevalence of above 90% in this group strongly suggests such a connection.

To date three main modalities are used to investigate CRS and biofilms: Confocal, electron and light microscopy ^(4,6,16-18). Light microscopy, which is cheap and readily accessible, has the drawbacks of low resolution and inability to visualize the individual bacteria, making it hard to differentiate between biofilms and planktonic bacteria. Scanning electron microscopy offers great resolution, but is expensive and labour intensive. It is also difficult to distinguish dead bacteria in the mucociliary blanket from

actual biofilms, which are bound irreversibly to the epithelial cells. We therefore speculate that this is the reason why Mladina et al. ^(16,17) found biofilms in almost all subjects, both patients and controls.

Ha et al. ⁽¹⁹⁾ concludes that confocal microscopy is best suited for detection of biofilms in the sinonasal mucosa. Confocal microscopy features the ability to distinguish between bacteria with intact cell membranes at the time of preparation, indicating vital bacteria, and dead bacteria with compromised cell membranes. Additionally, it provides the opportunity to scroll into the depth of the tissue sample, the z-axis, and allows an insight into the three dimensional properties of a suspected biofilm. On the downside, confocal scanning laser microscopy is expensive and labour intensive, and also not widely available.

In conclusion, this study strengthens the mounting evidence of the role of biofilms in patients with CRS. The control group with nasal congestion without CRS had 56% biofilm positivity, indicating a role for biofilms in this group as well. Furthermore, the methodology of biofilm research in CRS should become more standardized in the future, and we suggest the merits of confocal microscopy.

Further studies are needed in this area, and we plan to investigate the role of biofilms in the setting of nasal polyps, inflammation associated with biofilm colonies and postoperative results between biofilm positive and negative subjects.

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Authorship contribution

KAD: Planned and implemented the study. Performed microscopy on all the samples. Analysed data and drafted the manuscript. Final approval of version to be submitted.

ØE: Planned and implemented the study. Participated in microscopy, and was responsible for the entire main database. Analysed data and reviewed the manuscript. Final approval of version to be submitted.

KFA: Planned and implemented the study. Handled the samples, anonymised and stored them. Reviewed scoring on difficult samples. Reviewed the manuscript. Final approval of version to be submitted.

VCO: Planned and implemented the study. Handled the samples,

anonymised and stored them. Reviewed scoring on difficult samples. Reviewed the manuscript. Final approval of version to be submitted.

GBH: Planned and implemented the study. Helped with logistics concerning the biopsies in the operating theatre. Reviewed the manuscript. Final approval of version to be submitted.

EBA: Planned and implemented the study. Responsible for establishing the protocol of biopsy-staining. Analysed data and reviewed the manuscript. Final approval of version to be submitted.

Conflicts of Interest

None of the authors report any conflicting interests.

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RESEARCH ARTICLE

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Causes of higher symptomatic airway load in patients with chronic rhinosinusitis

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Abstract

Background: Chronic rhinosinusitis display a variety of different phenotypes. The symptoms of disease are characterised by various signs and symptoms such as nasal congestion, nasal discharge, pressure sensation in the face and reduced or complete loss of smell.

In a patient population undergoing functional endoscopic sinonasal surgery (FESS) for chronic rhinosinusitis, we wanted to investigate the clinical features and explore if the presence of biofilm, nasal polyps or other disease characteristic could serve as predictor for the symptomatic load. A patient group undergoing septoplasty without disease of the sinuses was included as control.

Methods: The Sinonasal outcome test (SNOT-20), EPOS visual analogue scale (VAS) and the Lund-Mackey CT score (LM CT score) were used to examine 23 patients with chronic rhinosinusitis without nasal polyps (CRSsNP), 30 patient with nasal polyps (CRSwNP) and 22 patients with septal deviation. Tissue samples were collected prospectively during surgery. The cohort has previously been examined for the presence of biofilm.

