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Original article

S. *aureus* biofilm metabolic activity correlates positively with patients' eosinophil frequencies and disease severity in chronic rhinosinusitis

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ABSTRACT

Chronic rhinosinusitis (CRS) is a persistent inflammation of the sinus mucosa. Recalcitrant CRS patients are unresponsive to medical and surgical interventions and often present with nasal polyps, tissue eosinophilia, and *Staphylococcus aureus* dominant mucosal biofilms. However, *S. aureus* sinonasal mucosal colonisation occurs in the absence of inflammation, questioning the role of *S. aureus* in CRS pathogenesis. Here, we aimed to investigate the relationship between *S. aureus* biofilm metabolic activity and virulence genes, innate immune cells, and disease severity in CRS. Biospecimens, including sinonasal tissue and nasal swabs, and clinical datasets, including disease severity scores, were obtained from CRS patients and non-CRS controls. *S. aureus* isolates were grown into biofilms *in vitro*, characterised, and sequenced. The patients' innate immune response was evaluated using flow cytometry. *S. aureus* was isolated in 6/19 (31.58%) controls and 23/53 (43.40%) CRS patients of 72 recruited patients. We found increased *S. aureus* biofilm metabolic activity in relation to increased eosinophil cell frequencies and disease severity in recalcitrant CRS cases. Mast cell frequencies were higher in tissue samples of patients carrying *S. aureus* harbouring *lukF.PV*, *sea,* and *fnbB* genes. Patients with *S. aureus* harbouring *lukF.PV and sdrE* genes had more severe disease. This offers insights into the pathophysiology of CRS and could lead to the development of more targeted therapies.

Chronic rhinosinusitis (CRS) is a heterogeneous chronic inflammatory disease of the sinonasal mucosa associated with tissue remodelling, dysfunction of the sinuses' natural defence mechanisms, and induction of different inflammatory pathways. Patients with CRS are phenotypically classified into two broad categories based on the presence (CRSwNP) or absence (CRSsNP) of nasal polyps [1–3]. CRSwNP, as the severe phenotype, is typically associated with higher rates of recurrence, comorbid asthma, and worse quality of life. This phenotype is characterised by a type-2 eosinophilic inflammation and *Staphylococcus aureus* mucosal colonisation [2,4,5]. CRSsNP has long been considered a non-type 2 inflammation with predominant neutrophils. However, geographical variation in inflammatory cell infiltration and endotypes has recently been described [6,7].

Eosinophils, as a major hallmark of western nasal polyps, express a multitude of surface receptors facilitating their survival, growth, recruitment, and activation [8]. Stimulated eosinophils elicit their bactericidal response by the extracellular release of cytoplasmic granules. Eosinophil granule proteins, including eosinophil cationic protein (ECP), Major basic proteins (MBP1 and MBP2), eosinophil-derived neurotoxin, and eosinophil peroxidase (EPO), activate other immune cells and contribute to the toxicity to microorganisms through the generation of reactive oxygen species as well as the direct killing of bacteria. Eosinophil extracellular traps (EETs) are also thought to serve an important immune response against extracellular pathogens via a distinct pathway from suicidal cell death known as extracellular trap cell death, or ETosis [9]. These lead to airway epithelial damage, hypersensitivity, mucus secretion, and airway remodelling [7,10]. Neutrophils are also considered important components of the innate immune system due to their numerous activities in bacterial killing and sequestration. They apply three means for directly attacking

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microorganisms: phagocytosis, production of neutrophil extracellular traps (NETs), and release of soluble antimicrobials from their granules [11]. Mast cells and basophils, as type-2 innate inflammatory cells, contribute considerably to the ongoing sinonasal inflammation in the context of CRS, generating various inflammatory mediators and toxic granule proteins that can maintain the inflammatory response and promote sinonasal mucosal injury [12]. Mast cells have also been recognised as important mediators in allergic inflammation [13] and other inflammatory conditions such as psoriasis [14].

Despite substantial medical advances, at least 10% of patients with CRS do not respond to medical and surgical interventions and develop refractory disease. These recalcitrant CRS patients frequently have nasal polyps with tissue eosinophilia and often have comorbid asthma and *S. aureus* dominant mucosal biofilms [15,16]. Biofilm formation is one of *S. aureus*' strategies in providing increased resistance to antibiotics and host immune defences. Bacteria within a biofilm have intrinsic characteristics different from planktonic cells [17]. *S. aureus* virulence factors evade host innate immunity in different ways, including modifications of structural components and secretion of extracellular molecules, such as enzymes and toxins acting in concert to promote tissue adhesion, immune evasion, and host cell damage to create a microenvironment that offers even better survival [18–20].

While innate inflammatory markers and *S. aureus* biofilm have been extensively studied in CRS, little is known about their relationship and with disease severity. Here, we investigated *S. aureus* virulence factors and biofilm metabolic activity from *in vitro* grown clinical isolates derived from CRS and non-CRS control patients in relation to patients' innate immune responses and disease severity scores.

