Toll-like receptor activation by sino-nasal mucus in chronic rhinosinusitis*

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Abstract

Background: The sino-nasal disease chronic rhinosinusitis (CRS) is primarily an inflammatory condition that manifests in several ways. However, the aetiology of this complex disease is poorly understood. The aim of this study was to explore the association between toll-like receptor (TLR) activation, host immune response and sino-nasal mucus in healthy and diseased patients.

Methods: The activation of TLR2/1 and TLR4 by sino-nasal mucus from 26 CRS patients and 10 healthy controls was measured. In addition, 7 inflammatory cytokines, bacterial community composition and bacterial abundance within the sino-nasal mucus were measured using molecular and diagnostic tools.

Results: TLR activity was observed in 9/36 samples, including 2 healthy controls. There was a strong, positive correlation between members of the Gammaproteobacteria (*Haemophilus*, *Enterobacter*, *Pseudomonas*) and TLR2/1 and TLR4 activity. Bacterial abundance and cytokine (tumour necrosis factor) abundance were also positively correlated with TLR activity.

Conclusions: These findings suggest that a small proportion (20-30%) of individuals in each sub-group are more predisposed to TLR activity, which may be related to bacterial composition, diversity and abundance in the sinuses.

Key words: Human toll-like receptors, cytokines, sinusitis, microbiota, 16S ribosomal RNA

Introduction

Chronic rhinosinusitis (CRS) is fundamentally an inflammatory sino-nasal disease that persists for over 12 weeks, and occurs in >5% of the general population [1, 2]. CRS is generally classified into two broad categories based on phenotype: one with nasal polyps (CRSwNP) and one without (CRSsNP) [3, 4]. However, the aetiology of CRS is poorly understood. Many of the current theories hold that infectious microbial agents and their products initiate inflammatory changes in the host mucosa [5]. Research from our own group has suggested that an imbalance (dysbiosis) in the bacterial community structure may be linked to this process [6]. Recently, there has been an increased focus on the interface between host epithelial cells and the environment in the sino-nasal cavity of CRS patients [7, 8].

The airway epithelium plays an important role in innate host defence [7, 9], as it is the first point of contact between the mucosa and external environment. Pathogens are recognised by transmembrane receptors such as toll-like receptors (TLR) that are usually expressed on macrophages, dendritic cells, endothelial and epithelial cells [10, 11]. To date, 10 functional TLRs have been identified in humans, of which TLR2/1 and TLR4 play a prominent role in recognising components of Gram-positive bacteria (peptidoglycan and lipoteichoic acid) and Gram-negative bacteria (lipopolysaccharide (LPS)) [12]. The activation of these TLRs results in activation of the NF-κB pathway, which results in production and release of inflammatory cytokines that stimulate the host innate and adaptive immune responses. Although a number of studies have investigated TLRs in lower respiratory diseases, very few have focused on sino-nasal diseases. All 10 TLRs have been shown to be expressed in both

healthy and CRS sino-nasal mucosa [10], while mRNA expression of TLR2 and TLR4 is significantly greater in CRS cohorts compared with controls [13, 14]. Recent work has begun to explore the possible nature of interactions between the host and its associated microbiota [15, 16]. These previous studies have targeted specific groups of bacteria, which risks overlooking the role of other organisms in the community and their influences on host inflammatory responses. The existing literature reveals major discrepancies among different studies, which need to be addressed in the immediate future.

This study aims to investigate the complex interactions between the host and the microbial components associated with sino-nasal mucus by analysing host inflammatory cytokines, bacterial community structure, and bacterial abundance in sino-nasal mucus of patients with CRSwNP, CRSsNP, CRS with cystic fibrosis (CF), and healthy controls. In addition, the activation of TLR2/1 and TLR4 receptors was measured in vitro using transfected human embryonic kidney (HEK) cells. The findings from this study provide novel insights into the role that bacterial diversity and/or abundance play in influencing host inflammatory responses through TLRs in sino-nasal disease.

Materials and methods

Patients and sample collection

A total of 26 CRS patients were recruited for this study, of which 11 had nasal polyps (CRSwNP), 9 had no nasal polyps (CRSsNP) and 6 had cystic fibrosis (CRS/CF). In addition, 10 healthy control patients undergoing endoscopic sinus surgery for pituitary tumour resection were recruited. Clinical information for each patient is presented in Table 1. Written consent from the patients and ethical approval (NTX/08/12/126) from the New Zealand Health and Disability Ethics Committee was obtained for this study.

Nasal lavage samples were collected from the sino-nasal cavity of patients undergoing endoscopic sinus surgery by a single ORL surgeon (RGD). The mucus within the nasal cavity was gently sucked out into a sterile 10 mL Leukotrap® prior to surgical intervention. Samples were placed on ice and transported to the laboratory within 2 h of collection. A third of the volume of each sample was freeze-dried for 48 h before shipment to the University of Washington (Seattle) for further analysis. The remaining samples were handled and processed according to standard biological laboratory practices, and stored at -20°C until further analysis.

Genomic DNA extraction

Collected mucus was thawed on ice and 200 µL was pipetted into a sterile Lysing Matrix E tube (MP Biomedicals, Australia). A negative extraction control containing 200 µL sterile water was carried out in parallel. Genomic DNA was extracted as described previously [6]. In brief, cells were ruptured using a Qiagen TissueLyser II at 25 m/s for 2x40 s. Following centrifugation the supernatant was transferred to an AllPrep DNA/RNA Isolation Kit (Qiagen) spin column. Genomic DNA was extracted following the manufacturer's instructions and eluted in 30 µL of DNase-free water. The quality and quantity of genomic DNA were measured on a Nanodrop 3300 spectrometer and by using a Qubit® dsDNA HS (High Sensitivity) Assay kit (Life Technologies).