Results: Patients with CRSsNP and CRSwNP had significantly higher degree of symptoms compared to the septoplasty group (SNOT-20 scores of 39.8, 43.6 and 29.9, respectively, $p = 0.034$). There were no significant differences in the total SNOT-20 or VAS symptoms scores between the CRSsNP and CRSwNP subgroups. However patients with nasal polyps showed significantly higher scores of symptoms related to sinonasal discomfort such as cough, runny nose and need to blow nose ($p = 0.011$, $p = 0.046$, $p = 0.001$ respectively). Patients with nasal polyps showed a significantly higher LM CT score compared to patients without polyps (12.06 versus 8.00, $p = 0.001$). The presence of biofilm did not impact the degree of symptoms.

Conclusion: The presence of nasal polyp formations in CRS patients was associated with a higher symptomatic airway load as compared to patients without polyps. These findings suggest that nasal polyps could be an indicator of more substantial sinonasal disease. The presence of biofilm did not impact the degree of symptoms, however, as biofilm seem to be a common feature of chronic rhinosinusitis (89% in this cohort), it is more likely to be involved in the development of the CRS, rather than being a surrogate marker for increased symptomatic load.

Keywords: Chronic rhinosinusitis, Nasal polyps, Endoscopic sinus surgery, SNOT-20, Vas, Lund-Mackey CT score, Biofilm

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Background

Chronic rhinosinusitis (CRS) is a major contributor to disease in central Europe with prevalence of 10.9% [1]. CRS patients with or without nasal polyps are a heterogeneous group with various phenotypes and characteristics such as allergic rhinitis, eosinophilic inflammation, Samters triad, fungal allergy, *Staphylococcus aureus* superantigens, biofilm formation and some patients without any apparent predispositions. They contribute to a whole range of different clinical setting ranging from severe life threatening disease as intracranial and intraorbital infections, to mild or low-grade chronic inflammation with various degrees of morbidity.

Eradication of chronic rhinosinusitis with medical treatment or surgery can be difficult, one reason for this might be the formation of biofilm in the sinonasal cavities. Bacteria are found in two different states. One state is characterized by free-floating bacteria with rapid bacterial growth, the planktonic state. This represents the traditional understanding of bacterial existence. In the other state, called the biofilm state, the bacteria are organized in a microbial community, often surrounded by an extracellular polymeric matrix. Within the biofilms the bacteria communicate with each other in a process known as quorum sensing. This communication is facilitated by several classes of molecules, amongst them peptides. Quorum sensing can influence a wide range of bacterial actions by regulating genetic expression. One example of this is switching back to the planktonic state. Bacteria in a biofilm can also exchange genetic material, especially genes involved in antibiotic resistance. Biofilms are usually heterogenic, consisting of many different strains of bacteria and/or fungi.

The precise distribution of biofilm in the CRS population has been debated, but there seems to be clear evidence of increased biofilm formation in the CRS patient ranging from 15 to 100% [2–4]. As reported earlier by this group, CRSwNP express a higher prevalence of biofilm as compared to CRSsNP. Biofilm was also found in 56% of the control patients without CRS but with septal deviations. Thus, the definite role of biofilms in CRS is still unclear.

One of the most obvious differences in the CRS groups is the presence or absence of nasal polyps. Previous European studies have shown that 77% of nasal polyp patients are characterized by an eosinophilic inflammation mediated by T helper 2 cells [5]. The reasons for this T-cell activation is still unknown, most likely both extrinsic and intrinsic factors are involved. Studies have indicated that nasal polyps may impact the degree of symptoms and the effect of surgery [6]. The eosinophilic component in CRSwNP, is also present in patients with bronchial eosinophilic hypersensitivity, and there are reports indicating a correlation between

asthma and rhinitis [7]. An association between CRS and asthma has been shown, but it is still unknown if patients with CRSwNP are more predisposed to develop asthma when compared to patients with CRSsNP. However, patients with CRS are at higher risk to develop symptoms from the respiratory tract such as bronchial asthma, which have led to the concept of united airway disease.

The goal of this study was to investigate the clinical features and objective findings in a Norwegian CRS cohort previously examined for the prevalence of biofilm [8], and explore if biofilm, nasal polyps or other disease characteristic could serve as predictor for the symptomatic load in such patients.

