

**SAFETY AND EFFICACY OF ANTI-MICROBIAL PHOTODYNAMIC THERAPY
(aPDT) IN PATIENTS WITH RECALCITRANT CHRONIC RHINOSINUSITIS: A
PILOT STUDY**

by

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Safety and efficacy of Anti-Microbial Photodynamic Therapy (aPDT) in patients with
recalcitrant chronic rhinosinusitis: a pilot study

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Abstract

Introduction: This pilot study intended to test the feasibility of addressing problems of recalcitrance with management of patients with Chronic Rhinosinusitis (CRS). The study explored the level of response and determined the safety of a novel treatment option for recalcitrant CRS patients.

Methods: The primary outcome was to assess the efficacy of antimicrobial photodynamic therapy (aPDT). Improvements with Alsaleh-Javer Endoscopic Symptom score (AJESS) by 1-point; Sinonasal Outcome Test 22 (SNOT-22) by 9-points and culture swab results over a 45-day period. The Secondary outcome was to evaluate the safety of the aPDT. Presence of adverse events, increase in pain and discomfort scores, smell test (UPSIT) and Nasal Mucociliary test (Saccharin test), were determined over the same period.

Results: A total of 7 participants completed the pilot study. There was a slight male preponderance (1.3:1, M: F) and age ranged from 47 to 78 years with mean age \pm SD, 60.3 years \pm 10.1. Two (2) out of the 7 participants (28.6%) had co-morbid conditions (Asthma), while all of the 7 participants were non-smokers. Patients with a clinical diagnosis of Chronic sinusitis without polyps (CRSsNP) accounted for more than half of the participants (57.2%). Mean AJESS reduction was maximal between completion of the aPDT treatment (day 7) and one-week post-treatment (day 14), 3.00 to 1.50 points. While mean SNOT-22 scores reduction was maximal between day-0 (44.53) and day-7 (35.57), about 9 points. A marked reduction in laboratory cultures within a week of treatment completion (days 7 and 14) noted but not sustained. No adverse events recorded, with mild-moderate discomfort noted with treatment. The NMCT results showed significant improvement in 2/7 participants (28.6%), while smell test did not record appreciable improvement.

Conclusion: This study showed that aPDT was able to improve the endoscopic scores (AJESS) and sinus-specific questionnaire (SNOT-22) by at least 1 point and 9 points or 1 MCID, respectively. No adverse events noted identified during the study period. Olfaction (using UPSIT), showed dysfunction not worsened with the procedure. Future research may involve sinonasal microbiota transplants use via nasal lavage post aPDT to treat refractory CRS patients from suitably healthy donors.

Lay Summary

This study examined the effectiveness and safety of aPDT, a novel treatment to improve the well-being of patients who had recalcitrant sinusitis not treatable using the commonly available means of treatment. The main observation was that the aPDT treatment option is safe and shows promise in improving patient's complaints and quantity of infection in the short-term but had minimal effect on the sense of smell. We also noted that this effect was not sustained and the possibility of using other additional treatment options for longer term control ought to be considered.

Preface

The clinical data collection was conducted at the St Paul's Sinus Centre and the False Creek Surgical Centre, Vancouver BC, while the laboratory cultures were examined at the Vancouver General Hospital (VGH). The mucus samples collected were processed and evaluated at the Centre for Microbial diseases and Immunity research, UBC, Vancouver BC.

This prospective pilot study was conducted under the approval of the University of British Columbia – Providence Health Care Research Ethics Board (H17-00614). I was the lead researcher who was responsible for executing the original concept, consenting patients for the study, coordinating the collection, processing and storage of all samples. The laboratory bench research portion of the study was done under the guidance of a member of the Centre for Microbial diseases and Immunity research, UBC, Vancouver BC. I performed the data analysis and received assistance with sequencing results of the 16S rRNA from the bioinformatics unit of the Centre for Microbial diseases and Immunity research, UBC, Vancouver BC.

Dr. Amin Javer was the lead supervisor and helped develop the original concept of the study design and assisted with preparation of the research manuscript. Dr Bob Hancock was a co-supervisor who assisted with the sample data processing protocol as well as preparation of research manuscript. Parts of Chapter 3 was presented at 73rd Annual meeting of the Canadian Society of Otolaryngology-Head and Neck Surgery, June 1-4, 2019, Edmonton, Ab (Adebola SO, Trimble MJ, Derikvand S, Hancock REW, Javer AR. Examination of bacterial and fungal species within

the sinus cavities of patients with chronic rhinosinusitis (CRS): Use of confocal microscopy and clinical data to understand CRS).

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List of Abbreviations

AJESS - Alsaleh-Javer Endoscopic Symptom Score

aPDT - Antimicrobial Photodynamic therapy

CLSM – Confocal Laser Scanning Microscopy

CNS – Coagulase-Negative Staphylococcus

CRS - Chronic Rhinosinusitis

CT Scan - Computerized Tomography Scan

CRSwNP - Chronic Rhinosinusitis with Nasal Polyps

CRSSNP - Chronic Rhinosinusitis without Nasal Polyps

FESS – Functional Endoscopic Sinus Surgery

GA2LEN - Global Allergy and Asthma European Network

Ig E – Immunoglobulin E

MCID - Minimal Clinically Important Difference

MLK scores – Modified Lund-Kennedy scores

NMCT - Nasal Mucociliary Clearance test

PROMs - Patient-Reported Outcome Measures

QOL – Quality of Life

SEM - Scanning Electron Microscopy

SLE – Systemic Lupus Erythematosus

SNOT-22 - SinoNasal Outcomes test

TEM – Transmission Electron Microscopy

Th2 inflammation - Helper T-cell type 2 inflammation

UPSIT - University of Pennsylvania Smell Identification test

VAS – Visual Analogue Scores

16S rRNA - 16S ribosomal ribonucleic acid

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Dedication

This research is dedicated to all patients with recalcitrant chronic rhinosinusitis. You are central to this research and an improvement in your clinical condition and quality of life will always be paramount to us.

Chapter 1: Introduction

The condition referred to as 'rhinosinusitis' describes an infection and resultant inflammation which involves the nose and sinus cavity. The term 'rhino' refers to the nose, however, due to the continuity of the mucosal lining of the nose into the sinuses, they tend to be classified together, hence the term 'rhinosinusitis'. The symptoms can be quite significant as to severely affect the patient's daily quality of life. These include colored nasal discharge, nasal blockage, reduced or absence of smell perception, post-nasal drainage, facial pain, pressure and headaches. The causes of sinus infections occur mainly from bacteria, viruses and fungi. Sinus infections and inflammation are described based on their duration, hence 'chronic' for cases lasting for 12 weeks or longer and 'acute' less than twelve weeks. Viral infections usually improve within a 5-10-day period whilst inflammation that lasts beyond the 10-day period and resolves prior to the 12-week mark is classified as acute bacterial rhinosinusitis (ABRS). Fungal causes often occur and are subclassified into invasive and non-invasive with the non-invasive group further sub-classified into mycetomas (fungal balls) and allergic fungal rhinosinusitis (AFRS). While the use of antibiotics is useful in the ABRS group of patients, this is not the case for chronic rhinosinusitis or fungal rhinosinusitis(1). Supportive measures such as irrigation with saline, topical steroids and decongestants have been proven to be of use for the chronic conditions(2). However, most chronic sinusitis patients (approximately 11-15% of the population) will eventually need to undergo endoscopic sinus surgery to open the infected and inflamed sinuses, which then allows topical medications via rinses to enter the sinuses and heal the inflamed mucosal lining. The success rate of surgery with good postoperative care is in the range of 80-85%. However, approximately 15-20% of patients who undergo surgery will require ongoing care and about half of them will become

recalcitrant, where the sinus involved will become chronically sick with an altered microbiome, and chronic inflammation will ensue. These recalcitrant patients make up the group that is of interest and addressed in this thesis.

This pilot study intended to test the feasibility of addressing the problem of recalcitrance in the management of patients with Chronic Rhinosinusitis (CRS). The purpose of the study was to explore the level of response as well as to determine the safety of a novel treatment option for a selection of recalcitrant CRS patients. It was postulated that the knowledge derived from this study would result in better treatment outcomes, especially for the patients who had failed the standard of treatment care (medical and surgical) in their management. The pilot study utilized a prospective quantitative study methodology for this group of patients who were recruited from an out-patient clinic.

This chapter provides an overview regarding the background and context, to provide an insight into the basis for the study. This is followed by a review of the literature concerning CRS disease condition, as well as some of the instruments utilized in carrying out the study.

1.1 Background and context

Chronic rhinosinusitis in adults refers to inflammation of the nose and paranasal sinuses lasting greater than 12 weeks, and having at least 2 of the following symptoms: including nasal blockage/obstruction/congestion, nasal discharge (anterior/posterior nasal drip), facial pain/pressure and/or reduction or loss of smell together with objective findings of either endoscopic signs of disease or relevant Computerized Tomography (CT) scan changes.(1, 2)The

term 'rhinosinusitis' is used rather than 'sinusitis' because the condition often involves inflammation of the contiguous nasal mucosa. It is further categorized phenotypically, based on the absence or the presence of nasal polyps (CRS without nasal polyps, CRSsNP; or CRS with nasal polyps, CRSwNP). CRSwNP is mostly due to Type-2 inflammation (eosinophilic, with IL-5, IL-13 from mast cells and innate lymphoid cells) while CRSsNP is secondary to Type-1 inflammation (neutrophilic, with Tissue Growth factor signaling, TGF- β). A study of the phenotypical characteristics of CRS patients in western population, recorded a relative prevalence of approximately 4535 (82%) and 990 (18%), for CRSsNP and CRSwNP respectively.(3) Studies have shown that 70-90% of CRSwNP in Europe and North America show nasal polyps with eosinophilia.(4) In contrast mixed inflammatory patterns have been reported in Asian population where CRSwNP are said to have eosinophilic and non-eosinophilic ratios of nasal polyps being almost equal (50:50). (5)

Epidemiologic data on the prevalence of CRS suggests varying results, depending on the geographical location at which the studies were conducted. A multicenter European study (Global Allergy and Asthma European Network project, GA2LEN) that sampled adults aged 15-75 years in 12 countries reported the overall prevalence of CRS to be 10.9% (C.I 6.9 – 27.1). Conversely, a prevalence of 5.51% was reported in South America, while Canadian studies have obtained prevalences ranging from 3.4% in male to 5.7% in female participants.(6, 7) The disease has been reported to affect 1 in 8 adults in the USA and constitutes a huge socioeconomic burden.(8) These include costs of disease management (clinic appointments, drugs prescribed and surgical interventions), and other economic costs (absence from work, reduced work efficiency). The overall yearly cost in the USA for CRS was reported to be about US \$1539 per patient.(9) An

estimate for the cost of US office-based physician visits resulting from a diagnosis of CRS in 2000 was recorded as US \$11.6 million.(10)

The presence of co-morbidities with CRS have also been reported. This is especially true in the case of Asthma, a disease associated with high correlation with CRS. A triad of asthma-nasal polyp-aspirin intolerance (Aspirin Exacerbated Respiratory Disease, AERD) has been recorded as having the highest correlation with CRS with prevalence ranging from 13% - 53%.(11, 12) A population-based study investigating CRS and the incidence of Asthma over a 12-year period, reported a prevalence of 6.0% (C.I, 5.4-6.7%).(13) Thus, a suggestion was made to provide an increased awareness of caregivers to this co-morbidity. Also, another study recorded a prevalence of asthma in patients with CRSsNP and CRSwNP as 36% and 56% respectively.(3)

The causes of recalcitrant sinus disease can be thought of as being multifactorial and involve four main considerations.(14) First, diagnosis-related (incorrect diagnosis, presence of local symptoms with or without systemic disease); second, disease-related (exogenous, endogenous or genetic factors); third, treatment-related (inadequate treatment) and fourth, patient-related factors (compliance).

1.2 Pathophysiology of Chronic Rhinosinusitis (CRS)

The accepted central theme regarding the pathophysiology of CRS revolves around the presence of inflammation.(1) While acute rhinosinusitis has been proven to derive its inflammation due to the presence of bacterial infection, the etiology of CRS appears multi-dimensional. The presence of an infectious agent, that is different from the microbes causing acute rhinosinusitis is generally

agreed to be the causative factor. Therefore, taking a swab or culture of the involved sinonasal cavity, often results in the presence of a mixed flora being obtained (normal body flora in addition to possible bacterial pathogens). It is now felt that the main cause of chronic inflammation may be the result of a dysbacteriotic microbiome, resulting in the formation of biofilms with its by-products that may be responsible for ongoing mucosal injury.(15)

The ability of the epithelial lining of the sinonasal cavity to perform its function of respiration and smell, lies in the integrity of its cellular junctional complex. This consists of tight junction (zonula occludens), adherens junction (zonula adherens) and desmosomes (macula adherens). The presence of ongoing inflammatory changes results in acantholysis due to the failed attempts at repair. This vicious cycle results in epithelial-to-mesenchymal transition which has been described as a hallmark in the pathogenesis of CRS.(16)

The presence of biofilms in CRS has been used to explain the ability of the micro-organisms to be able to withstand treatments and thus predispose to recalcitrance. Bacteria tend to occur in two main forms: as Biofilms (constitute over 90%) and as Planktonic forms. Biofilms refer to highly organized microbial colonies which are encapsulated in a resistant matrix. Its development stems from the adherence of planktonic bacteria forms into micro-colonies. This adhesion becomes strengthened with the extracellular matrix made up of polysaccharides, nucleic acids and proteins.(17) The presence of cyclic di-guanosine monophosphate (c-di-GMP) enhances its development by cell-to-cell adhesion as well as quorum sharing (refers to the ability of the bacterial organisms to interact via cell density-dependent signal transduction process).(18) Thereby enhancing the ability of the bacteria to thrive despite the existence of host-mediated defenses and

medical treatment. The biofilms thus possesses a 10 -1000 fold higher antimicrobial resistance compared to planktonic bacteria.(19) This has been observed in both pre and post-operative CRS patients.(20) Specifically, based on study by Bendouah et al, biofilms formed by *Staphylococcus aureus* or *Pseudomonas aeruginosa* have been associated with poor outcomes, despite patients with such infections undergoing surgical intervention for CRS and nasal polyposis.(21) Biofilms are not only limited to the nose and paranasal sinuses but have also been reported in most other chronic conditions such as chronic tonsillitis,(22) cystic fibrosis(23) as well as cholesteatoma.(24) Prosthetic apparatus such as orthopedic prostheses, urinary catheters, central venous catheter tips have also been documented to be colonized by biofilms.(25)

The role of fungal organisms as a primary cause of CRS has been implicated, which led to the fungal hypothesis of CRS. This suggests that an excessive non-Immunoglobulin E (Ig E) mediated host response to common airborne fungi is a primary pathogenic trigger of both polypoid and non-polypoid CRS, which might only vary in intensity.(26) This postulate might not be correct given recent evidence showing that the effect of fungi might be contributory (especially in patients such as Allergic Fungal sinusitis patients) rather than causative. Overall, the use of topical antifungal agents for the routine treatment of CRS has not been supported by current evidence.

1.3 Microbiology of Chronic Rhinosinusitis (CRS)

A review of 7 studies between 1991 and 2002 by Meltzer et al, recorded coagulase-negative *Staphylococcus* (CNS) as the most common aerobic organism isolated in 5 out of the 7 studies, usually together with *Staphylococcus aureus* and *Streptococcus viridians*.(27) The presence of bacteria recovered from swabs obtained from the middle meatus of healthy subjects suggest that

these organisms might be commensals, only suggestive of being a source of infection when associated with significant number of organisms. The above stated study also reported the presence of Gram-negative enteric rods which were not normally found in the middle meatus of healthy subjects. This suggests a colonization or secondary infection occurs due to defective host immunity. However, more recent research suggests that CRS maybe as a result of dysbacteriotic microbiome as well as other factors such as host factors, physiology.(15) Such defects might be secondary to ineffective mucociliary function manifested in conditions such as cystic fibrosis or CRSwNP.

While there appears to be an agreement concerning the possible microbiology in acute rhinosinusitis (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*), CRS microbiology appears quite broad. This might be related to the difficulty of summarizing the wide array of organisms recovered from the investigated sinuses. Factors associated with such difficulties include, the variety of sampling methods (ranging from aspiration, to irrigation, swab, biopsy); the sterility of the nasal cavity and sinuses where the endoscope passes prior to reaching the sinus under investigation; variations in the sinuses being sampled (ethmoidal bullae, maxillary antrum, middle meatus); a lack of quantification of bacteria; previous or current antibiotic use and variations with patient selection (patient and culture factors).

1.4 Treatment of Chronic Rhinosinusitis (CRS)

The treatment of CRS often involves the use of surgical and medical/pharmacological agents.(27) The aim of surgery is to correct anatomical barriers thereby re-establishing the natural drainage pathway in the sinuses by widening its natural drainage, and allowing for a more effective natural

drainage pathway. This is carried out endoscopically (endoscopic sinus surgery) after appropriate patient selection and adequate pre-operative workup and after failure of appropriate and maximal medical therapy. Medical therapy involves the use of topical and oral steroids, topical and oral antibacterial and antifungal agents, immunotherapy and other treatment modalities (antileukotrienes and manuka honey rinses). Both treatment modalities tend to complement one another to ensure successful treatment outcomes.