16S rRNA gene sequencing

The V3-V4 region of the bacterial 16S rRNA gene was amplified from the genomic DNA using primers 341F and 806R [17]. These primers have been recommended due to their broad taxonomic coverage [18], and their suitability for investigating the sino-nasal microbiota was examined in silico using Probe Match in the Ribosomal Database Project [19]. For this study, 16S rRNA gene amplicon sequencing was conducted using a dual-indexing (two-step PCR) approach with Nextera technology on the Illumina MiSeq platform (2x300 bp paired-end reads). Primers contained overhanging Illumina adaptors, and were used in equimolar concentrations (0.2 µM) in each PCR reaction. Other components of the reaction included genomic DNA template (~10 ng), dNTPs (0.2 mM), HotStar PCR buffer (x1), $MgCl_2$ (2 mM), 0.5U HotStar DNA polymerase (Qiagen) and PCR-certified water to a final volume of 25 µL. PCR amplification was performed in an Applied Biosystems Mastercycle gradient PCR machine with an initial denaturing step of 95°C for 15 min, followed by 35 cycles of denaturation (95°C for 30 s), annealing (55°C for 30 s), and elongation (70°C for 40 s), with a final elongation step at 70°C for 3 min. Each sample was amplified in two separate reactions which were then pooled. Amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter Inc.) as per the manufacturer's instructions, and eluted in 20 µL of DNase-free water. Purified PCR products were quantified using the Qubit dsDNA HS Assay kit (Life Technologies), and qualitatively checked on Agilent High Sensitivity DNA chips (Agilent Technologies). The negative extraction control produced no amplifiable or quantifiable PCR product and thus could not be sequenced. Sequencing was performed by a commercial sequencing supplier (Macrogen, South Korea) on the Illumina MiSeq. Raw sequences have been uploaded to the NCBI Sequence Read Archive (BioProject number: PRJNA308506).

Bioinformatic analysis of 16S rRNA gene amplicon sequences

Raw sequences were analysed using a combination of USEARCH and QIIME software packages [20, 21]. USEARCH was used to merge paired-end sequences, trim adaptors and primers, remove chimeras, and filter out sequences of <200 bp. The remaining high-quality reads were assigned into operational taxonomic units (OTUs) based on 97% 16S rRNA sequence similarity. Abundance data were re-incorporated into the dataset by mapping

Table 1. Patient information for this study.

Group	Patient No.	Revision	Age	Euro-pean	Sex	PO* score	Smoker	Asthma	Aspirin sensiti-vity	Anti-biotic	Pred-nisone	LM†
Control	1	N	46	N	F		Y	N	N	N	N	
	2	N	52	Y	F		Ex	N	N	N	N	
	3	N	68	N	F		N	N	N	N	N	
	4	N	48	N	F		Ex	N	N	N	N	
	5	N	84	N	F		N	N	Y	N	N	
	6	N	69	Y	F		Ex	N	N	N	N	
	7	N	61	Y	F		N	N	N	Y	N	
	8	N	54	Y	F		N	N	N	N	N	
	9	N	40	Y	F		Ex	N	N	N	N	
	10	N	53	Y	M		Ex	N	N	N	N	
CRSsNP	1	N	23	Y	F	15	N	N	N	N	N	10
	2	Y	67	Y	F	2	Ex	N	N	N	N	18
	3	Y	53	Y	M	11	Ex	N	Y	N	N	9
	4	N	33	Y	F	24	N	Y	N	N	N	20
	5	Y	58	Y	M	15	N	N	N	N	N	16
	6	N	28	Y	M	18	N	N	N	N	N	9
	7	Y	37	Y	M	5	N	N	N	N	N	16
	8	Y	41	Y	F	13	N	Y	N	N	Y	21
	9	N	19	Y	F	21	N	Y	N	N	N	14
CRSwNP	1	N	39	Y	M	15	N	Y	N	N	N	20
	2	N	18	N	M	11	N	Y	N	N	N	16
	3	N	37	Y	M	20	N	Y	N	N	N	13
	4	N	37	N	M	11	Y	N	N	N	N	20
	5	Y	66	N	M	18	Y	Y	N	N	N	23
	6	N	38	Y	M	22	Y	N	N	N	N	21
	7	N	31	Y	F	12	Y	Y	N	N	N	15
	8	N	43	Y	M	19	N	Y	N	N	N	24
	9	Y	25	Y	F	25	N	Y	N	N	N	22
	10	Y	71	Y	F	20	Ex	Y	N	N	N	23
	11	N	50	Y	F	18	N	N	N	N	N	14
CRS/CF	1	Y	21	Y	M	17	N	N	N	N	N	21
	2	Y	22	Y	M	7	N	N	N	N	N	24
	3	Y	37	Y	F	20	N	N	N	Y	N	12
	4	Y	50	N	F	17	Ex	Y	N	N	N	13
	5	Y	31	Y	F	21	N	N	N	Y	Y	19
	6	Y	32	Y	F	19	N	N	N	Y	Y	16

*PO: pre-operative symptom; † LM: Lund-Mackay; EX: ex-smoker; Y: yes; N: No

the original sequence files with each unique OTU. The resulting dataset was converted into a biom table which could be further recognised by QIIME. Sequences were evenly subsampled to 3500 sequences per sample, and alpha diversity of the bacterial communities was estimated for each sample using the 'observed species' and Chao1 richness metrics, and Shannon and Simpson diversity indices were used to measure richness and evenness. Rarefaction curves were built for each sample using the 'observed species' metric with a total of 100 permutations performed (Figure S1; online version).