Methods

Eighty-seven patients undergoing primary nasal surgery were included from 2010 to 2012 at Akershus University Hospital. 62 patients were diagnosed with CRS according to EPOS (2007) and underwent FESS. 27 of these patient were without polyps, 35 patients had accompanying polyps. 25 patients with nasal stenosis due to septal deviation were included as control, these patients underwent septoplasty. Of the 87 patients included in the study 12 patients (4 CRSsNP, 5 CRSwNP and 3 septoplasty patients) had not fully completed the questionnaires and thus were omitted from the statistical analysis. The patient demographics of this cohort is described in detail in a previous report by this group [2]. The study was approved by the hospital board and the Regional Ethical Committee (reference number 2009/1720b). Written consent was obtained from all patients, the study was performed according to the Helsinki declaration.

Data collection

Medical history including the duration of CRS, previous medical treatments (nasal- and/or systemic steroids), smoking-history, asthma and allergy were obtained. All patients underwent preoperative Computer Tomography (CT), and were subsequently scored according to Lund Mackay CT scoring system. CRS patients were asked to grade their quality of life and severity of disease using the EPOS 2007 visual analogue score (VAS) and the SNOT-20 questionnaire.

The data were analysed using SPSS 23 IBM. The results are reported as mean values. One-way analysis of variance, ANOVA, was used to compare biofilm formation, LM CT score, SNOT-20 and its subscore between the CRSsNP, CRSwNP and the septoplasty patients. Student's t-test was used to assess the significance of the mean differences for time to referral, VAS, LM CT score, SNOT-20 and its subscore between CRSsNP and CRSwNP. Pearson correlation was used to evaluate the

correlation between Lund Mackey CT score and SNOT-20/VAS, and correlation between SNOT-20 and VAS.

Results

Patients with CRSsNP and CRSwNP had significantly higher degree of symptoms compared to the septoplasty group (SNOT-20: 39.8, 43.6 and 29.9, respectively, ANOVA $p = 0.03$). There were no differences in the total SNOT-20 or VAS scores between the CRSsNP and CRSwNP groups (39.8 vs. 43.6, t-test $p = 0.455$, and 63 vs. 68.6, t-test $p = 0.265$, respectively). Regarding the SNOT questions related to nasal discomfort, patients with nasal polyps showed significantly higher scores as compared to patients without nasal polyps with regards to cough, runny nose and need to blow nose (t-test $p = 0.011$, $p = 0.046$ and $p = 0.001$, respectively). The association of nasal polyps and cough ($p = 0.011$) and need to blow nose ($p = 0.001$), remained statistical significant also when controlling for the presence of allergy as a potential confounder. The statistical significance for the third parameter, runny nose ($p = 0.046$), became insignificant when controlling for allergy in the cohort ($p = 0.248$). No statistical association was found between allergy and this symptomatic parameter ($p = 0.053$). The results are summarized in Table 1 and Fig. 1.

The mean duration of CRS symptoms prior to evaluation at our clinic was 79 months. The CRSsNP had a mean duration of CRS symptoms of 99 months prior to evaluation, while the CRSwNP had an average duration of 59 months. This difference did not reach statistical significance (t-test $p = 0.325$). The mean Lund Mackay CT score for the CRS group was 10,29. The CRSwNP patients had a higher LM CT score compared to the CRSsNP patients (12.06 vs. 8.00, t-test $p = 0.001$).

Biofilm formation was found in 81% of the patients with chronic rhinosinusitis without polyps, in 97% of the patient with polyps, and in 56% of the patients undergoing septoplasty as reported previously [2].

There was a significant correlation between the VAS- and SNOT-20 scores Pearson's correlation coefficient of 0.525 ($p = 0.001$).

Discussion

In this study we looked at differences in a patient population undergoing primary nasal surgery at Akershus University Hospital (Ahus). Ahus is a regional centre for otorhinolaryngology with patients referred from general practitioners and consultants. We examined the clinical differences between patients operated for CRS with or without nasal polyps and patients operated for septal deviation.