According to current guidelines, glucocorticoids (topical and short-term oral medications) and antibiotics, mainly long-term use antimicrobials (macrolides and doxycycline) are recommended for recalcitrant chronic sinusitis patients that have failed medical and surgical management. The challenges include the observation of failures with these medications especially glucocorticoids, even with CRSwNP patients.

Individual variations in responses are captured by the concept of endotypes which represents a subtype of a disease entity with discrete functional, phenotypic and outcome characteristics. Endotypes of CRS have been proposed based on cytokines such as IL4/5 and 13 which also include: non-type Th2; moderate type Th2 and severe type Th2.(28) Endotypes may also refer to certain classifications such as the type 2 cytokine-based approach; eosinophil-mediated approach; Ig E-based approach and cysteinyl leukotriene-based approach. Due to their discrete mechanisms and clinical presentation, endotypes might assist clinicians to better assess prognosis, improve patient selection for specific treatment options, and determine the risk for comorbid conditions such as asthma.(29)

1.5 Treatment intervention (Use of Antimicrobial Photodynamic Therapy, aPDT)

Photodynamic therapy (PDT) refers to a photochemical method that uses cytotoxic reactive oxygen species (ROS) generated from a non-toxic photosensitizer (PS) in the presence of light waves and ambient molecular oxygen to destroy susceptible cells. This reaction occurs at specific wavelength dependent on the photosensitizer utilized.(30) The production of superoxide and hydroxyl radicals from these reactions will result in the destruction of the cells being targeted.(31) Antimicrobial Photodynamic Therapy (aPDT) refers to the application of these oxygen radicals for clinical use enabling destruction of microorganisms including bacteria. It has been shown to be lethal against all classes of microorganisms: Gram-positive, Gram-negative bacteria, fungi, viruses, parasites and even spores.(32)

Antimicrobial Photodynamic therapy (aPDT) has been used in dentistry as a therapeutic tool for a several years and is now being utilized for other anti-infective indications. A range of photosensitizing (PS) molecules have been used with phenothiazinium dyes (methylene blue, toluidine blue, and PP904) being the most common. No matter the location of the topical infection, the mechanism of action of aPDT remains the same (the generation of ROS using PS in the presence of ambient molecular oxygen). In addition to its antimicrobial effects, aPDT also demonstrates a marked anti-inflammatory effect.(33) aPDT has been shown to effectively treat the polymicrobial biofilms involved in CRS.(34)

Various investigators have performed initial aPDT studies of CRS. In a small study of 23 post-surgical CRS patients indocyanine-green was used as a photosensitizer and excited with an 810 nm laser. Patients demonstrated clinical improvement with no adverse effects.(35) In a large study

of 140 patients (65 acute and 75 chronic) that were treated with 0.1% methylene blue, there was a significant improvement in more than 50% of participants.(36) There have been numerous other studies showing the efficacy of PDT for the treatment of various infections(32) including periodontitis,(37,38) wound infection,(39) oncology(40,41) dermatology(42), and gynecology.(43)

1.6 Management outcomes

1.6.1 Clinical tools

1.6.1.1 Endoscopic Symptom Scores (ESS)

The diagnosis of CRS can be made fairly challenging by the presence of related differential diagnosis such as allergic rhinitis, vasomotor rhinitis, anatomic nasal dysfunction (such as, nasal septal deformity). Direct visualization using endoscopy (the first-line confirmatory test) and radiological intervention with the aid of Computerized Tomography (CT) scanning (especially for patients with prolonged or complicated clinical course), has been quite helpful.(2) The presence of similar symptomatology to CRS, of the above listed differentials of CRS, often requires the use of instruments that are reproducibly able to make distinctions and also are able monitor the progression of sinus disease. The use of endoscopic symptom score (ESS) represents a useful tool for documenting findings of endoscopic findings from the various parts of the nose and paranasal sinuses.

A variety of endoscopic symptom scores have been described in the literature. The Lund-Kennedy endoscopy scoring grade was one of the earliest universally accepted endoscopic symptom scores and continues to remain widely utilized.(44) This was based on the visual presence of discharge,

scarring, polyps, edema and crusting. This was found to be particularly useful in patient's clinical status, pre and post endoscopic sinus surgery especially in patients with CRSwNP. Another relatively new grading was proposed by Durr et al, and described as being simpler is based on presence of discharge, inflammation and polyps/edema (DIP).(45) It was associated with substantial test-retest and interrater reliability in the post-endoscopic sinus surgery population.

An improvement, proposed by Psaltis et al, of the Lund-Kennedy Endoscopic Symptom Score (Modified Lund-Kennedy, MLK), which was derived based on a comparison between the Lund-Kennedy, DIP and Perioperative sinonasal ESS.(46) This modification of the Lund-Kennedy score excluded both scarring and crusting as components of the score. It was found to be correlate with patient-reported outcome measures (PROMs) utilized, which were Visual analogue scores (VAS) and Sinonasal Outcomes Test (SNOT-22).

A major shortfall in the current modified MLK scoring system is that it scores each side of the nose as a whole without separating the inflammation within each of the different and independent sinuses. With the current understanding in sinus anatomy and physiology, this is grossly inadequate. Therefore, a scoring system was developed at the St. Paul's Sinus Centre to highlight the importance of scoring each sinus independently. This was based on a previously validated scoring system, the Philpott-Javer endoscopic scoring system for patients with Allergic Fungal Rhinosinusitis.(47) The Alsaleh-Javer Endoscopic Symptom Score (AJESS) is utilized extensively at the St Paul's Sinus Centre, Vancouver BC with good results. It scores each sinus independently based on the presence of edema and polyposis (mild to severe disease) on a scale of 0 (no edema/polyposis) to 5 (moderate/severe polyposis). Each level of increase in inflammation is given

an additional value of 1 point. In addition to this, points are added for presence of crust, recirculation of mucous, synechia, purulence, mucin, and access into the sinus. An additional score of 1 point is given for mild vs 2 points for moderate/severe disease (e.g. slight purulence would add a score of 1 to the total vs thick purulence would add a score of 2 to the sinus being evaluated). (Appendix C) This scoring system is currently undergoing validation for formal publication.

1.6.1.2 Sinonasal Outcomes Test (SNOT-22)

The desire to measure outcome metrics that assesses the change in health status following health interactions from a patient's perspective informed the creation of the SinoNasal Outcomes test (SNOT-22). Outcomes questionnaires are often referred to as Patient-reported outcome measures (PROMs) and have strong validation in research and clinical settings. This differs from instruments that measure process metrics, that indicate the level of adherence to best practice guidelines but are not necessarily a measure of health outcomes. In the rhinologic literature, SNOT-22 is the most commonly used sinus-specific quality of life (QOL) outcomes instrument.(48,49)

This instrument evolved from the original twenty questionnaire SNOT-20. The additional questions on the sense of taste/smell and nasal obstruction resulted in a 22-questionnaire instrument.(50) SNOT-22 consists of 22 questions which are rated on a Likert-scale of between 0 to 5, with 5 representing the worst score for each domain. The total score ranges from 0 to 110 and higher recorded scores represent poor QOL. The generally accepted minimal clinically important difference (MCID) is a change of 8.9 (approximately 9). While this score tends to improve following treatment interventions (such as surgery), the presence of standardized parameters that serve as a basis for comparison are often useful.

The SNOT-22 scores post sinus surgery could be influenced by a variety of factors as identified in the meta-analysis study conducted over an 8-year period (2008 to 2016) by Soler et al.(51) These factors include; baseline SNOT-22 score, asthma prevalence and duration of follow up.

1.6.1.3 Smell test

Olfaction is one of the most important nasal functions for man. The study by Hummel et al(52), provides data on the correlation of normal olfactory threshold with age. They interestingly, also found better olfactory discrimination in females compared with men. Largely, the testing of olfactory function is more research-related than clinical. A variety of tests have been utilized and one of the most popular and widely available is the *University of Pennsylvania Smell Identification test* (UPSIT, under the tradename, Smell Identification Test).(53) It consists of 4 booklets that each contain 10 pages of encapsulated scratch and sniff odors, making it a total of 40-items tested. Consequently, patients are graded in terms of severity as being anosmia, severe, moderate and mild microsomia. (details seen in Appendix B). The test is simply an identification test and is not a test for smell discrimination or threshold.

Another popular and more intensive test for olfaction is the *Sniffin' sticks* test which is centered on nasal chemosensory function-based odor-emitting devices. The score involves testing for three main parameters, namely, threshold (T), discrimination (D) and odor identification (I).(50) The subsets of parameters are then collated together to yield a TDI sum score. Recent European studies involving 9139 healthy subjects, established hyposmia at a TDI score of <30.75.(54) As noted, earlier age appears to play a role as with olfactory function. Findings recorded show that, while

20-30-year-old performed best, children within the first decade and adults in the eighth decade of life, scored only half as well.

1.6.1.4 Nasal Mucociliary Clearance Test (Saccharin test)

The anatomical position of the nose and paranasal sinuses exposes it to the environment through the anterior nares. These therefore serves as a first line of innate defense for the body. This function is enhanced by the presence of a mucociliary clearance system. The system is composed of the cilia which are projections of epithelial cells in the mucosal epithelium, that beats (wave in a concerted fashion) towards the natural ostium (sinuses) and then towards the nasopharynx and lower respiratory tracts. The other component of the system is the mucus layer, made up of an outer gel layer (which traps the foreign particles including microbes) and the inner sol layer into which the cilia are located. This system coordinates an efficient ‘conveyor belt’ that assists in the removal of inhaled substances.

The function of this natural mechanism can be affected by a number of factors. These include, age, anatomical factors (such as septal deviation), congenital conditions (Kartagener syndrome, a form of primary ciliary dyskinesia), the presence of inflammation and infection, social habits (smoking), physical activities and some medications (enhanced by salbutamol and reduced by sodium chloride) and environmental conditions (such as the temperature, altitude and humidity).(55)

The initial nasal mucociliary clearance (NMC) test using saccharin was carried out by Andersen et al in 1974.(56) A modification was later made by Rutland et al in 1984(57), which is described as the gold-standard. It consists of the placement of saccharin granules on the surface of the inferior meatus. The popularity of saccharin (benzoic sulfimide) as a test tool was due to its potency, being

300-500 times as sweet as sucrose. Hence, the time taken to taste a sweet sensation at the tongue base, is recorded as an approximate test of the mucociliary function. Usually a period of up to 20 minutes is accepted as normal but variations exist due to demographic characteristics.(58) A study in Spain recorded the upper and lower limits of normal as being between 6-36 minutes, respectively.(59) The duration of NMC is described as normal (up to 20 minutes); prolonged (21 to 30 minutes); severely prolonged (31 to 60 minutes) and grossly prolonged (> 60 minutes).

1.6.2 Laboratory tools

1.6.2.1 Confocal Laser Scanning Microscopy (CLSM)

The need to be able to understand the ultrastructure of the sinonasal environment has resulted in the use of microscopy to obtain a better view of what is actually happening on the mucosal membrane surface. The possible role of biofilms in the pathogenesis of CRS has made this need even more important as it provides microorganisms (both bacteria and fungi) the ability to evade treatment modalities resulting in disease chronicity.

For sinus disease, the methods previously utilized have largely involved the use of the electron microscopic techniques. This involves of electron microscopy occurs as either transmission electron microscopy (TEM) or scanning electron microscopy (SEM). Ferguson et al(60) demonstrated the presence of biofilms in patients with bacterial CRS using TEM. The result revealed the *Pseudomonas aeruginosa*, surrounded by glycocalyx, which were refractory to culture-directed antibiotics. This was highly suggestive of the role that biofilms played in CRS. Sanclement et al(61) also demonstrated the presence of biofilms in the specimens of the mucosa of patients undergoing surgery for CRS, by using SEM and TEM. Biofilms were absent in the

controls (patients without CRS) and overall demonstrated better microscopy using TEM as opposed to SEM. The study carried out by Cryer et al(62), using SEM revealed patients biofilms containing *Pseudomonas aeruginosa*, which is a prominent biofilm former.

The confocal scanning laser microscopy (CLSM) refers to a technique that utilizes laser-based point illumination at high power with precise wavelengths. The key feature of CLSM is its ability to analyze samples at varying focal depths and to combine all images into a 3-dimensional representation. It has the advantage of being able to image live biofilms at several timelines in a non-destructive manner, and thus provide an 'optical biopsy'. CLSM can also be utilized with a variety of dyes to determine the biofilm composition and whether cells are alive or dead. Psaltis et al(20,63) showed that the major advantages of CSLM compared to conventional electron microscopy is the avoidance of flaws that occur with tissue propagation, orientation and analysis.

1.6.2.2 Microbiome analysis (16S rRNA sequencing)

The sequencing of 16S ribosomal ribonucleic acid (rRNA) is a useful tool in identifying bacteria at the level of species and gives information that helps in delineating between related species. The 16S rRNA gene codes for the RNA component of the 30S subunit of the bacterial ribosome, and the sequence of this gene contains both conserved and variable regions the latter of which vary according to bacterial species. Sequencing these variable regions after PCR amplification represents an important tool in bacterial taxonomy classification, serving as a phylogenetic marker. The use of 16S rRNA in phylogenetics was first described by the work of Carl Woese et al.(64) The technique has the major advantage of being able to measure phylogenetic relationships across different taxa and possessing universal distribution. It is not without its own drawbacks, the key

of which is the inability to discriminate between closely related species correctly and it may also exaggerate bacterial estimates.

1.7 Objectives

I proposed here to study the safety and efficacy of the aPDT treatment in recalcitrant CRS sinus cavities associated with biofilms. I aimed to:

1. Determine if the use of aPDT therapy improves endoscopy scores by at least 1-point over a 45-day period, as measured using the Alsaleh-Javer Endoscopic Symptom Score (AJESS).
2. Determine if the use of aPDT therapy improves the patient's well-being and specific quality of life score by at least 9-points in the 45-day period, using the Sino-Nasal Outcome Test 22 (SNOT-22) questionnaire.(48,51)
3. Determine if the use of aPDT changes the type of microorganisms in cultured sinus secretions and swabs, over the 45-day period.
4. Determine the safety of aPDT therapy based on:
 - a. Patient reported adverse events using the Visual Analog Scale (VAS) for Pain and adverse event questionnaire
 - b. Change in olfaction based on the UPSIT smell test.(53)
5. Determine if aPDT eliminates the biofilm seen in sinus mucus aspirates using confocal laser scanning microscope (CLSM).(63)
6. Determine if aPDT therapy improves the functionality of sinus cilia using the Nasal Mucociliary Clearance test (Saccharin test).(56,57)
7. Determine the mechanism of aPDT using 16s rRNA sequencing to examine changes in sinonasal microbiota over the 45-day period.(64)

1.7.1 Research Questions

1. Does aPDT treatment of refractory CRS patients improve patient outcomes within 45-day period?
2. Determine the safety of aPDT for refractory CRS patients.

1.7.2 Hypothesis

I hypothesized that endoscopic mucosal inflammation, discharge, and recurrence of polypoid edema as quantified by the Alsaleh-Javer Endoscopic Symptom Score (AJESS), as well as biofilm formation, can be significantly improved after treatment with aPDT in a cohort of refractory CRS patients.

1.7.3 Justification

The burden of Chronic Rhinosinusitis (CRS) constitutes a huge financial burden to any healthcare system, despite variations between countries.(10,65) This makes the issues regarding its prevention and possible control of crucial importance to healthcare delivery at all levels. The failure of the standard of care (current medical and surgical therapies) not only contributes to prolongation of the disease with its consequent morbidities, but may also result in further extension of the infection to involve intracranial and orbital regions. The resultant effect maybe cavernous sinus thrombosis and intracranial and orbital abscess formation.(66)This creates a justification for exploring other viable alternatives.

A study carried out by Biel et al(34), which was performed in-vitro was conducted on maxillary sinus biofilm. This demonstrated a >99.99% reduction in CRS polymicrobial biofilm after a single aPDT treatment. The study reported here which is an in-vivo study, aimed at determining the safety and efficacy of aPDT in patients with recalcitrant CRS, attending out-patient clinics at the St Paul's Sinus Centre and the False Creek Health Centre.

Chapter 2: Materials and Methods

2.1 Study design and background of study site

The study was a prospective pilot study conducted on participants with recalcitrant CRS. This cohort had failed to resolve their clinical issues despite medical management involving advanced endoscopic sinus surgery and maximal standard of care medical management. The diagnosis was based on sinus endoscopy, culture screening, endoscopic scores and SNOT-22 outcomes performed by an advanced team of subspecialist sinus physicians at St. Paul's Sinus Centre; and also, the presence of biofilm based on the physician's observations (a discolored and slimy film layering over the affected sinus mucus membrane).

The study was conducted at two (2) specialized Otolaryngology (Rhinology) facilities. The outpatient clinic of the St Paul's Sinus Centre, Vancouver, British Columbia. It is located in the downtown Vancouver area within the St Paul's hospital complex. The Sinus Centre is a 'Centre of Excellence' where cutting-edge diagnostic and therapeutic procedures as well as research, are conducted, offering a diverse range of state-of-the-art services to British Columbians and beyond. The other facility used was the False Creek Healthcare Centre, also located in the downtown area of Vancouver and is considered an extension of the work done at the St Paul's Sinus Centre with the same physicians providing services at both facilities.