Unique sequences from each representative OTU were taxonomically assigned against the SILVA 16S rRNA gene database (version 119) using the uclust algorithm in QIIME. Abundance data were re-populated to the dataset using the biom table. A phylogenetic tree was constructed using FastTree 2.1.3 [22]. Beta diversity measures (weighted and unweighted UniFrac, Bray-Curtis dissimilarity) were calculated based on the constructed phylogenetic tree. Bray-Curtis results are shown in the MDS plots and are representative of the results from all three beta diversity measures (Figure S2; online version).

Quantitative PCR to estimate bacterial abundance

To investigate the impact of bacterial load on sino-nasal disease and TLR activation, the relative proportions of bacterial and human DNA in the extracts of each sample were estimated as described previously [6]. In brief, quantitative real-time PCR was employed to calculate the total number of 16S rRNA gene copies in the proportion of bacterial DNA present in the genomic DNA extract (30 µL). For this purpose, primers bactin-F (nucleotide position, 393-413) and bactin-R (622-642) [23] were used to target the human beta-actin (ACTB) gene, and primers 8F and 341R [24, 25] were used to target the bacterial 16S rRNA gene. Standards (including a 10-fold dilution series) for each gene of interest were prepared. Amplification efficiency of each primer pair was calculated based on the respective standard curve using the formula: E = 10(-1/slope)-1 x 100%. Melting curve analysis was also performed to verify the specificity of the primer pairs, by using software 'dissociation curve' (Applied Biosystems). The reaction mix consisted of 7.5 µL of 1x QuantiTect SYBR Green master mix (Qiagen) with HotStar Taq, 0.5 µL of each primer (10 µM), the respective genomic DNA template (10 ng for 16S rRNA gene PCR; 1 ng for ACTB gene PCR) or prepared standard, and PCR grade water to a final volume of 15 µL. Thermal cycling conditions were as follows: 50°C for 2 min, activation step at 95°C for 15 min, followed by 40 cycles of denaturation (95°C for 15 s), annealing (60°C for 1 min), and elongation (72°C for 15 s). All samples including the non-template control and dilution series of standards were run in triplicate. Results were analysed using the ABI Prism 7900HT sequence detection system (Version 2.4).

Measurement of mucosal cytokines

Mucus specimens from healthy, CRS and CF patients were tested for the presence of IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A by Cytometric Bead Array (CBA) using a Human Th1/Th2/Th17 Kit (BD Biosciences). CBA is an effective approach for measuring multiple cytokines in small sample volumes such as mucus [26, 27]. Mucus samples were thawed on ice, centrifuged briefly to remove any particulate material, then supernatants were immediately transferred undiluted into CBA assay tubes and processed as per the manufacturer's instructions. CBA data were acquired on an LSR II with BD FACSDiva Software version 6.1.3 and analysed using FCAP Array™ version 1.0.1 (all BD Biosciences). Standard curves were produced using a 5-parameter logistic model with a fitting accuracy of >99.9% for all cytokines. Values below the manufacturer's minimum detection limits for this assay are reported as zero.

HEK293 cell TLR2/1 and TLR4 activation assays

HEK293 cell TLR2/1 and TLR4 activation assays were performed as previously described [28], with slight modifications. HEK293 cells were plated into 96-well plates at a density of 4 x 10^4 cells/well and transfected the following day. Transfections were performed using calcium phosphate precipitation of plasmids encoding the firefly luciferase, Renilla luciferase, combined with plasmids bearing human mCD14, human TLR2 and human TLR1 (TLR2/1 assays), or combined with plasmids bearing human mCD14, human TLR4 and MD-2 (TLR4 assays). The cells were allowed to grow 20-24 h post-transfection and test wells were stimulated in triplicate for 4 h at 37°C and 5% CO_2 with the indicated concentrations of mucus in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Mucus samples were prepared as stock concentrations of 25 mg/mL by adding ultrapure water to lyophilized patient samples, vortexing and sonicating for 5 min for maximum resuspension. Supernatants were collected following centrifugation at 4°C and stored at -80°C until stimulation, whereby stock solutions were serially diluted in DMEM containing 10% FBS to the indicated concentrations (50 pg/mL and 5 pg/mL). Following 4 h stimulations the reactions were terminated by washing the HEK293 cells with phosphate-buffered saline and lysing the cells with 50 µL of passive lysis buffer (Promega). The luciferase activity of 10 µL of each HEK293 cell lysate was measured using the Dual Luciferase Assay Reporter System (Promega). Positive controls using Escherichia coli LPS for TLR4 and synthetic triacylated lipoprotein (Pam3CSK4) for TLR2/1 were used. HEK cells transfected with reporter plasmids without either TLR4 or TLR2/1 were used as negative controls. Results are presented as increases in either TLR2/1 or TLR4 transfected cells compared with negative control cells. Data are expressed as fold increase of NF-κB-activity, which represents the ratio of NF-κB-dependent firefly luciferase activity to β-Actin promoter-dependent Renilla luciferase activity.

Statistical analysis

Study data were entered into an Excel database, and imported to SAS version 9.4 (SAS Institute Inc. Cary NC) and R version 3.2.2 (R Foundation for Statistical Computing) for final analysis. All statistical tests were two-sided at a 5% significance level. No imputation was considered on the missing data. Demographic and clinical characteristics of all patients were summarised by patient cohorts. Continuous variables were presented as mean, standard deviation, median and range. Categorical variables were presented as frequencies and percentages. Differences between groups were first assessed on individual variables of interest using non-parametric tests with small sample sizes. More specifically, the Kruskal-Wallis test was used on continuous variables and the Fisher's exact test was used on categorical variables. When an overall significant difference was found between groups, post-hoc pairwise comparisons were conducted with Bonferroni adjustment for multiple comparisons. Spearman correlation coefficients were used to investigate the association between important variables, within healthy control and CRS groups. Adjusted relative risk and 95% confidence intervals were estimated.