Nasal polyps increase the mass in the nose and mucopurulent stagnation thus as expected CRSwNP had significantly higher LM CT score compared to CRSsNP (12,06 vs. 8,00, t-test $p = 0,001$). As identified by other groups [9] we found a low correlation between the LM CT scores of the CRS patients and the SNOT-20 score (Pearson's correlation $r = -0.115$). The same was seen for the VAS and Lund Mackay CT scores (Pearson's correlation $r = -0.005$). Further analysing the subset of SNOT-20 symptoms in Table 1 only the "need to blow nose" subcategory showed a significant correlation to the Lund Mackay CT score ($r = 0.325$, $p = 0.018$). Subdividing the CRS groups into CRSwNP and CRSsNP did not reveal any statistical significant correlation between LM CT score and SNOT or VAS values.

One reason for the low correlation between the LM CT score and SNOT-20 scores could be the slow and

Table 1 Mean VAS and SNOT scores for the different patient categories

	Septoplasty	CRSsNP	CRSwNP	P-value (ANOVA)	P-value (t-test)
Number of patients	22	23	30		
Time to referral (months)		99	59		0.325
VAS score		63	68.6		0.265
SNOT-20 (total score)	29.9	39.8	43.6	0.034	0.455
SNOT (subscore)					
Need to blow nose	2.23	2.35	3.5	<0.001	0.001
Sneezing	1.27	1.57	1.87	0.29	0.425
Runny nose	1.86	2.17	2.93	0.02	0.046
Cough	1.27	1.09	2.10	0.02	0.011
Post-nasal discharge	1.73	1.48	2.3	0.19	0.077
Thick nasal discharge	1.73	2.3	3.07	0.02	0.119
Facial pain/pressure	1.27	2.43	2.03	0.04	0.355

One-way ANOVA was applied to calculate the significance of differences between CRSsNP, CRSwNP and septoplasty patients. The student's t-test was used to identify differences between CRSsNP and CRSwNP

Fig. 1 Clinical differences between CRSsNP and CRSwNP

gradual development of disease, that makes a true baseline difficult to perceive for the patients.

The low correlation between SNOT-20 and Lund Mackay CT score is well illustrated by one of the included patients with a high Lund Mackay CT score of 20 and a relatively low SNOT-20 score of 16 and VAS 52 (Fig. 2).

Fig. 2 CT picture of patient illustrating an individual variability between CT score and SNOT-20 score

Previous studies have shown that biofilm formations give rise to worse postoperative outcome [10]. There are only a few investigations into preoperative clinical differences between CRS patient with or without biofilm formation. Li et al. [11] found increased SNOT-20 symptom score and increased nasal- and sinus-related symptoms including need to blow nose, cough and postnasal discharge in their biofilm groups compared to the patients without biofilm. Due to the large prevalence of biofilm among our patients (CRSsNP 81,5%, CRSwNP 97,15% and control patients 56%) we were not able to investigate further any difference in clinical features between the biofilm positive and negative groups.

We have earlier shown [8], that CRSwNP and biofilm formation seems to be closely related. In populations with biofilm formation it is thus expected that the degree of polyp formation and symptomatic load is increased compared to patients without polyps and biofilm.

Even though we found increased SNOT-20 and VAS score in the patients with CRSwNP compared to the CRSsNP patients the differences were not significant. However, when looking at the nose related individual SNOT-20 questions, we found a significant increased score in the polyp patients regarding need to blow nose (2,35 vs. 3,5 t-test $p = 0,001$), runny nose (2,17 vs. 2,93 t-test $p = 0,046$) and cough (1,09 vs. 2,1 t-test $p = 0,011$). As allergic rhinitis also cause nose related symptoms we re-analyzed the association of nasal symptoms and