2.2 Eligibility criteria and outcome measures

2.2.1 Inclusion criteria

1. Patients over the age of 19 years
2. Diagnosed with CRS (with/without polyps) and have previously undergone functional endoscopic sinus surgery (post-FESS).
3. Patients who had continued to fail despite receiving ‘maximal medical treatment’. This was defined as patients who,
 - a. had received topical nasal steroids in any form (spray/rinse/atomized) for at least 3 months;
 - b. had at least one course of oral corticosteroids treatment (1 mg/kg for 2 weeks tapering dose)
 - c. had received at least one course of antibiotics treatment either based on culture and sensitivity of nasal secretions or had received:
 - long-term dose macrolide therapy (clarithromycin 500mg twice a day for 2 weeks, then 250mg once a day for 4 weeks) for at least one trial, or
 - had received a course of Itraconazole 100mg twice daily for 6 weeks with liver function monitoring for at least one trial (for Allergic fungal sinusitis, AFRS patients).
4. Patients with deteriorating SNOT-22 scores or who had not improved after surgery and appropriate maximal medical management.

2.2.2 Exclusion criteria

1. Patients with a clinical diagnosis of sinonasal tumors.
2. Patients with autoimmune diseases affecting the upper airway e.g Systemic Lupus Erythematosus (SLE), Sjögren's syndrome, Systemic Sclerosis etc.
3. Immune-compromised patients, and impairment in mucociliary function (e.g., cystic fibrosis, Kartagener syndrome)
4. Patients unable to speak English.

2.3 Outcome measures and safety evaluation

2.3.1 Primary outcome measures

Primary Aims. Assess the efficacy of aPDT based on the criteria stated below:

1. Improvement in endoscopic score. An improvement was defined as a reduction in Alsaleh-Javer Endoscopic Symptom Scores (AJESS) by at least 1 point during the 45-day period;
2. Improvement in disease-specific quality of life assessments, where a reduction in the Sino-Nasal Outcome Test 22 (SNOT-22) questionnaire score of 9 or greater within the 45-day post aPDT therapy period was defined as clinical success in this study.
3. Improvement in culture swab results within the 45-day period.

2.3.2 Secondary outcome measures

2.3.2.1 Secondary aim. Evaluate the safety of aPDT in CRS patients

The safety of aPDT was examined by identifying any adverse events, including serious adverse events (defined as any untoward adverse event/adverse reaction that at any dose resulted in death, was life threatening, required inpatient hospitalization or prolongation of existing hospitalization, resulted in persistent or significant disability/ incapacity, or resulted in other medically important events) for all study subjects. Assessment of adverse events was based on clinical history, adverse event questionnaire, patient report, VAS questionnaire, reduction in olfaction based on the UPSIT smell test, and any inflammation or mucosal changes on endoscopy as a result of aPDT (administered on days 0 and 7) at study visits 14, 21, 30 and 45 days.

2.3.2.2 Secondary aim. Investigate aPDT mechanism with CLSM and 16S rRNA

This involved the use of metagenomics, CLSM and 16S rRNA sequencing to investigate the mechanisms through which aPDT can displace and eradicate CR- related pathogens (mainly *Staphylococcus aureus*, *Pseudomonas spp*, *Escherichia coli* and *Hemophilus influenzae*) from the chronically infected sinus cavity.

2.4 Ethical considerations

This prospective pilot study was conducted under the approval of the University of British Columbia – Providence Health Care Research Ethics Board (H17-00614). Personal information was preserved and no information identifying potential subjects was placed on the data collection sheets. Data excel sheets containing password-protected and anonymized outcomes data was stored at specific secure locations on a central computer. One set of Excel data sheet containing items identifying patient information was used and was stored on the centralized computer at all times. Another sheet that was stripped of all identifying information was used for analysis.

2.5 Consent

A clear explanation of the research study was made to the participants before they were recruited into the study. Informed consent was obtained from all participants prior to the commencement of the study.

2.6 Disclosures

© *Sinuwave* was manufactured and provided by Ondine Biomedical Corporation, Vancouver B.C.

The authors did not receive any financial compensation for this study.

2.7 Sample collection and handling

All study procedures and their respective timelines are described in Figure 2.1

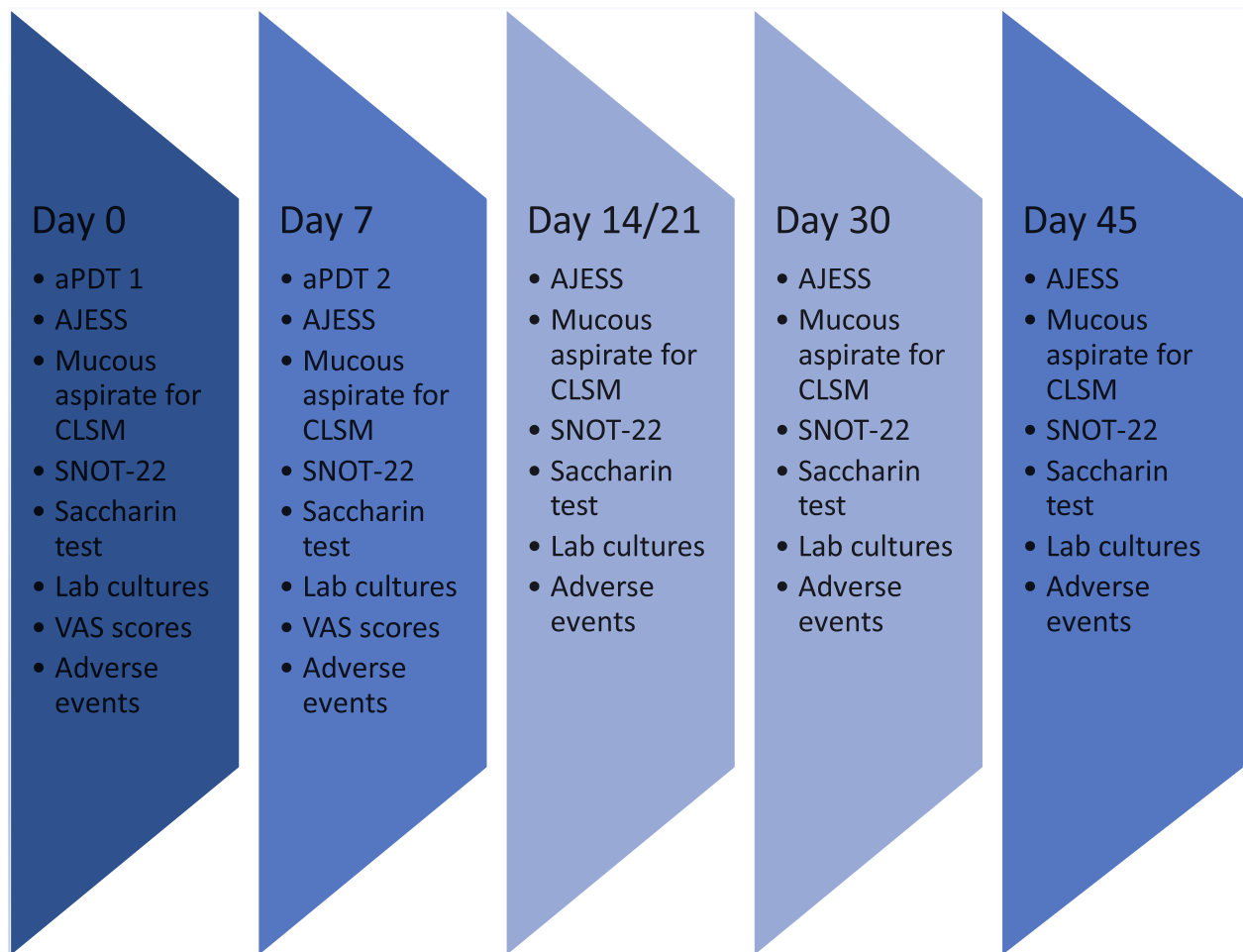


Figure 2.1 A summary of the study procedures and their respective timelines.

Patients who met the inclusion criteria and had been consented to participate in the study then underwent the following steps, prior (Day 0, Figure 2.1) to undergoing the aPDT procedure.

1. Baseline SNOT-22 questionnaire was completed by the participant and scored by the investigator afterwards (Appendix A).
2. UPSIT test was also administered to the participant in order to determine their smell (cranial nerve 1) function (Appendix B).
3. Nasal mucociliary clearance test (NMCT) was carried out with the aid of Saccharin (a few granules) applied to the inferior meatus of the nasal cavity of interest with the aid of a clean wooden applicator which had been moistened with saline. The time taken for the saccharin to be tasted by the patient was recorded as the test result. For the purpose of this study, the duration of NMC was described as normal (up to 20 minutes); prolonged (21 to 30 minutes); severely prolonged (31 to 60 minutes) or grossly prolonged (> 60 minutes).
4. AJESS was determined with the aid of a 3mm 30-degree endoscope and each of the sinus cavities of interest (maxillary, ethmoid, frontal and sphenoid) were scored based on the presence of edema and polyposis (mild to severe disease) on a scale of 0 (no edema/polyposis) to 5 (moderate/severe polyposis). Each score was given a value of 1. Additional scores were given for the presence of crust, recirculation of mucous, synechia, purulence, mucin, being narrow. A score of 1 point (lower case symptom representation, mild disease) and 2 points (upper case symptom representation, moderate/severe disease). (Appendix C).
5. Sinus mucosal aspirate was obtained from the involved sinus walls and cavity with the aid of a sterile suction trap from suspected areas. These samples were processed aseptically, transported to the Centre for Microbial Diseases and Immunity Research at UBC, and stored as per the laboratory protocol (Appendix D). The following tests were conducted on the aspirate: a. Analysis for detection of Biofilm using Confocal Scanning Laser Microscope (Appendix E); b. DNA extraction. The mucus samples from the pre- and post-aPDT treatments were received and stored at -80C. The samples were thawed and their DNA extracted using the QIAmp PowerFecal Pro DNA Kit (Qiagen, Germantown, MD, USA). The samples were initially disrupted in a Bead Ruptor 24 (Omni international, Kennesaw, GA, USA) at power setting 6, for 2X 30 s increments. The samples were then processed according to the protocols outlined in the manual. The DNA was quantified on a Nanodrop 1000 (ThermoFisher Scientific, Waltham, MA, USA); c. 16S Ribosomal RNA (rRNA) sequencing. 16S amplicons were sequenced using the Illumina MiSeq which was executed by the Integrated Microbiome Resource (IMR) Centre at Dalhousie University, Halifax, NS. The Human Microbiome Project mock community HM-782D (BEI Resources, ATCC, Manassas, VA) and *Pseudomonas aeruginosa* PA01 were used as positive controls; d. 16S Ribosomal RNA (rRNA) analysis. Data analysis was executed using the Qiime2 (version 2018.8). Sequence quality was high with a random sampling of

9963 reads out of the entire set of 2.3 million reads. Quality control was carried out using DADA2 with filtering of lower quality regions of the sequence by using a cut-off of position 270 for forward reads and 220 for reverse reads. There were no significant drops in the number of reads obtained during any of the stages.

6. Laboratory culture swabs were obtained from the involved sinus and sent for microscopy, culture and sensitivity. This was processed for each participant and a record of the quality and estimate of the quantity of organisms recovered (bacteria and fungi) was made afterwards.
7. A Visual Analogue Score (VAS) for pain and discomfort was recorded before and after each aPDT procedure. The VAS was on a scale of 0 to 10, 0 - No pain; 1-2 Mild pain, 3-5 Moderate pain, 6-8 Moderately severe and 9-10 Severe/Worst pain possible. The form of pain included pricking pain, stinging pain, cramping pain, throbbing pain and stabbing pain. While the VAS for discomfort was also rated from 0 to 10. (Appendix F)
8. Presence of any serious adverse events which are graded from grade 1 (mild) to grade 5 (death) were recorded. In cases where this occurred, the outcome of such adverse events, form of intervention given, relationship with intervention and time of resolution were recorded. (Appendix G)

2.8 Anti-Microbial Photodynamic Therapy (aPDT)

This was administered to participants who met the inclusion criteria and had clinical evidence of biofilm formation. The procedure, performed using the instruments shown in Figures 2.2 and 2.3, was electronically video recorded for a comparison with post procedure results. Two treatments were given over a one-week period (time 0 and 7 study days respectively). Each treatment lasting for eight minutes. The aPDT procedure steps are as stated in Appendix H.

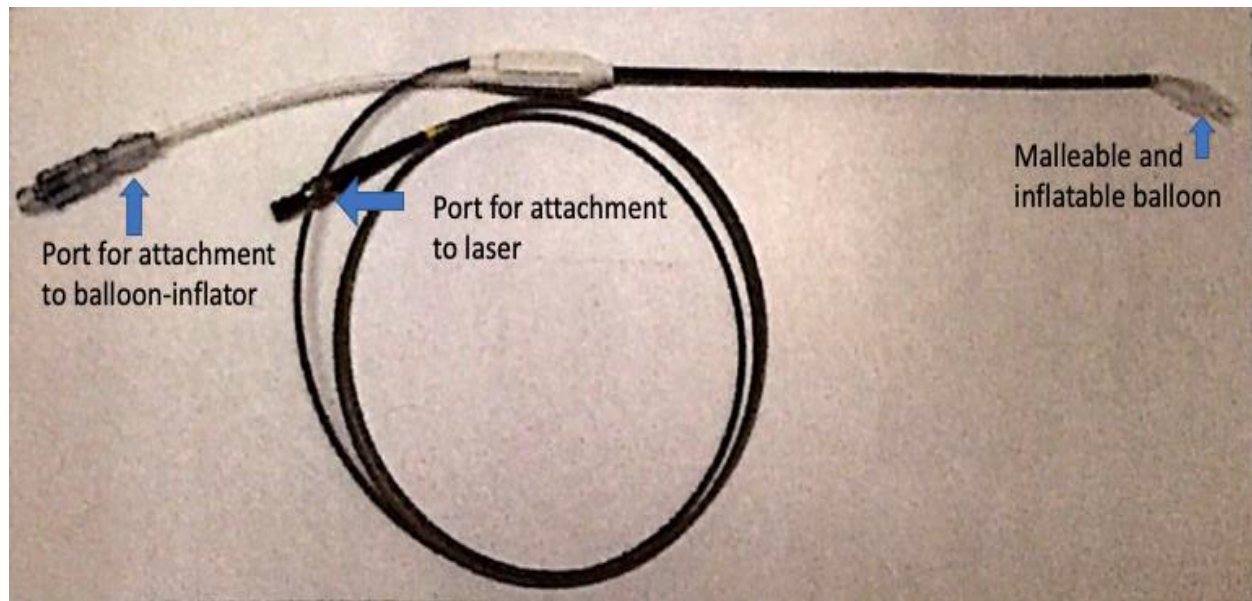


Figure 2.2 Sinuwave Light Delivery Catheter

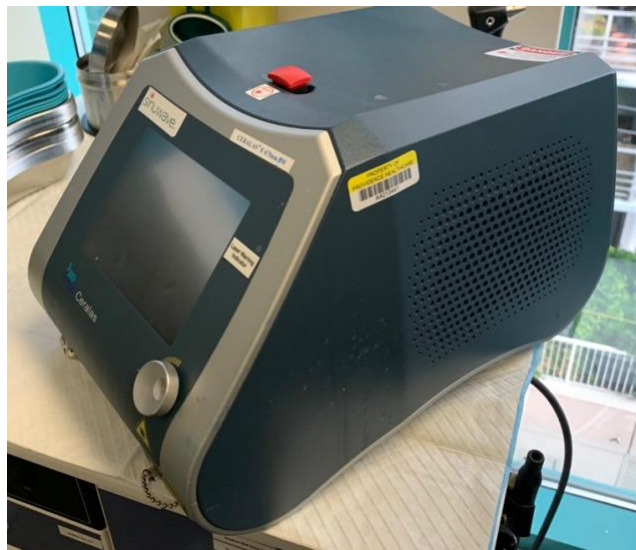


Figure 2.3 Sinuwave Laser Console

2.9 Sample handling in the laboratory

Sinus aspirate samples from a previously-diagnosed patients identified sinus with recalcitrant CRS disease were collected under aseptic conditions with the aid of a sterile suction trap. This trap was connected to a suction system and visualization was enhanced with the aid of 3mm 30-degree rigid nasal endoscope. A culture swab was also taken from the same area in the same sinus cavity. This was sent for culture in the routine hospital laboratory (Vancouver General Hospital, VGH microbiology laboratory). The results were obtained after one to four weeks, depending the organisms cultured (bacteria and fungi).

The sinus aspirates were divided into two equal parts. One part was fixed with 4% formaldehyde within a sterile Eppendorf tube. This was allowed to fix adequately for at least 24 hours after collection and stored in 4C fridge. The other part was placed in -80C freezer unwashed. This later had DNA extraction carried out and 16S rRNA sequencing done. Details of the sinus preparation protocol as stated in (Appendix D).