Results

Characteristics of the cohort

Subjects chosen for this study were grouped into four categories based on sinus disease status, presence of nasal polyps, and co-morbidity with cystic fibrosis (Table 1). Members of the control group were, overall, significantly older ($p<0.01$, Dunn's test) than those in the CRS group. Interestingly, 90% of individuals in the control group were female, while males and females were more equally distributed in the CRS sub-groups. Another important clinical factor only observed in the CRS cohort was asthma. There were no significant differences in the Lund-Mackay or pre-operative symptom scores among the three CRS sub-groups.

Bacterial diversity and abundance in nasal mucus

A total of 660,881 high-quality 16S rRNA gene sequence reads were used in this study after quality trimming. The number of reads for each sample varied from 8,838 to 31,309. The overall bacterial diversity (OTU richness) of the samples was low compared with that of many other human body sites (e.g. skin, intestine). The number of OTUs across all samples was 243. Bacterial richness and evenness were compared between patients with CRS without nasal polyps (CRSsNP), CRS with nasal polyps (CRSwNP), CRS with cystic fibrosis (CRS/CF), and healthy controls. All measures (Chao1, Observed OTUs, Shannon, Simpson) showed significant differences ($p<0.05$, Kruskal-Wallis test) between the CRS/CF group and all others (Figure 1A-D). Interestingly, no significant differences in bacterial richness or evenness were observed among CRSsNP, CRSwNP, and healthy controls.

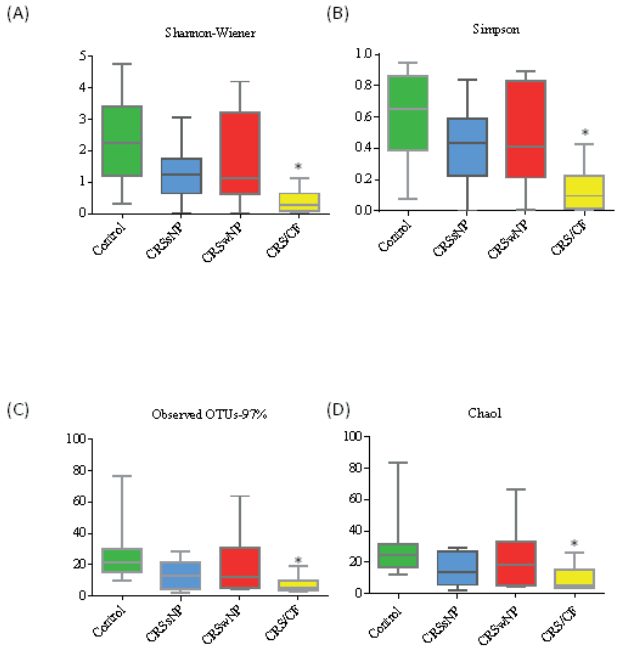

Figure 1. Box and whisker plots comparing bacterial community diversity between CRSwNP, CRSsNP, CRS/CF and healthy control individuals. (A) Shannon-Wiener, (B) Simpson, (C) observed OTUs-97%, and (D) Chao1 indices were calculated for the four groups of samples. Significant differences ($p<0.05$, Kruskal-Wallis test) between the CRS/CF and all other groups are displayed on the plots with an asterisk.

Bacterial diversity resembled that previously reported for the human sino-nasal cavity, with considerable variation observed among individuals. Variation based on bacterial community composition between the four groups (CRSsNP, CRSwNP, CRS/CF and control) was measured using a multivariate analysis ('adonis' function in R package). The differences between these four groups explained 11.9% of the variation in this data set, while the majority of the variation remained unexplained. This indicates that disease status does not play a major role in accounting for the variation observed in bacterial community composition among individuals in this study. Overall, 22 dominating genera were found across all 36 samples, with only one or two dominating in each sample, including members of the genera *Pseudomonas, Haemophilus, Enterobacter*, and *Staphylococcus* (Figure 2B). A specific bacterial community or causative bacterial agent of CRS was not observed in this study. However, patients with CRS/CF were generally dominated by members of a single genus (such as *Pseudomonas, Staphylococcus* or *Fusobacterium*). Interestingly, the control cohort also harboured the same OTUs or genera as the CRS cohort, but generally had greater bacterial community diversity (Fig. S1). Bray-Curtis MDS plots (Fig. S2) reveal that TLR activated samples tend to cluster together at a broad phylum/class level and at genus level. However, no