polyps while controlling for the presence of allergy. The association of nasal polyps and cough ($p = 0.011$) and need to blow nose ($p = 0.001$), remained statistical significant also when controlling for the presence of allergy as a potential confounder. The statistical significance for the third parameter, runny nose ($p = 0.046$), became insignificant when controlling for allergy in the cohort ($p = 0.248$). To further understand if allergy alone was associated with an increased runny nose, we examined the association between allergy and runny nose directly and found no statistical association between allergy and this symptomatic parameter ($p = 0.053$). Our interpretation of these supplementary analysis is that nasal polyps is associated with increased nasal symptoms, however for runny nose the relationship between nasal polyps, runny nose and allergy is not completely revealed as allergy does impact the statistical significance of nasal polyps driving such symptoms. As allergy alone is not associated with runny nose, it strengthens the impact of nasal polyps on this specific symptomatic finding. Further investigations are necessary to better understand the complete clinical presentation of such patients and underlying biological phenomena.

Nasal discharge, postnasal drip and cough are also symptoms often seen in patients with asthma. There is evidence of a link between asthma and CRS giving rise to the hypothesis of united airways. The exact molecular

pathways are still unclear [12] but specially the eosinophilic nasal polyp inflammations share similarities to eosinophilic asthma with a Th-2 inflammatory response pathway. In our population we found a higher number of patients with asthma in the CRS groups (33,3%) compared to the patients with septum deviation (8.7%). We did not find any significant difference in the prevalence of asthma between the CRSwNP and CRSsNP as other investigators have reported [13, 14]. Håkansson et al. 2014 [15] found a 25% under diagnosis of asthma in the CRS group. This may explain the reason for the low number of asthma patients in our cohort as we did not test our patients for asthma but based the diagnosis on medical history and use of asthma medication. Further investigations into these connections are needed.

The VAS score from the EPOS document is a fast and easy tool for assessing severity of sinus symptoms. In our study we found a Pearson's correlation coefficient of 0.525 ($p = 0.001$) between the VAS score and the SNOT-20 score for our CRS patients (Fig. 3). In a busy practice pressed for time the VAS score gives valuable information regarding patient sinus disease.

Conclusion

Chronic rhinosinusitis with nasal polyps gives an increased symptomatic load compared to chronic rhinosinusitis without nasal polyps. This is specially seen in

Fig. 3 Scatterplot illustrating the correlation between VAS and SNOT-20 score

relations to symptoms of the united airways as cough, need to blow nose and postnasal discharge. The finding of nasal polyps should therefore alert the physician to expect increased sinonasal discomfort and thus help in the prioritization of medical and surgical treatment. Due to the high prevalence of biofilm formation we were not able to find specific clinical features related to biofilm formation.

Additional file

Additional file 1: Data containing patient disease classification, allergy status, asthma status, LM CT score, VAS score, SNOT score and biofilm status. (XLS 28 kb)

Abbreviations

CRS: Chronic rhinosinusitis; CRSsNP: Chronic rhinosinusitis without nasal polyps; CRSwNP: Chronic rhinosinusitis with nasal polyps; LM CT score: Lund Mackay CT score; NP: Nasal polyps; SNOT-20: Sino-Nasal-Outcome-Test-20; VAS: EPOS Visual analogue scale; vs: Versus

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Availability of data and materials

Spreadsheet containing our data is included in the Additional file 1. For additional information contact author.

Authors' contributions

ØE participated in the planning of the study, scored the CT-scans, collected the preoperative data, operated the patients, participated in the statistical calculation and wrote the first draft of the manuscript. KAD participated in the planning, collected the preoperative data, analysed the material for biofilm, operated the patients and reviewed the manuscript. FD supervised the statistical calculation and reviewed the manuscript. KF and VCO participated in planning the study, helped prepare the biopsy and reviewed the manuscript. GBH participated in planning the study, operated the patients, collected samples and reviewed the manuscript. EB planned and supervised the study design, operated the patients, participated in collecting samples and reviewed the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The study was approved by Akershus University Hospital board and the Norwegian Regional Committees for Medical and Health Research Ethics (REC) (reference number 2009/1720b).

All patients included in the study filled out a consent form prior to inclusion.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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