Chapter 3: Results

3.1 Socio-demographic and clinical characteristics

A total of 7 participants completed the pilot study. There was a slight male preponderance (4 men and 3 women) The age ranged from 47 to 78 years with mean age \pm SD, 60.3 years \pm 10.1. Two (2) out of the 7 participants (28.6%) had co-morbid conditions (Asthma), while all of the 7 participants were non-smokers. Patients with a clinical diagnosis of Chronic sinusitis without polyps (CRSSNP) accounted for 4/7 of the participants (57.2%) while those with Allergic Fungal Rhinosinusitis (AFRS) made up the remaining 3/7 (42.8%). The sinuses involved in treatment with the aPDT were the ethmoid and maxillary sinuses, with the maxillary sinus being the most involved (4/7, 57.2%). Both right and left maxillary sinuses were treated for 2 participants respectively. Conversely, the left and right ethmoidal sinuses were treated with aPDT for 2 and 1 participants respectively (3/7, 42.8%). The details are shown in **Table 3.1**.

Parameters	n = 7	%
Age range		
45 – 54	2	28.6
55 – 64	3	42.8
65 – 74	1	14.3
>75	1	14.3
Gender		
Male	4	57.2
Female	3	42.8
Co-Morbid condition		
Asthma	2	28.6
Nil	5	71.4
Diagnosis		
CRSsNP	4	57.2
AFRS	3	42.8
Sinus Involvement		
Right maxillary antrum	2	28.6
Left maxillary antrum	2	28.6
Right ethmoid	1	14.2
Left ethmoid	2	28.6

Table 3.1 Showing the Socio-demographics and clinical parameters of the study participants

3.2 Mean AJESS changes over the 45-day study period

The mean AJESS for all the 7 participants showed a reduction of 0.71 points from day 0 (3.71) to day-7 (3.00), which was between the 1st and 2nd treatment days. The changes between the completion of the aPDT treatment (day 7) and one-week post-treatment (day 14) showed a further reduction of 1.50 points (from 3.00 to 1.50). There was however an increase in the AJESS score between days 14 and 30 (0.64 points, from 1.50 to 2.14 points respectively). The final study period of day-45 revealed a reduction of 0.14 points, when compared with day-30 findings and an overall 1.71-point reduction compared to pre-treatment. The details are in **Figure 3.1**.

Statistical analysis was carried out to determine the significance between the AJESS and study timelines. Utilizing the Skillings-Mack (SM) test gave a p-value of 0.014 (statistically significant) was obtained. The SM test is a generalization of the Friedman test, which is a nonparametric test comparing paired groups. The test is quite useful with incomplete data as we had in our data set. To identify the exact timeline that was significant, a paired t-test was carried out. The timeline between day 7 and 14 had t-value of 2.468 (CI 0.009, 2.562) and a p-value of 0.049 (statistically significant). **Table 3.2**

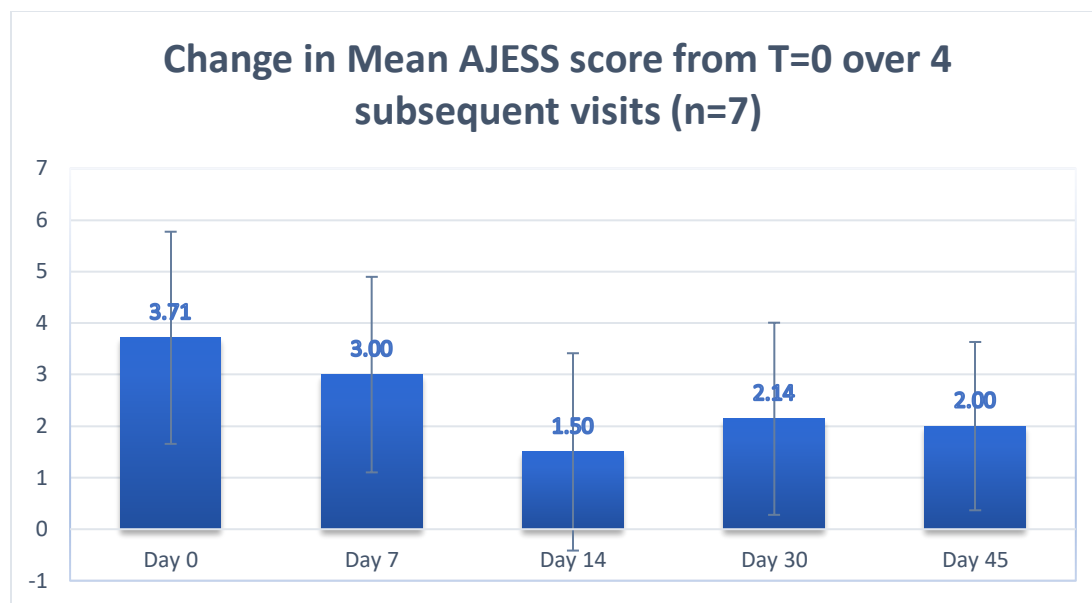


Figure 3.1 Showing changes in the mean AJESS scores over the 45-day study period, n=7

Study timeline	Paired t-test	95% Confidence Interval (CI)	p-value
Day 0 and Day 7	1.867	- 0.267, 1.980	0.111
Day 7 and Day 14	2.468	0.009, 2.562	0.049**
Day 14 and Day 30	-1.333	-1.620, 0.477	0.230
Day 30 and Day 45	0.186	-1.740, 2.025	0.825

Significant p-value - **

Table 3.2 Showing paired t-test and p-values for mean AJESS scores over the 45-day study period, n=7

3.3 Mean SNOT-22 score changes over the 45-day study period

The mean SNOT-22 scores for all the 7 participants showed a reduction of about 9 points from day-0 (44.53) to day-7 (35.57), which was between the 1st and 2nd treatment days. The changes between the completion of the aPDT treatment (day 7) and one-week post-treatment (day 14) showed a further reduction of 5.77 points (from 35.57 to 29.80). There was however an increase in the SNOT-22 scores between days 14 and 30 (7.20 points, from 29.80 to 37.00 points respectively). The final study period of day 30-45 revealed a reduction of 3.14 points, as compared with day-30 findings and an overall 10.57-point reduction compared to pre-treatment. The details are in **Figure 3.2**.

Statistical analysis between the SNOT-22 and study timelines was also carried out using the Skillings-Mack test, where a p-value of 0.208 (not statistically significant) was obtained.

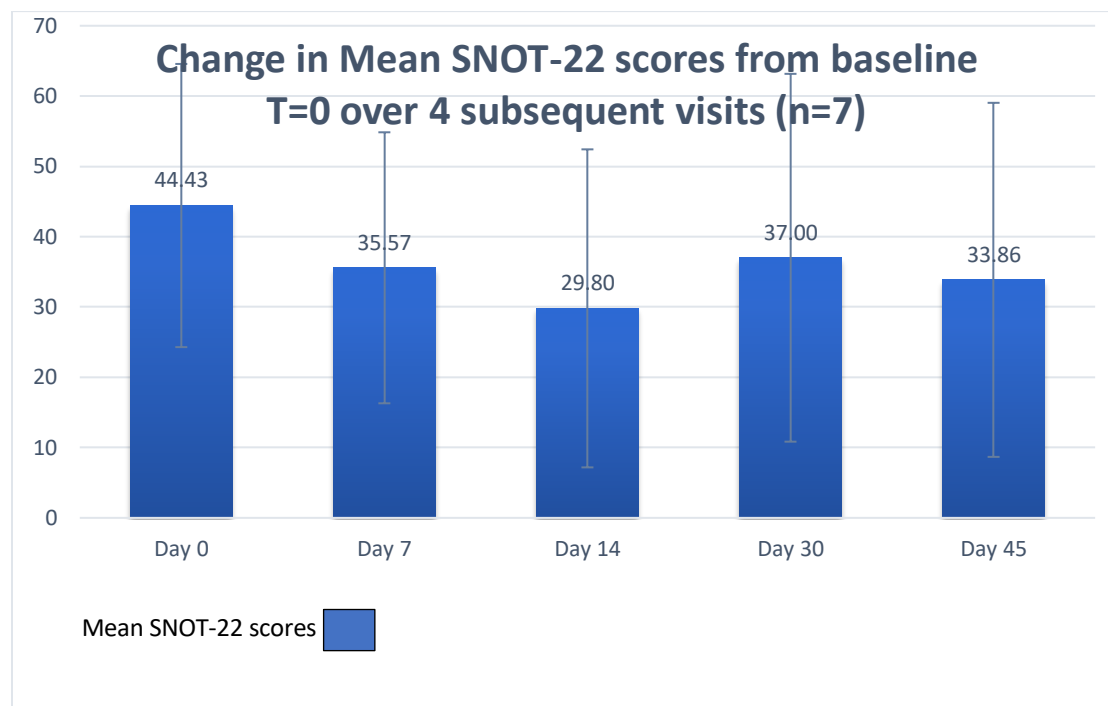


Figure 3.2 Showing changes in the mean SNOT-22 scores over the 45-day study period, n=7

3.4 aPDT safety based on Visual Analog Scale (VAS) for pain and discomfort with the adverse event questionnaire

The study recorded no adverse events. However, the pre and post-aPDT treatment findings of the pain questionnaire revealed that the level of pain ranged from mild pain for the pre-treatment group (0.54 to 1.38 points) to mild to moderate pain for the post-treatment group (1.79 to 2.29 points). Stabbing pain was associated with the highest difference between the pre and post-treatment pain status (1.52 points, from 0.77 to 2.29). This was followed closely by Stinging pain (1.50 points, from 0.54 to 2.14), then pricking, throbbing and cramping pain (1.17, 0.91 and 0.87 points) respectively. Details are seen in **Figure 3.3.1**.

One of the participants who appeared to be a far outlier with overly excessive pre and post-treatment pain scores. Excluding this one patient's data (n=6) resulted in an appreciable reduction in the pre-treatment range of pain VAS scores range (n=7, 0.54 – 1.38 points and n=6, 0.00 - 0.73 points) and for the post-treatment range of pain VAS scores range (n=7, 1.79 – 2.29 points and n=6, 1.00 - 2.16 points) respectively. Details are seen in **Figure 3.3.2**.

The VAS scores for the participant's discomfort level for the pre and post aPDT treatment groups revealed a difference of 2.28 points (from 1.30 to 3.58 points). Details are seen in **Figure 3.3.3**.

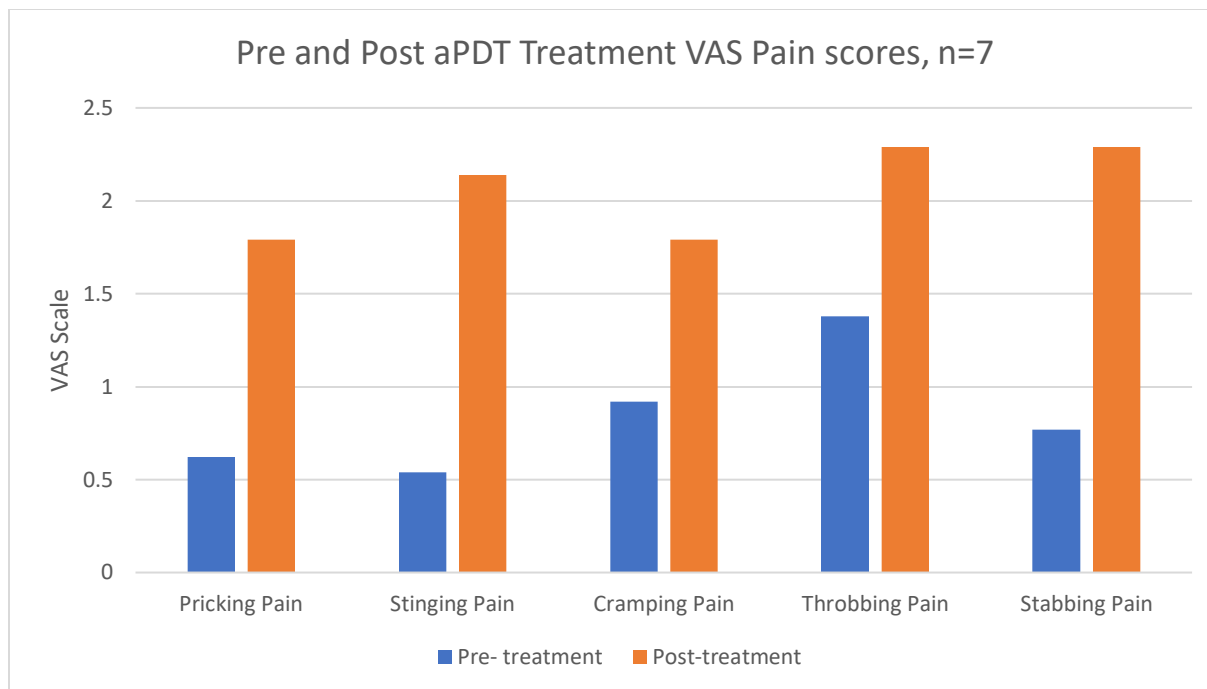


Figure 3.3.1 Showing pre and post aPDT Treatment VAS Pain scores, n=7

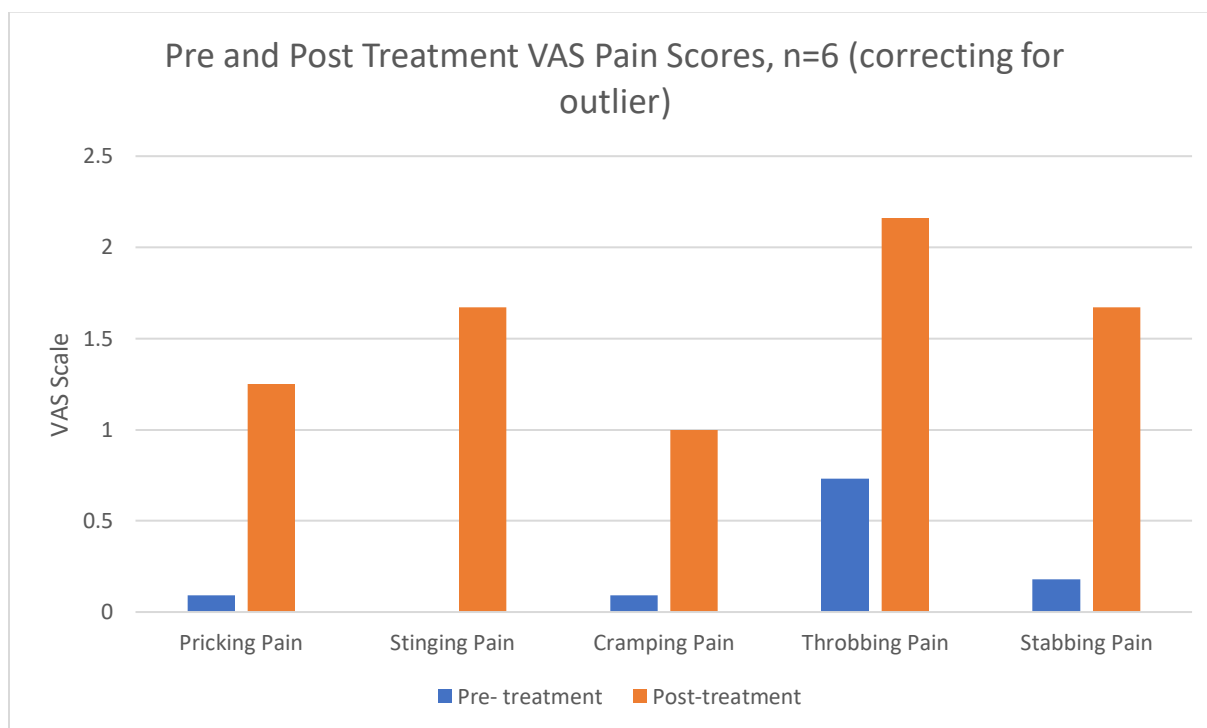


Figure 3.3.2 Showing pre and post treatment VAS Pain scores (correcting for an outlier), n=6

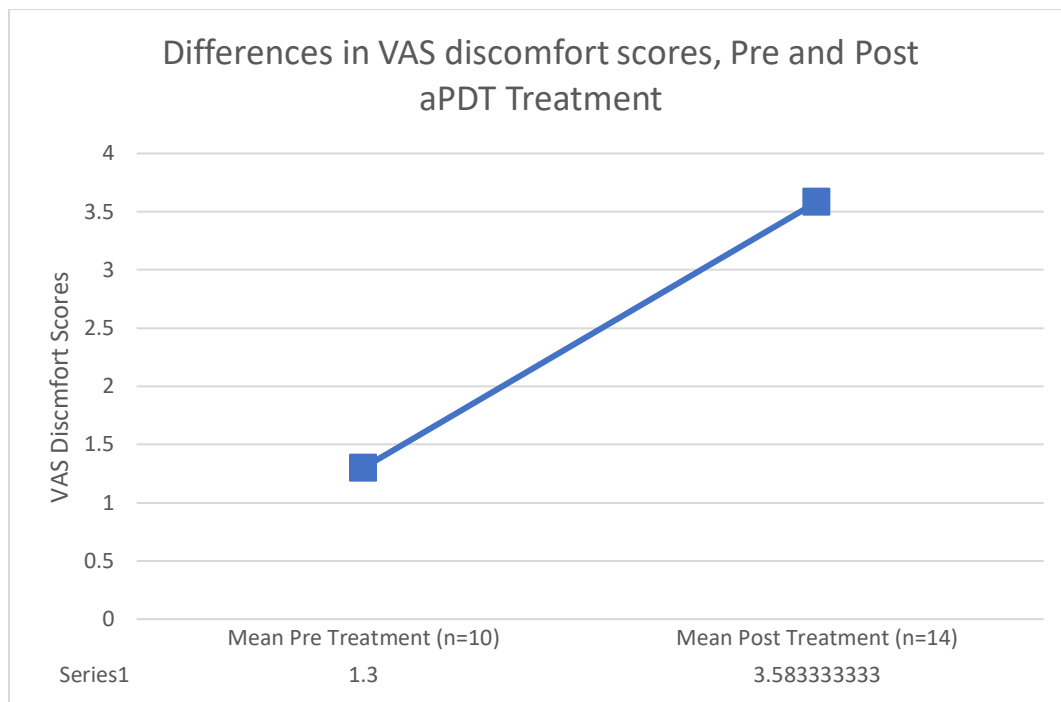


Figure 3.3.3 Showing differences in VAS discomfort scores for pre and post aPDT Treatment, n=7

3.5 Nasal Mucociliary Clearance Time findings, pre and post-aPDT Treatment

The findings of the Nasal Mucociliary Clearance Time (NMCT) showed that participants timing ranged from normal to severely prolonged. While participants 1, 3, 6 and 7 (PO1, PO3, PO6 and PO7) had NMCT within the normal range, participants 2, 4 and 5 (PO 2, PO4 and PO5) were either prolonged or severely prolonged. Participant 4 and 5 (PO4 and PO5) showed the most significant improvement (from 25 to 7 minutes and 24 to 4 minutes respectively) between the pre and post aPDT treatment period. There was minimal or no changes for the remaining participants. Details are seen in **Figure 3.4**.