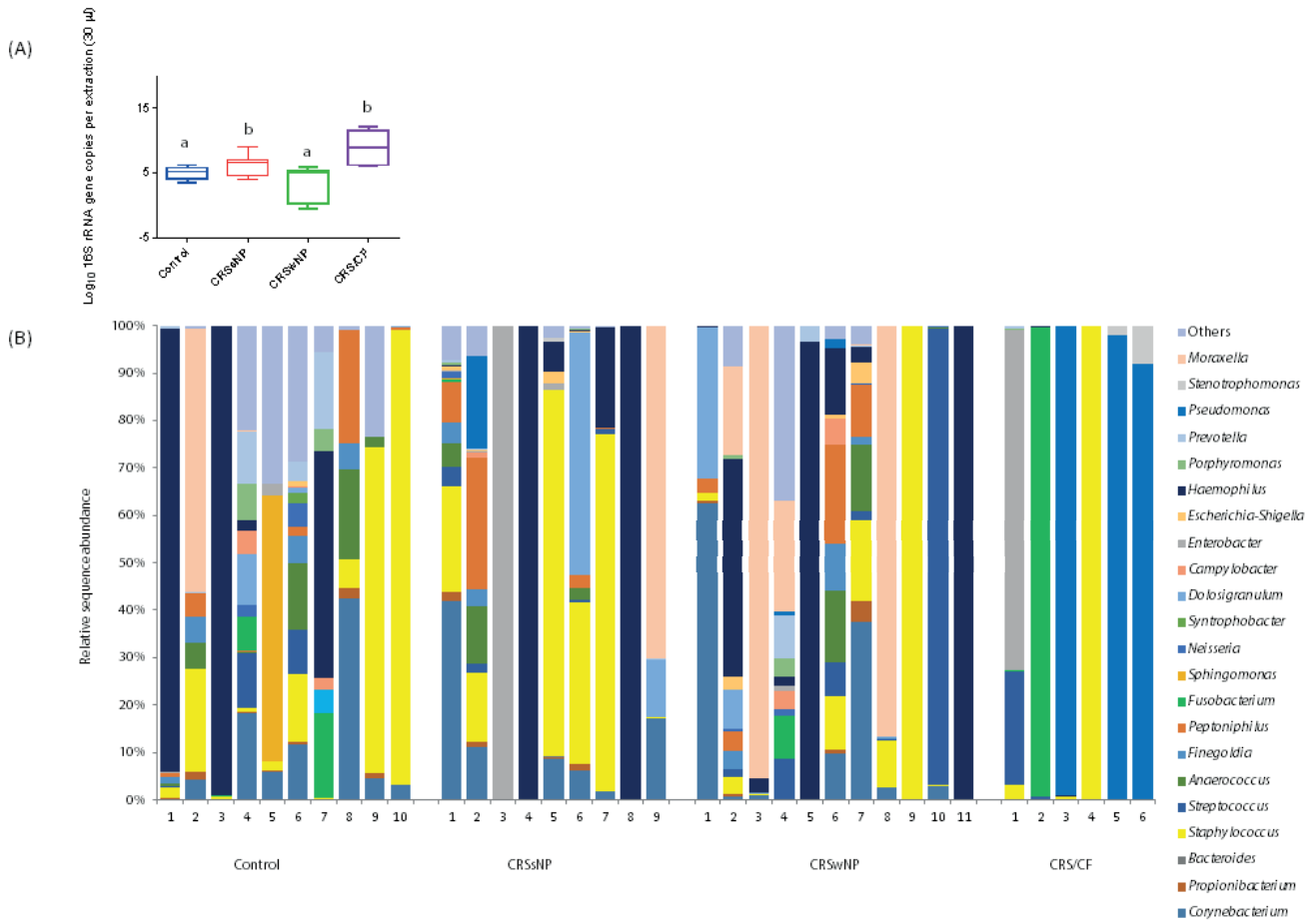

Figure 2. 16S rRNA gene-based bacterial community (A) abundance and (B) composition at genus level in this study. Abundance is displayed as box and whisker plots, which show the median, lower quartile and upper quartile for each sub-group of this study. Bacterial abundance of CRS/CF (b) and CRSsNP (b) groups was significantly higher ($p<0.05$, Dunn's test with Bonferroni adjustment for multiple comparisons) than CRSwNP (a) and healthy controls (a). 16S rRNA gene copy numbers are used as a proxy for bacterial abundance in qPCR. Bacterial composition, displayed as bar graphs for each individual, are represented as relative sequence abundance.

obvious clusters between the four sub-groups of this study were observed.

Quantitative real-time PCR was used to estimate the bacterial load in each sample (Figure 2A). Patients with CRS/CF had significantly higher ($p<0.05$, Dunn's test with Bonferroni adjustment for multiple comparisons) bacterial load compared with healthy controls and CRSwNP. Interestingly, the CRSsNP cohort also had a higher bacterial load than CRSwNP and healthy control samples. There were no significant differences in bacterial load between the other groups. Again, as previously mentioned, inter-patient variability within cohorts was substantial and single patients may be accounting for the majority of the changes observed (Figure 2A).

Inflammatory cytokine profiles in nasal mucus

To determine the type of host inflammatory responses with CRS, inflammatory cytokine markers were measured in the sino-nasal lavage samples (Figure 3). Of the seven measured cytokines, IL-6 and TNF produced the highest signals for healthy controls (Figure 3A), CRSsNP (Figure 3B), CRSwNP (Figure 3C), and CRS/CF (Figure 3D). Low level measurements of IL-10 and IL-17A were also observed in one or two patients from each cohort. Correlations between patients that produced higher levels of TNF or IL-6 with disease severity and TLR activation will be explored in a following section.

TLR activation by sino-nasal mucus

Expression of TLRs in the sino-nasal tissue, together with host mechanisms that preclude engagement with the receptor, will significantly influence the inflammatory response. In this study, TLR activation by sino-nasal mucus was measured in vitro. Similar experimental models have been used previously to measure TLR activation by bacterial pathogens [29, 30]. Dunnett's test for multiple comparisons was performed to evaluate significant

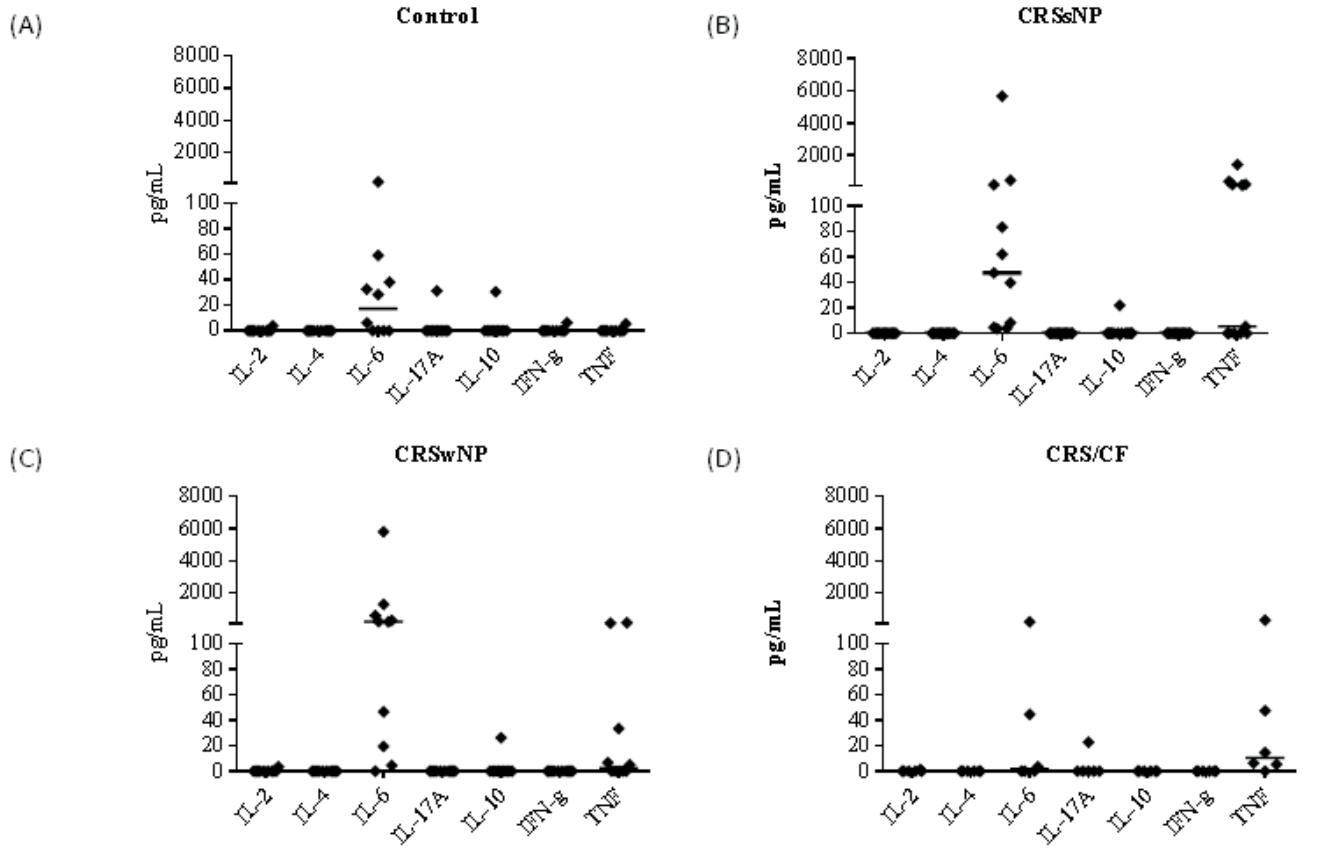

Figure 3. Measurements of pro-inflammatory cytokines IL-2, IL-4, IL-6, IL-17A, IL-10, IFN-γ, and TNF in the mucus samples of 36 individuals. Patients are split into four groups based on clinical diagnosis: healthy control (A), CRS without nasal polyps (B), CRS with nasal polyps (C), and CRS with cystic fibrosis (D). Horizontal bars represent the median value for each of the measured cytokines.

activation of TLRs compared with a negative control. Activity of TLRs was not consistent across all patients within each group. A small proportion of the patients had either TLR2/1 (CRS/CF-5,6) or TLR4 (Control-3; CRSsNP-8; CRSwNP-5,11) activation and in some cases both of these TLRs (Control-7; CRSsNP-3,4) were activated (Figure 4). The potential microbial triggers of TLR activity for these patients were investigated further.

Correlation between TLR activation and other significant factors

Of the 26 CRS samples in this study, seven exhibited measurable TLR activity, while two of the 10 healthy control samples also displayed activation. The CRS samples with TLR activation were further broken down into sub-groups where 3 patients without polyps, 2 patients with polyps and 2 with CF displayed activity. There were no measured clinical confounders (such as asthma, antibiotics, or aspirin sensitivity) associated with the patients that were positive for TLR activity. The microbiota of TLR activate samples were largely composed of a single Gram-negative genus belonging to the class *Gammaproteobacteria* (Figure 5A). In particular, members of the genera *Haemophilus, Pseudomonas* and *Enterobacter* dominated (>90% of the sequence reads) the CRS bacterial community of these TLR activated samples (Figure 5B). *Haemophilus* also played an important role in the two TLR activated control samples, along with *Fusobacteria* (20%) to a lesser extent. Interestingly, the two TLR activated CF/CRS patients behaved slightly differently to other CRS samples in that they were predominantly composed of *Pseudomonas* and not *Haemophilus*, and displayed measured activity with only TLR2/1 receptors. Furthermore, Gram-positive organisms (such as *Staphylococcus, Corynebacterium*, and *Streptococcus*) were positively correlated with non-TLR activated CRS samples.

Bacterial abundance was elevated in TLR activated compared with non-activated samples within each CRS sub-group (Figure 5C). Interestingly, this difference was not observed in activated versus non-activated TLRs in the healthy cohort. However, the significance of this finding cannot be tested due to the small sample size of TLR activated samples.

TNF and IL-6 were detected in negligible amounts in control samples. In contrast, TLR activated CRS samples correlated positively with TNF, indicative of an elevated host inflammatory response amongst these cohorts (Figure 5D). However, IL-6 did not correlate with the activity of the two measured TLRs in this study.