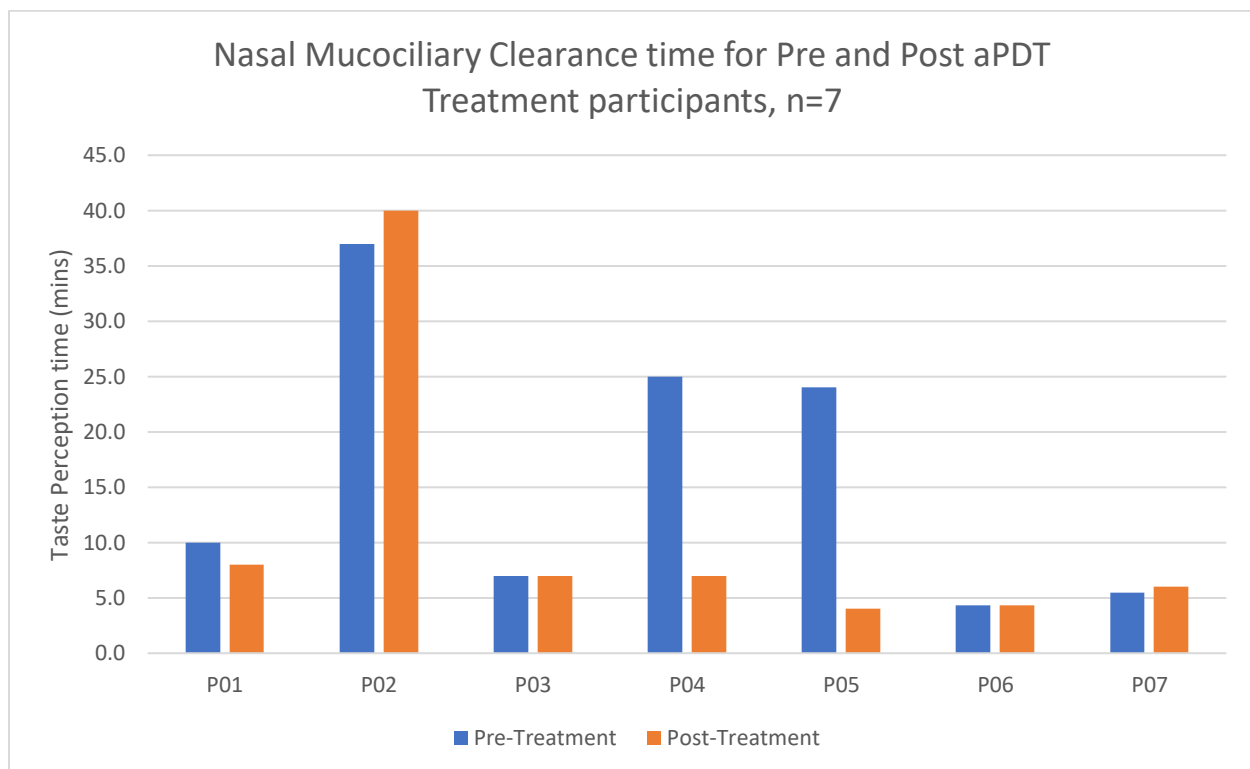


Figure 3.4 Showing the Nasal Mucociliary Clearance time for Pre and Post aPDT participants, n=7

3.6 Smell Test results using UPSIT, pre and post- aPDT treatment

Results of UPSIT from the participants showed that all the participants had a smell disorder that ranged from mild microsmia (diminished sense of smell) to total anosmia (loss of smell). Mild and moderate anosmia were reported in participants 1 and 6 (PO1 and PO6) respectively, while participant 4 (PO4) had severe anosmia and total anosmia was found for participants 2, 3, 5 and 7 (PO2, PO3, PO5 and PO7). There were no appreciable changes in smell status between the pre and post aPDT treatment period in all participants. Details are as seen in **Figure 3.5**.

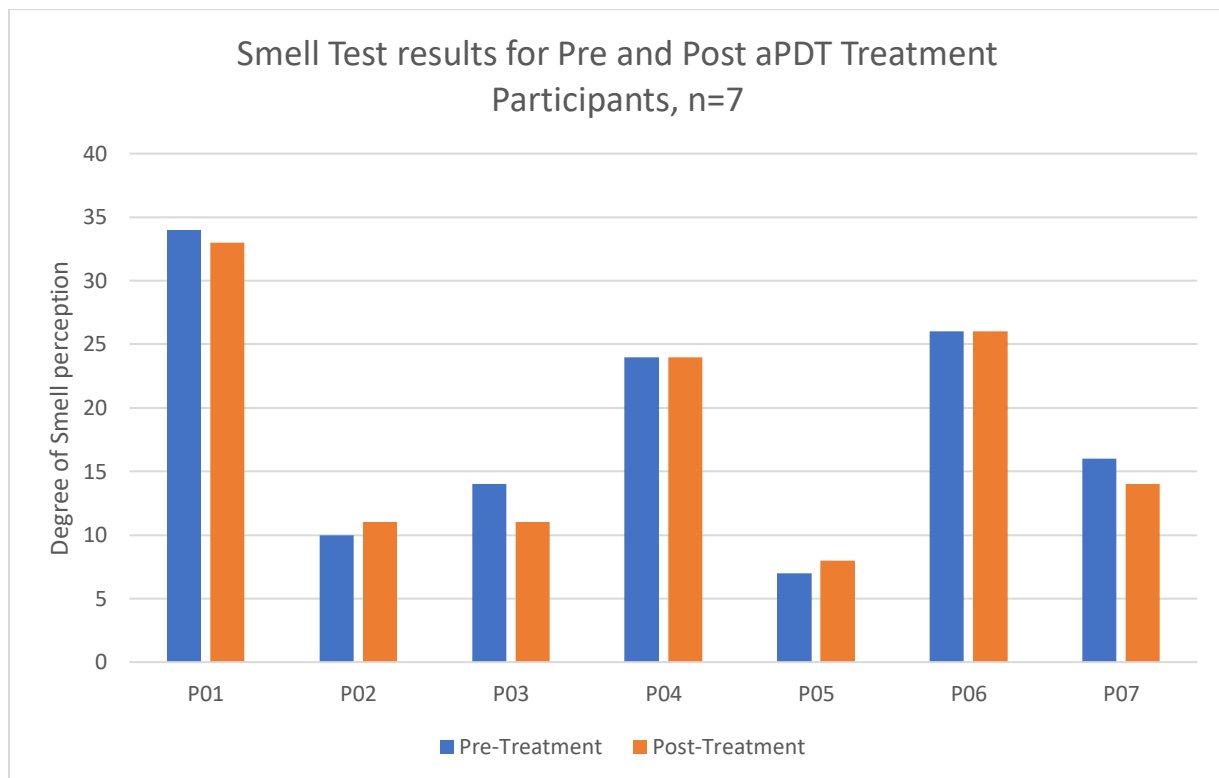


Figure 3.5 Showing the Smell test (UPSIT) results for participants, pre and post aPDT treatment, n=7

3.7 Laboratory Culture results, pre and post-aPDT treatment

The study observed a mixed growth of bacterial cultures within the sampled sinuses. The most common predominant bacterium cultured in the sinuses of the participants was *Staphylococcus aureus*, which was cultured in participants 2, 5, 6 and 7. *Hemophilus influenzae* was cultured in participant 1 and *Staphylococcus lugdenensis* in participant 7. A comparison of the Pre and post-aPDT cultures showed a reduction in the quantity of bacteria which was most evident between study days 14 and 30. Thereafter on day 45, there appeared to be an increase in the organisms cultured except for participant 6, where no organisms were cultured. Details are seen in **Table 3.2**.

No fungi were isolated within the sinuses cultured.

Study Timelines	Patient 01	Patient 02	Patient 03	Patient 04	Patient 05	Patient 06	Patient 07
Day 0	<i>H. influenzae</i> , +3	<i>Staph. aureus</i> , +2	<i>Staph. aureus</i> , +1	<i>Gram +ve cocci</i> , +2	<i>Staph. aureus</i> , +2	No organisms isolated	<i>Gram +ve bacilli</i> , +2
Day 7	<i>H. influenzae</i> , +3	<i>Staph. aureus</i> , +3	<i>Staph. aureus</i> , +1	<i>Gram +ve cocci</i> , +1	Not done	No organisms isolated	<i>Staph. lugdenensis</i> , +2
Day 14	Not done	<i>Staph. aureus</i> , +2	Not done	<i>Gram +ve cocci</i> , +1	<i>Staph. aureus</i> , +1	<i>Staph. aureus</i> , +2	No Organisms seen
Day 30	<i>H. influenzae</i> , +2	<i>Staph. aureus</i> , +2	No organisms isolated	No Organisms isolated	<i>Staph. aureus</i> , +3	<i>Staph. aureus</i> , +1	<i>Staph. aureus</i> , +1
Day 45	<i>H. influenzae</i> , +3	<i>Staph. aureus</i> , +3	<i>Gram +ve bacilli</i> , +2	<i>Gram -ve cocci</i> , +2	<i>Staph. aureus</i> , +3	No Organisms isolated	<i>Staph. lugdenensis</i> , +3

+1 – Mild Growth; +2 – Moderate Growth; +3 – Profuse Growth

Table 3.3 Culture results for the prominent bacteria recovered from the treated sinuses, pre and post-aPDT treatment, n=7

3.8 Confocal Laser Scanning Microscopy (CLSM) findings, pre and post-aPDT

The CLSM involved the use of BFL-Vancomycin (stained Gram-positive organisms), D-Polymyxin (stained Gram-negative) with a background bright field image, showing the host cells. All this was combined in the all panel to give a detailed picture of the CLSM images showing all its constituent stains. Details are presented in **Figure 3.6.1**.

Generally, the findings on CLSM were in agreement with the laboratory cultures for bacterial obtained from mucous aspirates from the sinuses. There were a few exceptions as seen in participant 1. In this patient, not all the organisms cultured (*Hemophilus influenzae*) were identified with CLSM. The details for participant 2 showing the CLSM findings for the different study times are seen in **Figure 3.6.2**.

Fungi was identified for participant 3 on days 0 and 30 days with the aid of Calcoflour white staining, but no fungal growth occurred in the clinical laboratory for this patient. Details as seen in **Figure 3.6.3**.

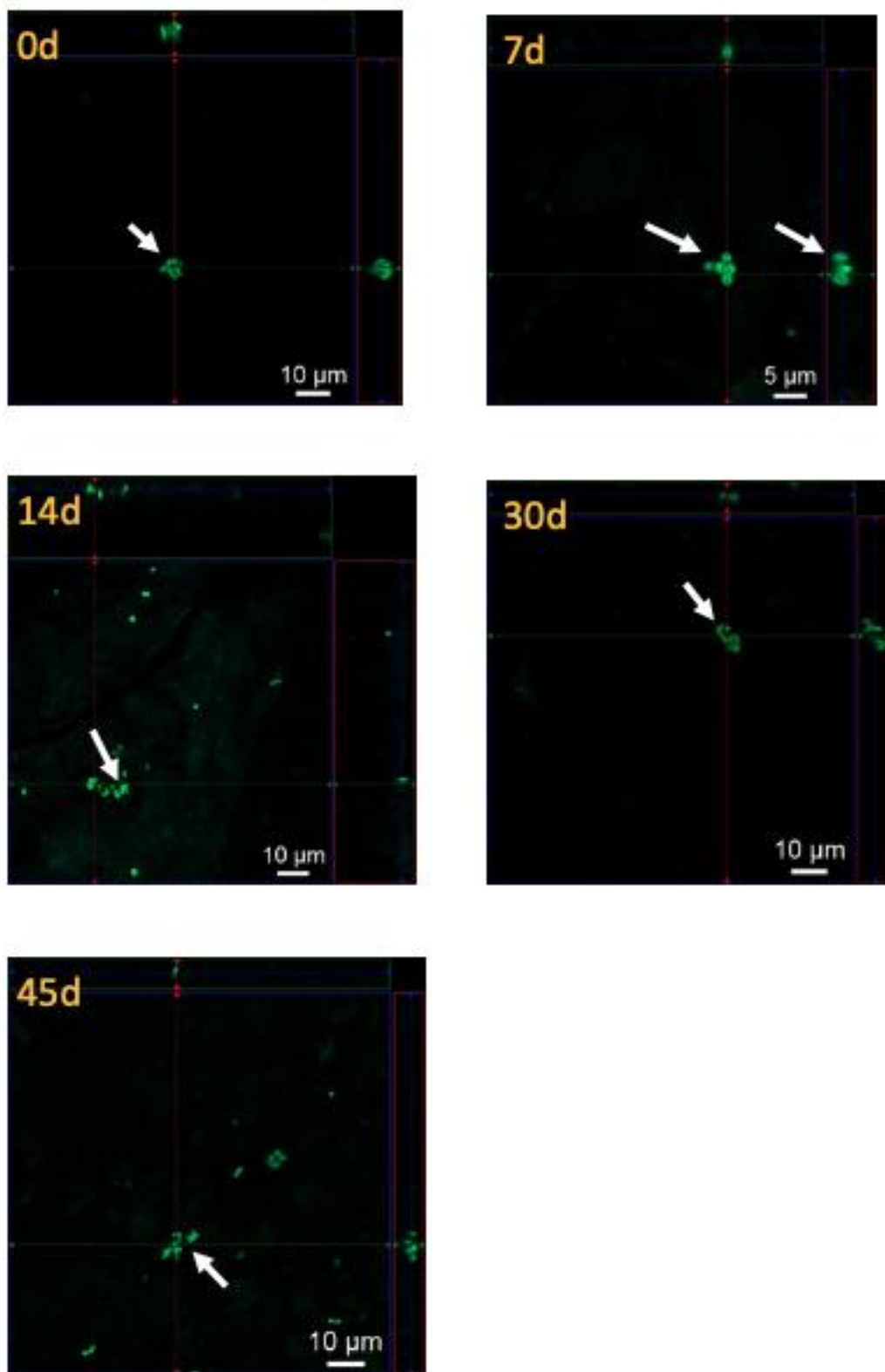


Figure 3.6.2 CLSM findings showing the presence of bacteria clustered in biofilms for participant 2

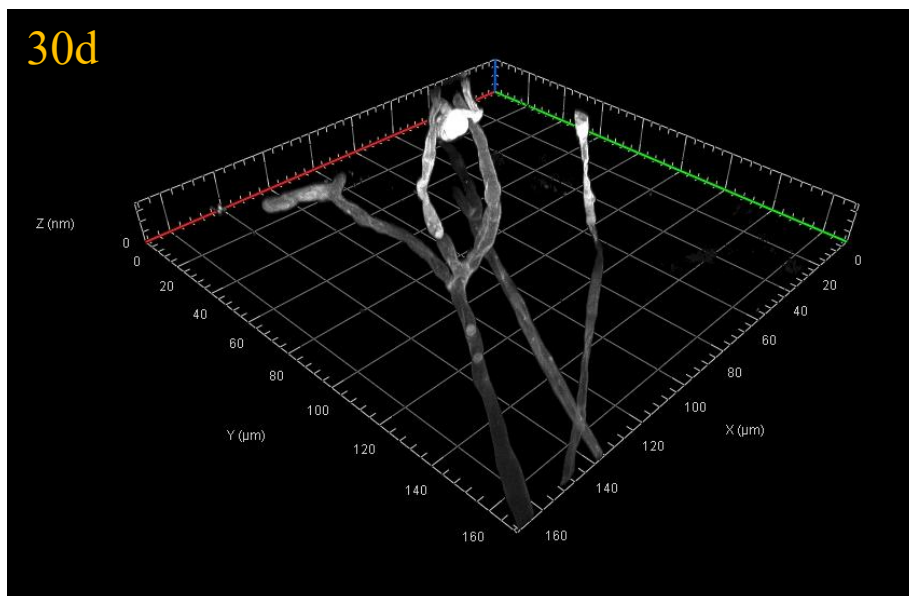
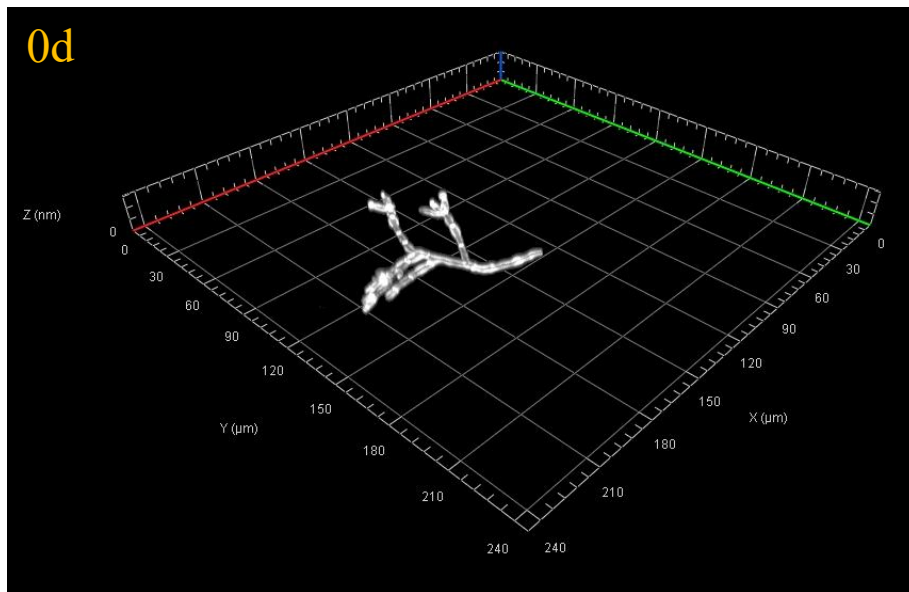


Figure 3.6.3 CLSM findings for participant 3, showing fungal hyphae

3.9 16S rRNA Sequencing findings, pre and post-aPDT

A comparison of the Shannon diversity index of the participants was made (this is an indicator of the range of abundance and evenness of species seen within a community, thereby giving a quantitative reflection of the different species present). This diversity index is therefore high with a Mock community and low with *P. aeruginosa* PA01 controls. A low diversity was recorded for participant 1. This participant had mostly a single species and found to be similar to the PA01 control (Shannon diversity index, 0.0). Participant 3 was the most diverse of the participants (Shannon diversity index, 3.0). A significant difference was observed in the Shannon index diversity between participants and controls using Kruskal-Wallis test of significance as well as the Pielou's evenness index (p-value 0.0002 and 0.001) respectively. Details as seen in **Figure 3.7.1**.

A test of the relationship that might exist between the Shannon diversity index and the study timelines did not show any significance with both the Kruskal-Wallis test (p-value 0.7) and Pielou's evenness index (p-value 0.6). This suggests that the biocomplexity of the bacteria present within the mucous samples pre-aPDT treatment is similar to the post-aPDT values. Details as seen in **Figure 3.7.2**

The results obtained from the 16S rRNA sequencing were organized into the respective micro-organisms at varying taxonomy levels. The bar chart depicting the Level 2 (phylum) order of the bacteria using colors, are displayed in reducing order of the quantity involved. Each individual microbiome of the study participants has been quite uniquely displayed. Details are seen in **Figure 3.7.3**.

For the level 7 (species), the mock community control showed a large diversity of bacteria (about 7 different species), while PA01 control had only *P. aeruginosa* present. *H. influenzae*, *Staphylococcus spp* and *S. epidermidis* were the most prevalent organisms found in participants 1, 5 and 6 respectively. There was no appreciable change in the diversity between pre and post-aPDT findings for these group of participants. For participant 2, *Fusobacterium* which was identified at day 0 (pre-aPDT), cleared up from the other consecutive timelines. However, *Staphylococcus species* as well as some *Haemophilus influenzae* became more noticeable subsequently. While both *Staphylococcus spp* and *S. epidermidis* were found in participant 3 pre-aPDT treatment, subsequent study post-aPDT timelines showed an increase in the quantity of *S. epidermidis*.

There appeared to be large quantities of *Fusobacterium* and *Prevotella* identified for participant 4 prior to the aPDT treatment which weren't resolved post-aPDT treatment. In the case of participant 7, the proportion of *Corynebacterium* and *Staphylococcus* identified between the pre and post aPDT treatment timelines seems to have been altered. There appeared to have been a change from being mostly *Staphylococcus spp.* pre-aPDT to being *Corynebacterium* predominant on day 7, then being *Staphylococcus spp. predominant* on day-14 and equal distribution in subsequent study timelines. Details as seen in **Figure 3.7.4**.

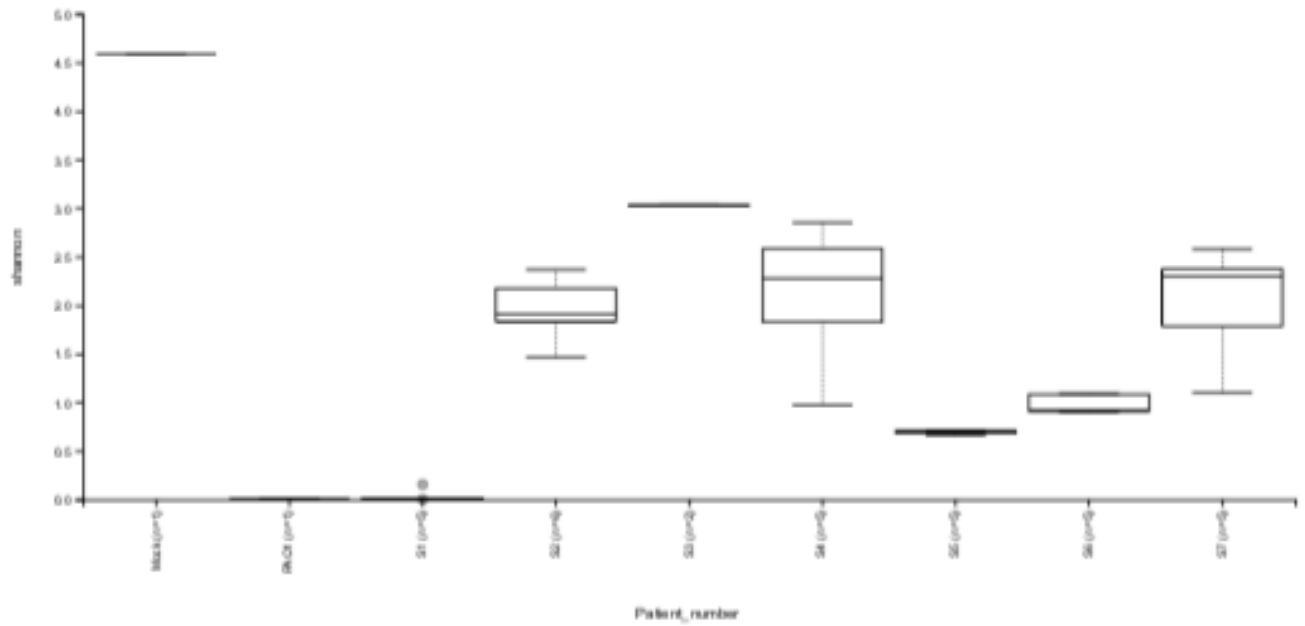


Figure 3.7.1 Boxplots showing comparison of the Shannon diversity index amongst participants and controls

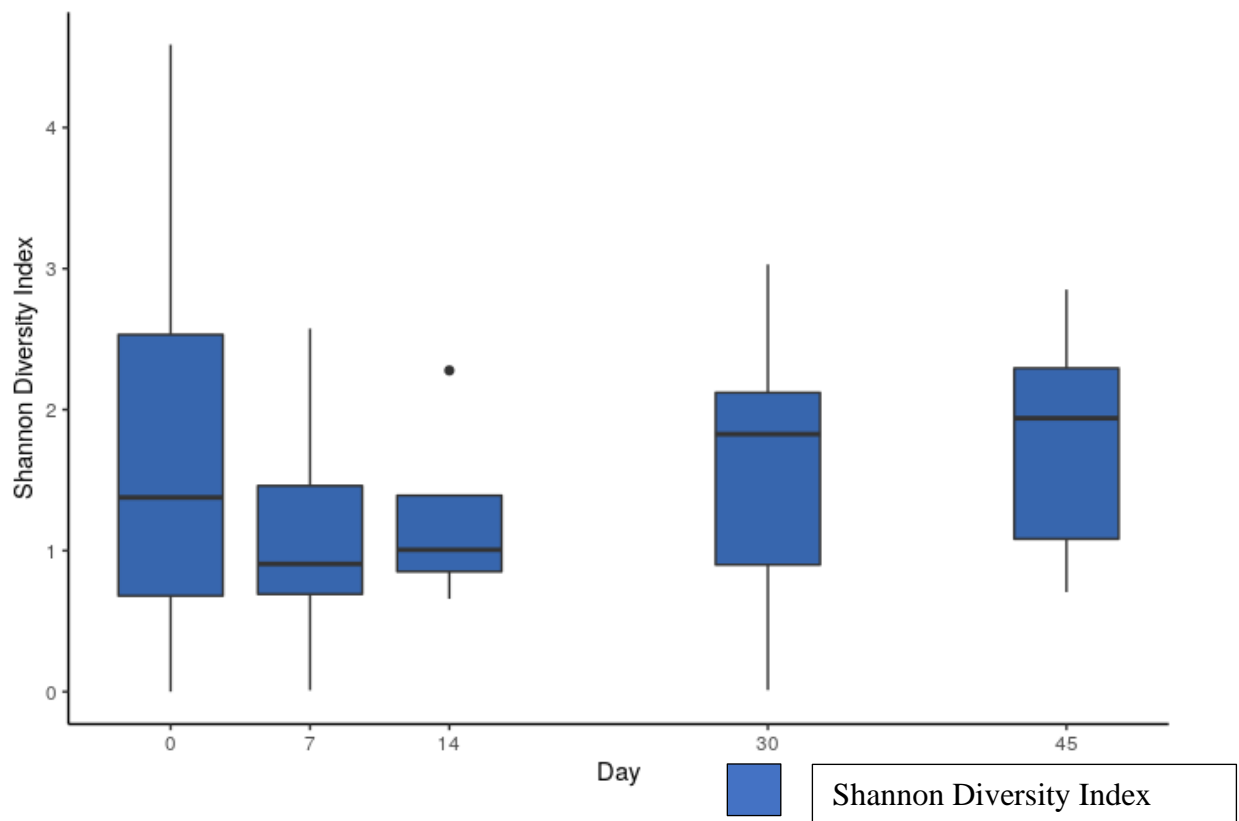


Figure 3.7.2 Boxplot showing comparison of Shannon diversity index and the study timelines

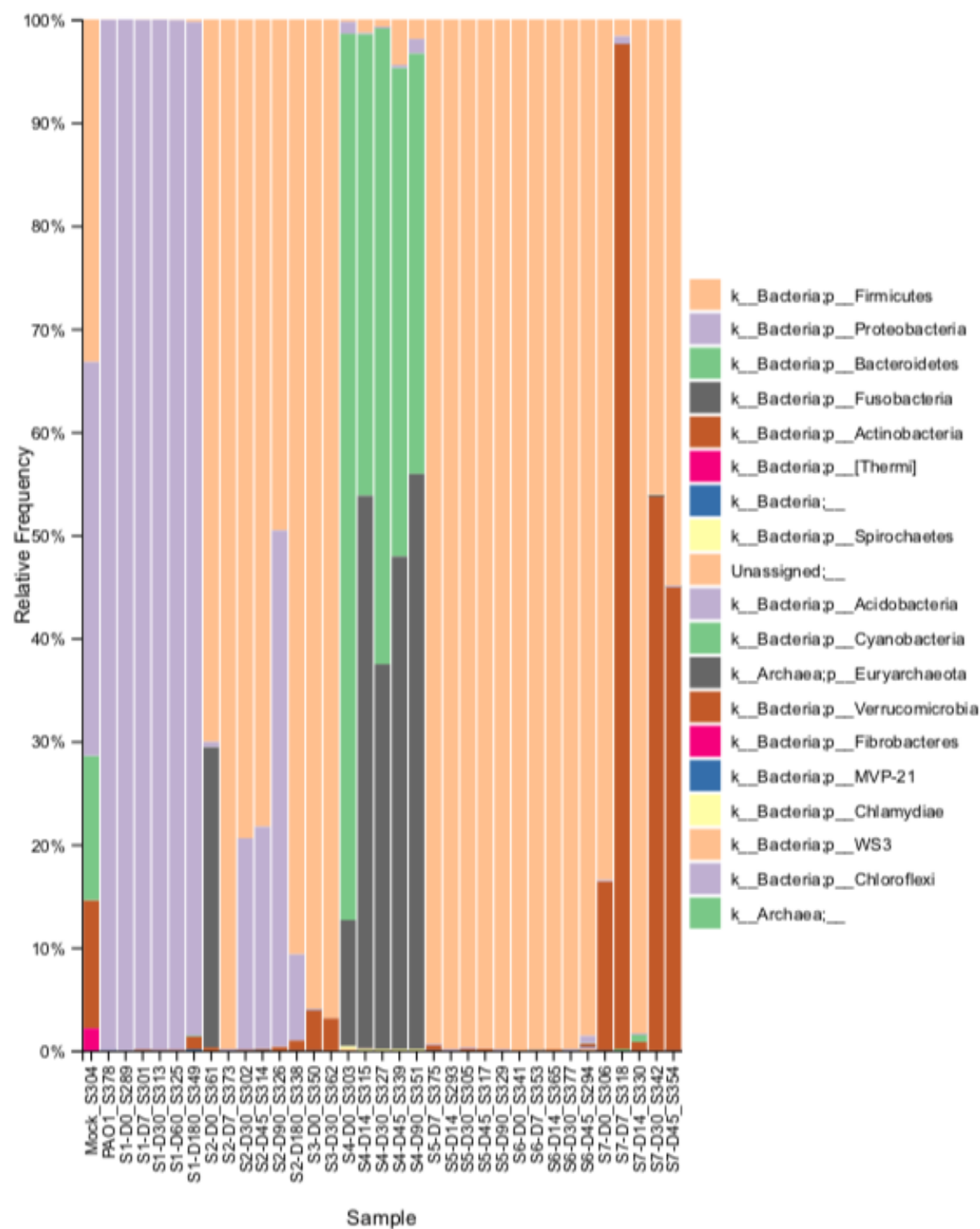
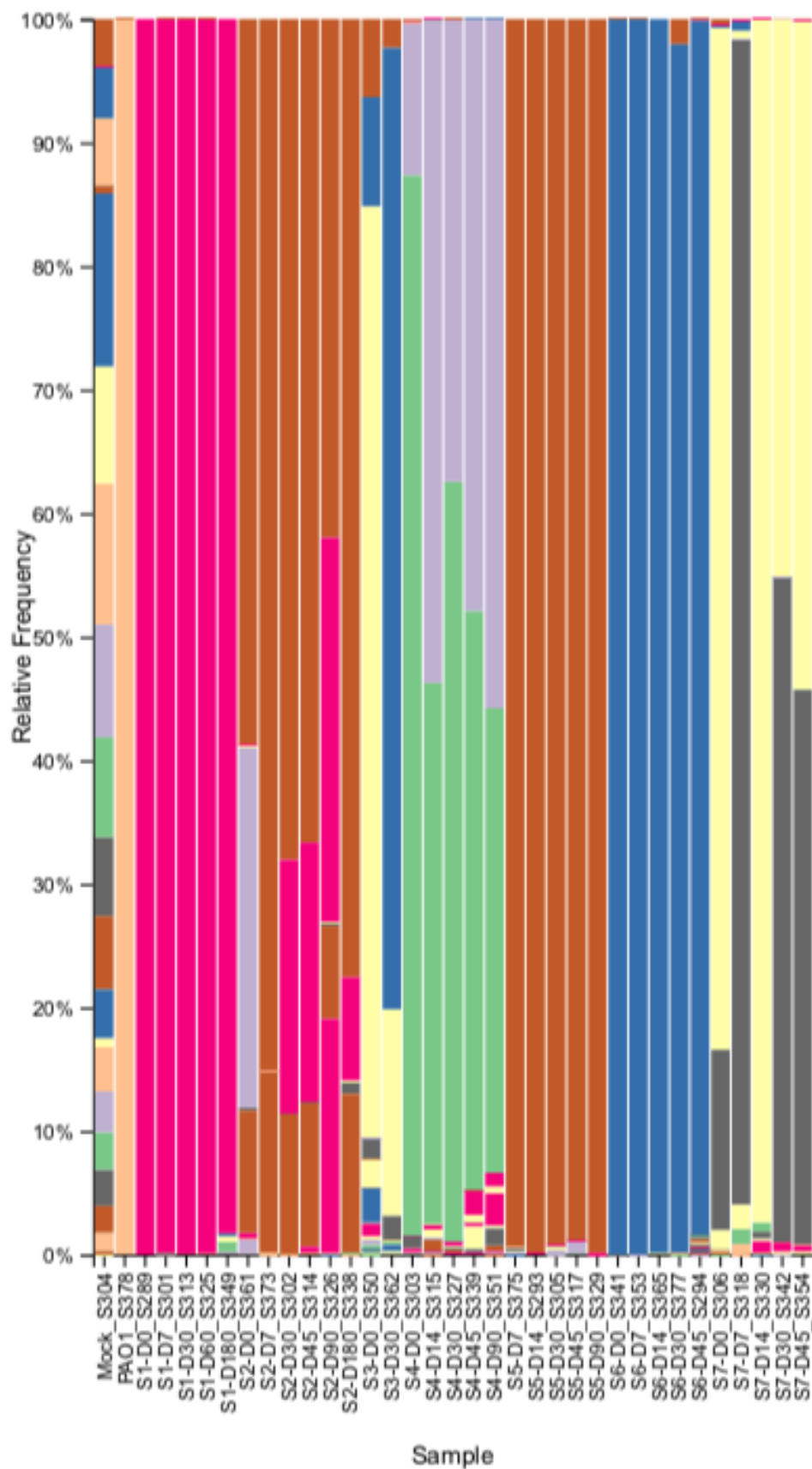