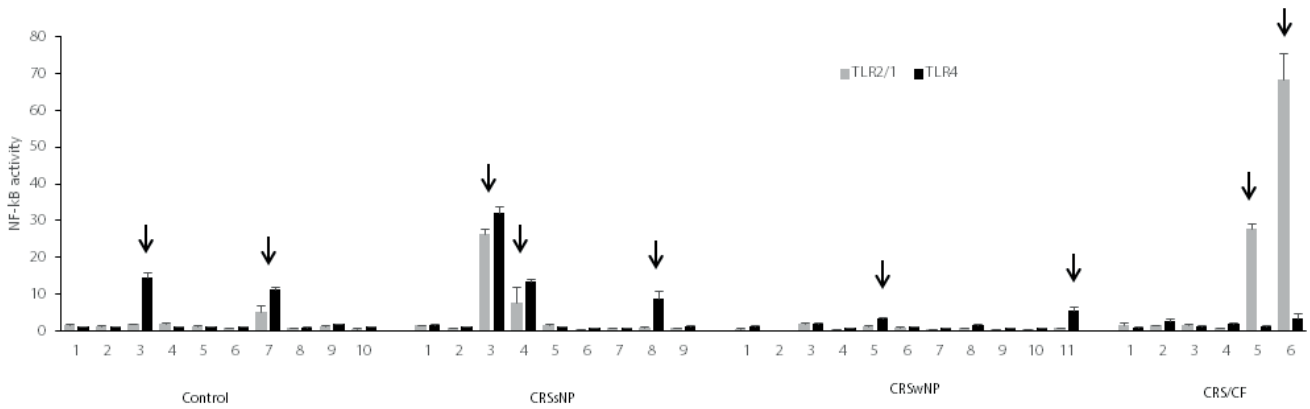

Figure 4. Toll-like receptor stimulation by sino-nasal mucus in vitro was recorded by measuring NF-κB activation. Measurements of NF-κB activity (mean ±SE) were recorded for each individual of this study for the two TLRs (TLR2/1-grey bars and TLR4-black bars) of interest at 50 pg/μL of mucus. Black arrows indicate samples that displayed elevated TLR activity over negative control.

Discussion

This study of a cohort of 36 individuals is the first to report on significant associations between the sino-nasal microbiota and TLR activation. Twenty-six patients with CRS and 10 healthy controls were chosen. Our results demonstrate that Gram-negative members of the Gammaproteobacteria dominate the bacterial community of both TLR2/1 and TLR4 activated samples. Furthermore, TLR activation in CRS cohorts was significantly associated with bacterial abundance and host release of the pro-inflammatory cytokine TNF. There were no patterns observed between clinical confounders (such as prevalence of asthma, antibiotic and prednisone usage or aspirin sensitivity) and TLR activity within the cohort of this study. However, with larger cohorts it is possible that these associations become more apparent. Mucus contains a range of toxins, tissue injury factors, damage-associated molecular pattern molecules and pathogens including viruses [31]. However, by specifically investigating the activation of TLR2/1 and TLR4 receptors, that are stimulated by components of Gram-positive and -negative bacteria, this study focuses on the sino-nasal bacterial interactions with TLR receptors. Patterns of bacterial interactions within the healthy control and diseased groups were observed and will be discussed further below.

TLR activation and host immune response

The results from the current study show that TLR2/1 and TLR4 were activated by the sino-nasal mucus layer of seven CRS samples, which could have directly influenced the elevated measurements of TNF in the mucus of these samples. In contrast, the two control samples showed no TNF activity. TNF has been suggested to play a synergistic role in the induction of chronic inflammation when in the presence of Th1 or Th2 cytokines [32]. IL-6 and TNF have previously been found to be significantly elevated in tissues of CRS patients relative to healthy nasal tissue [33]. Furthermore, treatment with corticosteroids reduced symptoms of disease and led to corresponding reduction in IL-6 and TNF, suggesting that these cytokines are correlated with inflammation. The possibility that some of the cytokines investigated in this study were not detected due to the diluted nature of nasal lavages must also be acknowledged.

Bacterial interactions with TLR

The strong positive correlation between TLR2/1 and TLR4 activity and members of the Gammaproteobacteria (*Haemophilus*, *Enterobacter*, *Pseudomonas*) in the CRS sub-groups, suggests that bacterial composition plays an important role in stimulating TLRs. However, the two control samples that also displayed TLR activity were dominated by *Haemophilus* but did not show mucosal inflammation. We conclude that other factors along with bacterial composition must be involved in TLR activation and chronic inflammation. Bacterial species richness and evenness (as measured by Shannon-Wiener, Simpson index, observed OTUs and Chao1) could also influence TLR activity, as the results from this study show that samples with TLR activity tend to have lower bacterial diversity than their counterparts. Another possible explanation is that some individuals are more prone to immune hyperresponsiveness to commensal microorganisms, as has been suggested previously [15].

Interestingly, organisms commonly implicated in CRS such as members of *Staphylococcus*, *Corynebacterium*, *Moraxella*, were found exclusively in patients without TLR activity in all sub-groups of this study [6, 34-36]. The association with a negative immune response suggests that it may play a role in downregulation of the host immune response. Evidence of *S. aureus* cells forming microcolonies within the tissue of the sinus mucosa without inducing a local immune reaction has been found previously [37]. Suppression of the host immune system may also be due to the constant exposure to microbial components in the mucus layer, resulting in a desensitisation effect [38].