Figure 3.7.3 Showing results of the taxonomic Level 2 (phylum) for the participants, n=7



 k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus;s__
 k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Haemophilus;s__influenzae
 k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus;s__epidermidis
 k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus;s__
 k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__
 k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium;s__
 k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__
 k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium;s__
 k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus;s__aureus
 k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Moraxella;s__
 k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides;s__
 k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium;s__acnes

Figure 3.7.4 Showing results of the taxonomic Level 7 (species) for the participants, n=7

Chapter 4: Discussion

This pilot study aimed at determining the safety and efficacy of anti-microbial photodynamic therapy (aPDT) as a treatment option for patients with recalcitrant chronic rhinosinusitis (CRS) disease. The study noted a slight male preponderance (1.3:1, M:F ratio). This appears slightly different from the findings of Chen et al(7) in the study that was conducted on the epidemiology of CRS in Canadians. They had a female preponderance in their study (5.7%:3.4%, F:M ratio), which was consistent across all age groups, however their focus was not on the recalcitrant CRS group of patients. Ference et al(67) attributes the gender disparity in CRS toward the tendency of the females to be more likely to report symptoms and have better health-seeking behavior compared with the male population. Other factors such as in anatomic size and tobacco usage have also been proffered as reasons for a gender difference. The index cases in my study had no participants who had used tobacco in any form previously, although exposure to second-hand smoking could not be ruled out. Our study cohort differs from the general CRS patients, in that they are recalcitrant to treatment and therefore make up a subgroup of CRS patients.

The age of the research participants ranged from 47 to 78 years with mean age \pm SD of 60.3 years \pm 10.1. The focus of the study was on ‘recalcitrant CRS’ participants, defined as patients who had failed the standard of care (medical and surgical treatment). This might explain why the mean age was in the 6th decade. Two (2) out of the 7 participants (28.6%) in this study population had co-morbid conditions (Asthma). Asthma, which is a clinical condition characterized by the presence of cough, shortness of breath, chest discomfort and audible wheeze, is a chronic respiratory condition. This affects a significant Canadian population aged 12 and older (about 8.1% or

approximately 2.4 million, in 2014).(68) The respondents from a Canadian National Population health survey (1998/1999) reported that over a 12-year period, 6% (95% CI, 5.4%-6.7%) prevalence of Asthma in CRS patients developed asthma.(13) The relatively significant proportion of Asthma co-morbidity recorded in this study (28.6%) lends credence to the need for its awareness amongst healthcare providers managing patients with upper and lower respiratory diseases.

Patients with a clinical diagnosis of Chronic sinusitis without polyps (CRSsNP) accounted for more than half of the participants (57.2%) while those with Allergic Fungal Rhinosinusitis (AFRS) made up the remaining 42.8%. AFRS patients are a subgroup of CRSwNP patients. The term ‘allergic’ in AFRS has been described as a misnomer and the term ‘reactive’ suggested due to the absence of a link with type 1 hypersensitivity (for which allergy is a classic example) in the etiology of the disease. It is said to constitute 7-10% of CRS patients.(47) Currently, the St. Paul’s Sinus Centre manages arguably the largest volume of AFRS cases within the province of British Columbia, Canada.

The sinuses undergoing treatment with the aPDT treatment in this pilot study were the ethmoid and maxillary, with the maxillary sinus being the most treated (4/7, 57.2). Since the most diseased sinus was chosen to be treated with aPDT, other diseased sinuses were excluded. This ensured that the clinical findings (such as endoscopic scores) as well as the laboratory findings (laboratory cultures and CLSM results) could be objectively compared from one patient to the other. A case series presented at a rhinology meeting by Al Felasi et al(69), involved both maxillary and ethmoidal sinuses as the index study but also had the frontal sinus as the most prevalent sinus treated (13/29). The protocol for their study involved a single treatment except for four

participants, who had two treatments. Their protocol was different from ours as we utilized a two-treatment protocol, each lasting eight minutes and given a week apart (days 0 and 7 respectively). Our decision for two treatments per patient was made based on our previous experience with this technology and the need to achieve maximal therapeutic benefits by ensuring a complete clearance of the microorganisms that may not have been eradicated by the initial treatment.

The most significant improvement in the Alsaleh-Javer Endoscopic Symptom Score (AJESS) was recorded between the completion of the aPDT treatment (day 7) and one-week post-treatment (day 14) period which showed a reduction of 1.50 points (from 3.00 to 1.50 points, p-value 0.049). Since the therapeutic objective was set at a 1-point reduction in the AJESS, it can safely be stated that there was a significant effect on the mucosal inflammation from the aPDT treatment in patients with recalcitrant CRS. This initial gain however, was not sustained, as there was an increase of 0.64 points at 30 days. Furthermore, improvement with a reduction of 0.14 points on day 45 might indicate that an addition of another stabilizing intervention may prolong the efficacy of the aPDT. The feeling is that complete eradication of all microbes from the involved sinus may leave it vulnerable to even more pathogenic organisms. An intervention to avoid this from happening is currently being considered by looking at the potential use of Sinonasal Microbiota Transplant (SNMT) therapy via nasal lavage from a healthy donor (preferably a first degree relative). The concept is to repopulate the sinonasal cavity with a healthy microbiome and in so doing, repair the thus repair the microbial dysbacteriosis that exists in these sinuses. The combination of the aPDT followed by SNMT would therefore result in a true ‘re-booting’ of the sinus microbiome. This research proposal has already received approval from Health Canada and is awaiting final ethics approval from University of British Columbia (UBC) ethics board.

The maximal mean SNOT-22 score improvement for all the 7 participants (9 points) was recorded between day 0 (44.53) and day 7 (35.57), the 1st and 2nd treatment days (p-value, 0.208, not statistically significant). The minimal clinically significant difference (MCID) commonly considered is a change of 8.9.(51). SNOT-22 is the most commonly utilized sinus-specific quality of life instrument, which is a form of patient reported outcome measure.(48) Hence it can safely be stated that using the SNOT-22 questionnaire as an outcome measure, the aPDT appears to be an effective tool for the treatment of recalcitrant CRS disease. The changes observed after the completion of the aPDT treatment (day 7) and one-week post-treatment (day 14) showed a further reduction of 5.77 points (from 35.57 to 29.80). While this second drop (improvement) may not meet the MCID value of 8.9, it is important to note that the aPDT therapeutic advantage appears sustained to some point immediately after its application. However, beyond this time point, an increase in the SNOT-22 scores was noted between days 14 and 30 (7.20 points). This was mirrored with the findings of the AJESS for the same treatment period suggesting that the largest aPDT therapeutic benefit was mainly short-term. The reduction of 3.14 points, seen between study period of days 30 and 45, appear to indicate a stabilization or ‘new-norm’ of inflammation and therefore symptom score.

The study did not record any adverse events. However, the pre and post-aPDT treatment findings of the pain questionnaire revealed that the level of pain ranged from mild pain for the pre-treatment group (0.54 to 1.38 points) to mild and moderate pain for the post-treatment group (1.79 to 2.29 points). Although, as noted previously, these differences related largely to one outlier participant. The fact that sinus pain is often associated with rhinosinusitis, makes the occurrence of mild pain in all the subjects prior to aPDT administration understandable. In fact guidelines from the

European Position paper on rhinosinusitis and nasal polyps, 2012 endorses the presence of pain in CRS.(4) This is largely as a result of pressure within the air-filled sinuses as well as the rich nervous system that is located within and in close proximity to the sinuses. Often the location of such pain can be an indicator to the sinus most affected especially with acute sinusitis. Stabbing pain was associated with the highest difference between the pre and post-treatment pain status (1.52 points, from 0.77 to 2.29). This was closely followed by Stinging pain (1.50 points, from 0.54 to 2.14), then pricking, throbbing and cramping pain (1.17, 0.91 and 0.87 points) respectively. These results from the pain questionnaire, indicate that pain attributable to aPDT alone was in the mild pain category. The VAS scores for participant' s discomfort for the pre and post aPDT treatment groups showed a difference of 2.28 points (from 1.30 to 3.58 points).

The absence of any adverse events, a mild pain profile and the mild-moderate discomfort experienced by the participants, demonstrates the tolerability and safety of this novel treatment modality. It must also be appreciated that the sensation of pain is very individual-based. This was noted particularly in one of the participants who had a very high pre and post-treatment pain VAS scores compared to the other participants which resulted in significant skewing of the data. In real life, such events do occur. Caregivers need to be aware and vigilant so as take appropriate action to ameliorate such effects and ensure the patient's safety.

The results of the Nasal Mucociliary Clearance Time (NMCT) using Saccharin, showed that participants timing ranged from normal to severely prolonged. While 4/7 (57.2%) of the participants had NMCT within the normal range (less than or equal to 20 minutes), the other three participants had either prolonged or severely prolonged results (23 – 37 minutes). The mucociliary

clearance function serves as an important innate defense mechanism by catching and clearing debris microparticles that are breathed in through the nose. It also moves mucus in a front to back direction allowing proper function of the nasal cavities. Conditions such as inflammation and infection, can impair these functions and result in a prolongation of the disease. Thus, patients with CRS become prime targets for poor mucociliary transport. This is further worsened with the presence of other conditions such as Cystic fibrosis and Bronchiectasis, which are known to affect ciliary function.(57) None of the patients in this index study had clinical evidence of any other disease other than CRS. It was therefore an interesting finding that two of the participants showed a remarkable improvement in the NMCT results, between the pre and post aPDT treatment period (from 25 to 7 minutes and 24 to 4 minutes respectively). Despite the small sample size, this result suggests that aPDT might have an important healing effect on the innate defense mechanism of the participants. This effect might be worth exploring further when a larger study is conducted.

Results of a smell test (University of Pennsylvania Smell Identification Test, UPSIT) in the participants, showed that all the participants had olfactory disorders ranging from mild microsmia to total anosmia. This is in keeping with a recent meta-analysis involving 1,956 participants with this disorder and background CRS disease (pre and post-surgery). They examined a set of studies that utilized accurate smell testing methodologies, revealing that abnormal baseline olfactory dysfunction ranged from 28% to 100% in this group of patients.(70) Factors such as the presence of polyps, type of surgery performed, degree of smell loss and the duration of recovery were factors identified which influenced olfactory outcomes.

This study identified total anosmia in 4/7 (57.2%) of the participants, while the rest were mild, moderate and severe anosmia respectively. The occurrence of this significant level of olfactory disorder in CRS participants is corroborated by Kohli et al(71) who conducted a meta-analysis looking at the prevalence of olfactory dysfunction in CRS patients. Their study reported a prevalence of 30.0% (using Brief Smell identification test) and 67.0% (using the 40-item Smell Identification Test). Another observation from that study was that participants with mixed group of data (CRSwNP and CRSsNP) and the polyp group demonstrated improvement in UPSIT scores. This finding might explain why in our study cohort that was made up of only CRSsNP (4/7) and AFRS (3/7) participants, there did not seem to be appreciable improvements in their smell status.

There were no appreciable changes in smell status between the pre and post aPDT treatment period in all participants. The study of Haxel et al(70) noted that recovery of olfactory function for post-sinus surgical patients appear to be impeded by previous sinus surgery and prolonged sinus disease. All of these factors are present in our cohort (hence their recalcitrant status), as well as the relatively short duration (45 days) may have been too early to obtain appreciable results with olfactory status.

The study observed a mixed growth of bacteria cultured within the sampled sinuses. While the microbiology in acute rhinosinusitis is quite predictable, the rather insidious nature as well as the challenges with quantification of the organisms cultured in CRS have made its management difficult. *Staphylococcus aureus* was cultured in 4/7 participants (57.2%), while *Hemophilus influenzae* and *Staphylococcus lugdenensis* were found in the others. These findings are similar to the study carried out by Brook, who found *Staphylococcus aureus* and anaerobic organisms

(*Prevotella* and *Peptostreptococcus spp*) as the most common isolates for CRS.(72) The presence of *Hemophilus influenzae* tended to be associated more with acute sinusitis, but can be found in CRS. However, *Staphylococcus lugdenensis*, a coagulase negative *Staphylococcus*, is presumed pathogenic whenever cultured as opposed to *Staphylococcus epidermidis* that can be considered a normal flora.(73) There is also a possible role of polymicrobial biofilms, whereby they develop various adaptative changes and are able to evade the host immune mechanisms.(72)

A comparism of the pre and post-aPDT cultures showed a reduction in the quantity of bacteria which was most evident between study days 14 and 30. An in-vitro study carried out on an maxillary sinus model and treated with methylene blue photosensitizer and 670nm non-thermal activating light was described to have achieved a >99.99% reduction in Methicillin-Resistant *Staphylococcus Aureus* (MRSA) biofilm after a single treatment.(74,75) Our current in-vivo study also noted a reduction, but not a total clearance of the bacterial biofilms. The sterilization of the sinuses may be a concept that could be difficult, if not impossible to achieve. Also, the benefit of this sterilization is questionable given the current knowledge regarding the advantages of the normal microbiome within the sinuses. Instead a correction of the dysbacteriosis, which would otherwise enable virulent organisms to multiply rapidly while suppressing normal commensals, may be a more favorable approach in managing the recalcitrant CRS.

The use of 16S rRNA sequencing for the identification of microbiome is quite superior to the use of laboratory cultures. For instance, participant 5 did not have any bacteria cultured either pre-aPDT treatment and the immediate follow-up period. However, with the use of the 16S rRNA sequencing, *Staphylococcus Spp.* was identified. This raises the question whether laboratory

cultures should be the main source of determination of bacteria present in CRS patients as well as monitoring their follow-up after treatment.

Three separate microbial identification methods; hospital laboratory culture, CLSM and 16S rRNA sequencing were utilized to obtain the greatest amount of information, as well as to compare and contrast the different techniques for future studies. While the hospital laboratory cultures gave a rough idea of the pathogenic organisms, many of the findings were non-specific and some organisms were missed compared to the other two methods. The CLSM on the other hand gave us an idea of the structure of the biofilms and assisted in confirming the presence or absence of etiologic organisms within the shortest period of time. The 16S rRNA sequencing served as the ‘final arbiter’, due to its ability to give a concise and clear result of the biocomplexity within the sinuses. While each component of the tests performed gave varying perspectives, the 16S rRNA provided the most extensive and complete information of the 3 tests in identifying the nature of the offending organisms. A major drawback of the 16S rRNA sequencing was the cost and duration it took to execute the test.

In the case of participant 7, the proportion of *Corynebacterium* and *Staphylococcus* identified between the pre and post aPDT treatment timelines appears to have varied significantly. The study of Chalermwatanachai et al, which looked at the microbiome of a cohort of CRSwNP with or without Asthma, reported the following findings: *Staphylococcus aureus* was most prevalent amongst the CRSwNP without Asthma group; *Escherichia coli* for the CRSwNP with Asthma group and *Propionibacterium acnes* for the controls.(76) However, Actinobacteria (such as *Propionibacteria* and *Corynebacterium spp.*) does not play the protective role one would have

expected against *Staphylococcus aureus*, despite its abundance. Though participant 7 was a CRSsNP patient, it appeared that the aPDT treatment enhanced *Corynebacterium spp.* activity but might not have been sufficient to inhibit the *Staphylococcus aureus* growth effectively. These findings further align with the suggested hypothesis of dysbacteriosis being an important etiological factor with CRS disease.

The Confocal Laser Scanning Microscopy (CLSM) technique used for this study utilized BFL-Vancomycin (Gram positive organisms), D-Polymyxin (gram negative organisms) with background bright field, showing the host cells. All of this was combined to give a detailed picture on the CLSM made up of all its constituent stains. Other techniques have been used to characterize the biofilms using the CLSM which include, LIVE/DEAD BacLight (Invitrogen Corp., Carlsbad, CA), fluorescence in situ hybridization (FISH).(77) These techniques tend to be complementary rather than one being better than the other and the choice of their use is often dependent on the objectives of the research protocol. I chose a protocol for the study which was quite specific for the organisms (Gram positive and negative) with differential staining, thereby making a clear distinction between eukaryotic and prokaryotic cells. The CLSM technique was also preferred to the scanning and transmission electron microscopy as it avoided the various drawbacks with tissue preparation and scanning. This was similar to the study by Psaltis et al(63) who conducted CLSM for patients with CRS.

Generally, the findings on CLSM were in agreement with the laboratory cultures for bacterial obtained from mucous aspirates from the sinuses. The processing time required for laboratory cultures could be a drawback as samples take time to be cultured on a plate which could take up

to 2-4 weeks to culture for fungal specimens. Basically, our CLSM protocols involved fixing with 4% paraformaldehyde for 24 hours after which they were rinsed and then analyzed. We also found that with the use of Calcofluor white for fungal staining, we were able to recover organisms from the mucus specimen that were often missed by the routine lab culture.

Chapter 5: Conclusion

This study showed that antimicrobial photodynamic therapy (aPDT) was able to improve the endoscopic scores measured using the Alsaleh-Javer Endoscopic Scores by at least 1 point. This was achieved between the completion of the aPDT treatment (day 7) and day 14 with average changes of 1.50 points. The study also noted that an improvement was attained in the sinus-specific quality of life measure which represented the patient's reported outcome and was measured using the SNOT-22, with scores changing by at least 9 points or one MCID. This occurred between the first and second treatment days (9 points).

There was a general reduction in the lab culture results for the various participants during the study period, mostly during the period after completion of the treatment and the next follow-up visit. The safety of the procedure was carefully monitored. No adverse events noted were identified except for mild to moderate pain and discomfort during the study period. Olfaction which was measured using the University of Pennsylvania Smell Identification Test (UPSIT) showed olfactory dysfunction which was not worsened or changed with the procedure.

Complete elimination of the bacterial and fungal biofilms was not achieved at any period of the study; rather a reduction was noted. The Nasal Mucociliary Clearance test (NMCT) showed significant improvement in a small number of participants 2/7 (28.6%), while others remained relatively unchanged.

The study was limited by the small sample size as well as the relatively short duration (45 days). Although, the results may not be generalizable at this point, they give useful insights to proceeding with a larger randomized-control study in the future.

Future research involving the use of sinonasal microbiota transplants from suitable healthy donors via nasal lavage post aPDT to treat refractory CRS patients, may enhance and prolong the short-term effect of aPDT seen in this study.

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Appendices

Appendix A

Disease-specific Quality of Life Questionnaire - SNOT-22

Sino-Nasal Outcome Test-22 Questionnaire

Below you will find a list of symptoms and social/emotional consequences of your nasal disorder. We would like to know more about these problems and would appreciate you answering the following question to the best of your ability. There are no right or wrong answers, and only you can provide us with this information. Thank you for your participation.

Please rate your problems, as they have been over the past two weeks.

Considering how severe the problem is when you experience it and how frequently it happens, please rate each item below on how 'bad' it is by circling the number that corresponds with how you feel using this scale.	No problem	Very mild problem	Mild or slight problem	Moderate problem	Severe problem	Problem as bad as it can be
1. Need to blow nose	0	1	2	3	4	5
2. Sneezing	0	1	2	3	4	5
3. Runny nose	0	1	2	3	4	5
4. Cough	0	1	2	3	4	5
5. Post-nasal drip (mucus into back of your nose)	0	1	2	3	4	5
6. Thick nasal discharge	0	1	2	3	4	5
7. Ear fullness	0	1	2	3	4	5
8. Dizziness	0	1	2	3	4	5
9. Ear pain/pressure	0	1	2	3	4	5
10. Facial pain/pressure	0	1	2	3	4	5
11. Difficulty falling asleep	0	1	2	3	4	5
12. Waking up at night	0	1	2	3	4	5
13. Lack of a good night's sleep	0	1	2	3	4	5
14. Waking up tired	0	1	2	3	4	5
15. Fatigue during the day	0	1	2	3	4	5
16. Reduced productivity	0	1	2	3	4	5
17. Reduced concentration	0	1	2	3	4	5
18. Frustrated/restless/irritable	0	1	2	3	4	5
19. Sad	0	1	2	3	4	5
20. Embarrassed	0	1	2	3	4	5
21. Sense of taste/smell	0	1	2	3	4	5
22. Blocked/congestion of nose	0	1	2	3	4	5

1-TOTAL _____

GRAND TOTAL: _____

Appendix B UPSIT CLASSIFICATION

Olfactory Diagnosis	Test Score
Probable Malingering	00 - 05
Total Anosmia	06 - 18
Severe Microsmia	19 - 25
Moderate Microsmia	
Male	26 - 29
Female	26 - 30
Mild Microsmia	
Male	30 – 33
Female	31 - 34
Normosmia	
Male	34 – 40
Female	35 - 40

Appendix C Alsaleh-Javer Endoscopic Sinus score (AJESS) July 2019 classification

Site	Score Each Side Separately	
Middle Turbinate/ Middle Meatus	Document letter (in upper or lower case) corresponding to the following features if present *: P Polypoid MT N Narrowed MM (lateralized MT) Lower Case Letter: Mild (one point) Upper Case Letter: Moderate/Severe (two points)	
Maxillary sinus	Grade each individual cavity using the following: (e.g. Right Ethmoid 1p) ▪ Document mucosal Inflammation score: 0 No oedema or polyposis 1 Mucosal oedema 2 Mild polypoid oedema 3 Mod/severe polypoid oedema 4 Mild polyposis 5 Mod/severe polyposis ▪ Document letter corresponding to the following features if present*: C Crust R Recirculation of mucous S Synechia P Purulence M Mucin N Narrow ostium Lower Case Letter: Mild (one point) Upper Case Letter: Moderate/Severe (two points)	
Ethmoid sinus		
Sphenoid sinus		
Frontal sinus		
Olfactory Cleft		
Total		

Appendix D SOP for Sinus mucus aspirate preparation

Receiving Samples

Materials:

- 1X PBS, pH7.4
- Formaldehyde waste bottle
- Sterile Eppendorf tubes
- 15 ml screw capped (blue) conical tubes
- 70% Ethanol
- Forceps

Protocol:

1. Receive the samples either prepared in formaldehyde on ice (for microscopy) or frozen (for DNA extraction).
 2. Place the frozen samples in the -80C freezer.
 3. For the formaldehyde treated samples, rinse 3x with 1X PBS, pH7.4
 - a. For the biopsy, using 1 ml pipette remove the formaldehyde and dispose into the waste bottle. Rinse 3x with 1 ml 1X PBS, pH7.4 (dispose rinses into the waste bottle as well).
- *** If the sample is loose, spin gently at 7,000 RPM for 4 min prior to each wash.
- b. For the mucosal sample, estimate the size of the sample.
 - i. Large (takes up most of the volume of the microfuge tube) using forceps rinsed in ethanol and dried with a kimwipe, remove the sample from the formaldehyde tube and place the sample into 10 ml 1X PBS, pH7.4 in a 15 ml blue screw cap conical tube. Dispose of the formaldehyde in the waste bottle
 - ii. Small (takes up less than 10% of the microfuge tube), treat identically to the biopsy (3x 1 ml washes)
 4. Place the rinsed samples at 4C refrigerator.

Sample Staining

Materials:

- Dyes
 - Dansyl Polymyxin (DPX)
 - BODIPY FL Vancomycin (BV)
 - Calcofluor White
- 1X PBS, pH7.4
- 10% Potassium Hydroxide
- Kimwipes
- Long coverslips (24 x 60mm)
- Clean petri plate

- Clean razor blade
- 70% ethanol
- Forceps

Protocol:

Sample Staining

- Bacteria (Dansyl polymyxin and BODIPY FL Vancomycin)

1. Remove the dyes from the freezer and thaw at room temperature in the dark (in bench). When thawed, place DPX in the dark on ice (aluminum foil covered lid). Leave the BV at room temperature
2. Add 250 ul 1X PBS, pH7.4 to an Eppendorf tube
3. Add sample to the PBS
 - a. Mucosal – using forceps (long ones are needed for samples in 15 ml tubes) pinch off a small piece and place into the tube.
 - b. Biopsy – try to pinch off a piece and place into the PBS. Otherwise, pull out the biopsy and place it onto the lid of the petri plate. Using a new razor blade each time, cut off a tiny piece of sample. Place the large sample back into the appropriate tube and place the tiny piece into the working sample tube. **Work quickly so the sample does not dry out.**
4. Add 20 ul DPX
5. Let sit for 20 min in the dark at room temperature (in bench or under aluminum foil)
6. Add 1 ul of working stock of BV and let sit for 10 min
7. Place the bottom coverslip onto a kimwipe. Using forceps, remove the sample from the stain and place onto the coverslip. Cover with a second coverslip.

- Fungal (Calcofluor white)

1. Place sample of interest onto a long coverslip. Suck and remove excess liquid around sample with a pipette tip.
2. Add 2-5 ul 10% potassium hydroxide to the sample (use 2 ul for a small sample and 5 ul for a large sample).
3. Add an equal amount of Calcofluor White (Sigma Cat# 18909)

Appendix E SOP FOR CLSM

1. Start up the microscope (consult the pictures on the desk for help)
2. Gently push back on the microscope's light source (large arm above the stage/objectives), till it stops against the plastic housing
3. Adjacent to the right-hand focus knob, press the first thumb button to lower the objectives. The microscope control panel will indicate, "nosepiece in the load position."
4. Check to see if the correct stage mount is place. If not, remove the stage mount, find the one with the sides that slide, line up the red dot at the corner and slide into place. Give the mount a slight tug to ensure proper alignment
5. When prompted to calibrate the stage, select confirmation to calibrate the stage from the computer software
6. At the microscope, look at the control panel and select "microscope" on the left. At the top, under the "objective" tab select 63x oil.
7. Place a small drop of oil onto the objective and set your coverslip onto the stage, sliding the sides to "lock" it in place.
8. If needed, gently raise the objectives until the oil comes into contact with the coverslip
9. In the software, select locate, just below the tab, click on the blue DAPI or the green 488 (depending on which dyes you're using) to activate the lamp and filters
10. Scan your image for a desired location.
11. When you have selected the location you'd like to image, select the acquisition tab, either "Dansyl Bodipy FL" or "Calcofluor white"

Appendix F VAS for pain and discomfort

Subject ID: _____

Date: _____

(A) Visual Analogue Score (VAS) for Discomfort:

Please draw a circle on the number on the scale that indicates your current level of discomfort since your last treatment with aPDT:

No Discomfort

Worst Discomfort

0-----1-----2-----3-----4-----5-----6-----7-----8-----9-----10

(B) Visual Analogue Score (VAS) for Pain:

Please draw a circle on the number on the scale that indicates your level of pain during your clinic visit procedure:

Type of Pain	Scale
Pricking Pain	No Pain Worst Pain 0-----1-----2-----3-----4-----5-----6-----7-----8-----9-----10
Stinging Pain	No Pain Worst Pain 0-----1-----2-----3-----4-----5-----6-----7-----8-----9-----10
Cramping Pain	No Pain Worst Pain 0-----1-----2-----3-----4-----5-----6-----7-----8-----9-----10
Throbbing Pain	No Pain Worst Pain 0-----1-----2-----3-----4-----5-----6-----7-----8-----9-----10
Stabbing Pain	No Pain Worst Pain 0-----1-----2-----3-----4-----5-----6-----7-----8-----9-----10

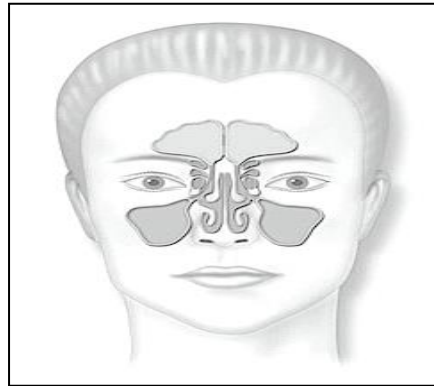
(C) Onset of Pain:

Please check when you first noticed pain after your clinic visit procedure in minutes (mins).

Less than 5 mins	5 to 10 mins	11 to 15 mins	16 to 30 mins	More than 30 mins
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

(D) Pain Location:

If applicable, mark where the pain is on the figure to the right.



Comments:

(D) Duration of Pain: *Check how long pain persisted in minutes (mins).*

Less than 5 mins	5 to 10 mins	11 to 15 mins	16 to 30 mins	More than 30 mins
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

(E) Visual Analogue Score (VAS) for Discomfort:

Please draw a circle on the number on the scale that indicates your level of discomfort during your clinic visit procedure:

No Discomfort

Worst Discomfort

0-----1-----2-----3-----4-----5-----6-----7-----8-----9-----10

Appendix G Adverse Event Questionnaire

Study ID _____

For days 0, 7, 14, 21, 30, 45

Subject ID: ____ - ____

Date: ____ / ____ / ____

Time: ____ : ____

How has your overall health been since the aPDT?

1. Location of SAE: _____

2. Age: ____ years

3. Gender: Male Female

4. Weight: ____ kg lbs

5. Height: ____ cm in

6. SAE term (provide diagnosis):

6a. If diagnosis is not known, symptoms:

7. Date of onset: _____ (dd/mm/yyyy)

8. What is the severity grade of the serious adverse event?

Grade 1: Mild

Grade 2: Moderate

Grade 3: Severe

Grade 4: Life-threatening

Grade 5: Death

9. Did the subject receive aPDT prior to this SAE?

Yes No N/A

10. Outcome of SAE:

- Ongoing at this time
- Resolved without sequelae
- Resolved with sequelae
- Death
- Present at death, not contributing to death

11. Date of resolution: _____ (dd/mm/yyyy) or Ongoing at end of Study

12. Seriousness criteria? (Check all that apply)

- Life-threatening
- Required hospitalization or prolongation of existing hospitalization
- Congenital anomaly
- Disabling/incapacitating
- Important medical event
- Fatal

If fatal:

12a. Date of death: _____ (dd/mm/yyyy)

12b. Primary cause of death: _____

12c. Was an autopsy performed? Yes No

13. Relationship to investigational product/study intervention:

- Related (*Associated with the use of the study intervention. There is a reasonable possibility that the experience may have been caused by the study intervention.*)
- Unrelated

14. If SAE is unrelated to investigational product/safety intervention, select all possible etiologies:

- Concurrent illness, disease, or other external factors, specify:
- Concurrent medication, specify:
- Secondary study procedure, specify:
- Accident, trauma, or other external factors, specify:
- Other, specify:

15. Did the subject receive any relevant concomitant medications in response to the SAE?

Yes No

15a. If yes, add each medication below:

16. Did the subject receive any treatments/procedures in response to the SAE?

Yes No

16a. If yes, list each treatment and procedure below:

17. Did the subject receive relevant laboratory or diagnostic tests in response to the SAE?

Yes No

17a. If yes, provide the name of the test and results with normal ranges and/or

supplemental exams below:

18. Narrative/Comments (provide a description of the serious adverse event including chronological clinical presentation and evolution of the serious adverse event and associated signs/symptoms):

19. Completion of form: printed names, signatures and date of signature

Person Completing Form (print name)

Person Completing Form (signature)

Date

Investigator (print name)

Investigator (signature)

Date

Appendix H SOP for aPDT Procedure

1. The device used for the aPDT was the Sinuwave™ Ceralas E® laser system (delivers homogenous laser energy at 670 nm.)
2. Nasal endoscopy carried out using a 3mm 30-degree rigid nasal endoscope to visualize the sinus cavity. Nasal debridement was then performed as required.
3. Three milliliters (3mls) of the photosensitizing agent (methylene blue) was sprayed over the surface of the affected sinuses with the aid of a curved suction under direct endoscopic visualization. Standard laser precautions were applied, and eye protection goggles provided to all personnel available at the session.
4. Sinuwave Light Delivery Catheter (Figure 2) made of a malleable yet firm material to facilitate access into the sinus to be treated, was utilized in connecting the device to the sinus cavity. The distal end of the catheter houses a balloon which surrounds the laser projecting tip. It facilitates equivocal assortment of the photosensitizing agent on the sinus wall mucosa, mechanical evacuation of purulent or mucinous discharge that is located in the sinus as well as equal distribution of the laser light to the surrounding mucosa. This is executed under endoscopic guidance.
5. The Sinuwave catheter balloon is inflated with saline in retrograde fashion (quantity of saline used for inflation was determined according to the sinus volume and patient tolerability). The inflation was executed via a port located at the proximal end of the catheter.
6. After connecting the catheter to the Sinuwave Laser Console (Figure 3), the sinus is illuminated using a low-level laser at 670 nm in 3nm wavelength. The laser varying power (0.5W to 4.90W) chosen is dependent on the amount of saline (<1ml – 15ml) inflating balloon of the laser catheter.
7. The catheter is left in place for a duration of 8 minutes.
8. At the conclusion of treatment, the balloon is deflated and removed.
9. The sinus cavity is then re-examined for the presence of any immediate adverse events.