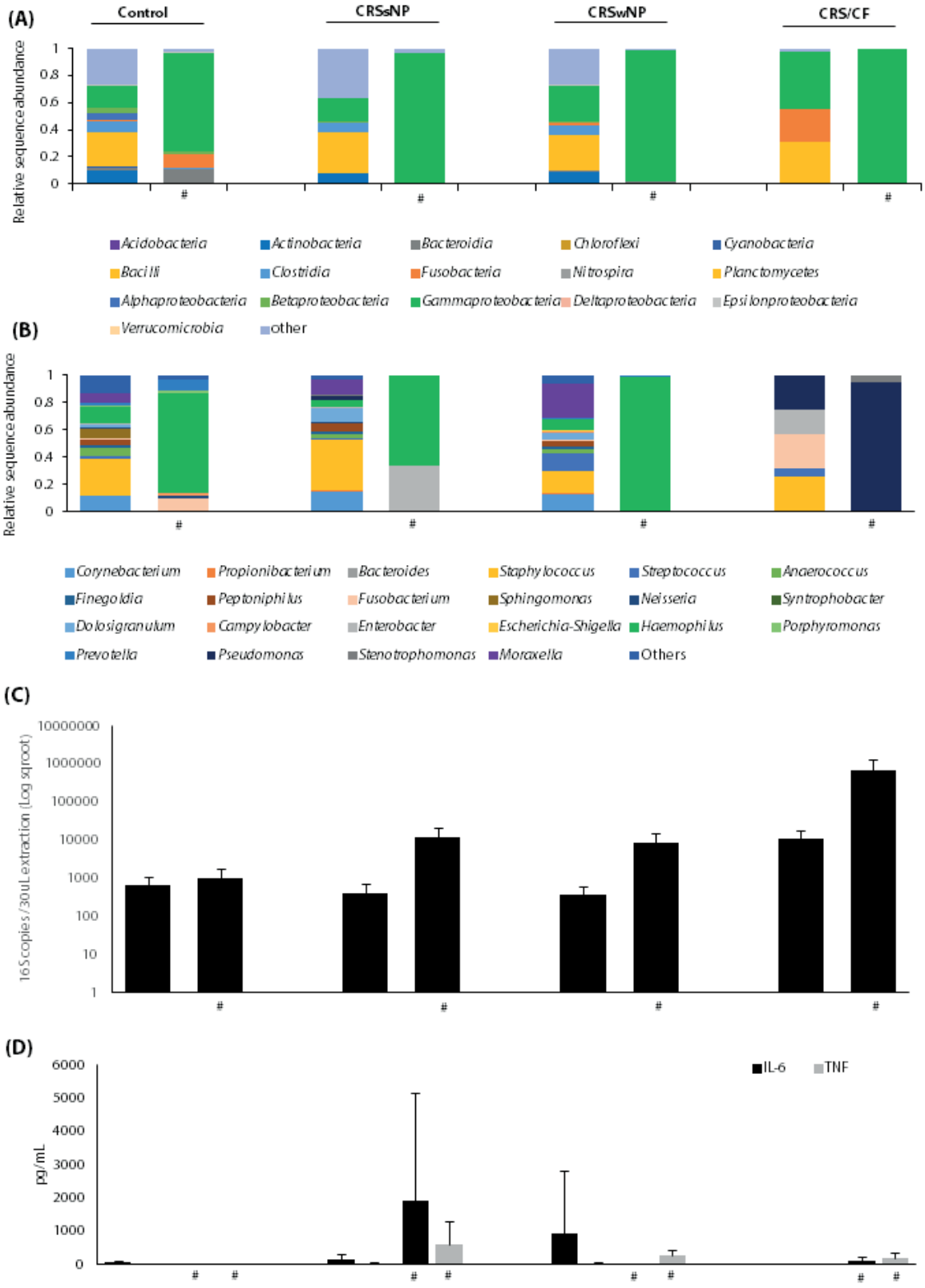

Figure 5. Samples that displayed TLR activity were averaged and displayed as bar graphs within each sub-group of this study. The bacterial community ((A) phylum/class level and (B) genus level), bacterial abundance (C) and cytokine measurements (IL-6 and TNF) (D) were re-evaluated based on these new groupings. The mean and standard deviation is displayed in graphs (C) and (D). # represents TLR activated groups of samples.

Bacterial burden in CRS patients plays a major role in TLR activation, as seen in this study. Within the TLR activated cohort, patients with CF have significantly higher bacterial abundance compared with CRSwNP and healthy controls. Several studies suggest that CF involves a defective epithelium or innate immune response due to CF transmembrane conductance regulator mutations, which lead to impaired mucociliary clearance and accumulation of pathogens [39]. The higher abundance of bacteria observed in TLR activated CF patients in this study is probably due to defective mucociliary clearance of the airway epithelial cells, though this requires further investigation. In addition, the antimicrobial therapies used for CF patients could be selecting for organisms that are resilient (*Pseudomonas aeruginosa*) or resistant (*S. aureus*) [40, 41].

One hypothesis holds that patients with a disrupted epithelial layer have TLRs that are more exposed to pathogens in the mucus layer, which then initiates the immune response and inflammation [7]. Disruption of the epithelial barrier has been observed previously among patients with asthma and CRS [42, 43]. In this study, we speculate that some patients with CRS are predisposed to TLR activation due to compromised integrity of the epithelial barrier, which then leads to a heightened immune response, resulting in inflammation. However, this requires further investigation.

Conclusion

In conclusion, the results of this study open a new perspective on CRS and possible microbial instigators of inflammation in this disease. We speculate that chronic inflammation in the sinuses is caused by multiple pathways and factors between the host and the environment, however by better understanding one such pathway (through TLRs), the complexities of such a disease can begin to be unravelled. Results from this preliminary study suggest that only a small proportion (20-30%) of patients from each subgroup display measurable TLR activity. Future studies would greatly benefit from larger cohorts to help solidify the positive correlations found between bacterial composition, bacterial abundance, TNF and TLR activity. In addition, future research in this area should focus on investigating TLR activation by clinical bacterial isolates of CRS patients, which will help strengthen the findings of this study.

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Authorship contribution

Conceived, study design and collection: K.B., R.D., R.G.D.
Performed the experiments: K.B., A.C., M.H., F.J.R.
Analysis and interpretation: K.B., F.J.R., Y.J.
Writing the report: K.B., F.J.R, Y.J., M.W.T., R.D.
Critical review of the paper: All authors

Conflict of interest

The authors declare no conflict of interest relevant to this study.

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