1. Methods

1.1. Study design and patient samples

Ethics approval for the collection, storage, and use of clinical isolates and patient samples from CRS and non-CRS control patients was granted by The Queen Elizabeth Hospital (TQEH) Human Research Ethics Committee, South Australia (HREC/15/TQEH/132), and all patients had signed written informed consent. A prospective study was conducted from January 2020 to December 2021 with patients recruited from The Queen Elizabeth Hospital, Memorial Hospital, and Calvary Hospital, Adelaide, Australia. The diagnostic criteria for CRS with and without nasal polyps (CRSwNP and CRSsNP respectively) were contented by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement (EPOS) on CRS [4]. Eligibility for the study included age $\geq$18 years and having CRS requiring endoscopic sinus surgery (ESS). The control subjects underwent endoscopic skull base surgery or septoplasty with no clinical or radiologic evidence of sinus disease. Exclusion criteria included the use of antibiotics or oral corticosteroids in the month before surgery. The severity of CRS was measured based on the completion of the Lund-Mackay (LM) [21], Lund-Kennedy (LK) [22,23], and patient-reported 22-item Sino-Nasal Outcome Test (SNOT-22) questionnaire [24]. A self-reported questionnaire was used to assess the status of asthma, aspirin sensitivity, gastro-oesophageal reflux disease (GERD), and diabetes mellitus. Sinonasal polyp or mucosal tissue samples were collected from the ethmoid sinuses of CRS patients and the ethmoid sinuses and middle turbinate of non-CRS control subjects during ESS. Tissue samples were immediately placed in Dulbecco's modified Eagle medium (Gibco, New York, USA) on ice and transported to the laboratory for processing. Nasal swabs were also collected from the middle meatus and placed in a sterile Amies transport medium (Sigma Transwab, MWE Medical Wire, Corsham, UK) on ice and immediately transported to the laboratory for processing.

1.2. Flow cytometry

Fresh sinonasal polyps or mucosal samples were washed with phosphate-buffered saline (PBS) and dissected into fine pieces before processing into a single-cell suspension by enzymatic digestion with 25 mg/ml of collagenase D (Roche Diagnostics GmbH, Mannheim, Germany), 10 mg/ml of DNAse I (Sigma-Aldrich, USA) and Hanks' Balanced Salt Solution (HBSS, Gibco; Thermo fisher scientific, USA) for 30 min at 37 °C. Cell suspensions were filtered through a 70 μm cell strainer and washed in PBS. Single-cell suspensions were used at a concentration of 4×10^6 cells/ml and stained with Fixable Viability Dye eFluor® 780 (BD Bioscience, San Jose, CA, USA) to exclude dead cells, followed by incubation with Fc Block (BD Bioscience) and fluorochrome-conjugated antibodies **(listed in** Table S1**)**. Subsequently, the cells were washed and resuspended in autoMACS Running Buffer (MACS Separation Buffer, Milteny Biotec, Germany). Multi-colour flow cytometry was performed using a BD FACS Canto II instrument (BD bioscience) and FACS Diva software (BD Bioscience). For each sample, at least 500,000 events were collected. Data were analysed using Flowjo software (FlowJo LLC, Ashland, USA). A summary of the innate immune cells analysed in this study is illustrated in Table S2, and the gating strategy is shown in Fig. S1.

1.3. Isolation of S. aureus

S. aureus was isolated from the nasal swabs of CRS patients and non-CRS controls on mannitol salt agar plates (Oxoid, Basingstoke, UK) and identified using MALDI-TOF (Bruker). All isolates were then screened for MRSA using chromogenic MRSA selective agar (CHROMID® MRSA SMART, bioMerieux, Australia). The *S. aureus* isolates were then stored at −80 °C in tryptone soy broth (TSB, Oxoid) plus 20% (v/v) sterile glycerol for further analysis.

1.4. Evaluation of S. aureus biofilm properties

Overnight cultures of *S. aureus* isolates grown on tryptic soy agar (TSA) plates were transferred into a sterile glass tube of 0.9% sodium chloride and adjusted to 1.0 ± 0.1 McFarland turbidity standard (approximately 3×10^8 CFU/mL). Next, the bacterial suspension was diluted into nutrient broth at 1:15 ratio, and 150 μL of the final suspension was transferred to flat-bottom 96-well microtiter plates and incubated at 37 °C for 48 h in the dark on a gyratory mixer (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm to form biofilms. To evaluate the metabolic activity of *S. aureus* biofilms, an alamarBlue [Resazurin (7-Hydroxy-3H-phenoxazin-3-one10-oxide)] assay was performed. Briefly, 48-h biofilms in black 96-well plates were washed twice with 200 μL per well 1 × PBS to remove planktonic cells and air-dried for 10 min. The wells were then stained with 200 μL of 10-fold dilutions of Alamar Blue cell viability reagent DAL1100 (Invitrogen, Thermo Fisher Scientific, USA) in nutrient broth. Subsequently, the plates were incubated at 37 °C on a gyratory mixer in the dark. The wells containing nutrient broth without bacterial culture were considered a sterility control. The fluorescence intensity of each well was then measured at different time points starting from 30 min and followed by 1h, 2h, 3h, 4h, and 5h using the FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany) at an excitation wavelength 530 nm and emission 590 nm until maximum fluorescence was reached. The experiment was repeated three times in six replicates. ATCC 25923 was used as a biofilm-forming reference strain of *S. aureus*. The values of *S. aureus*

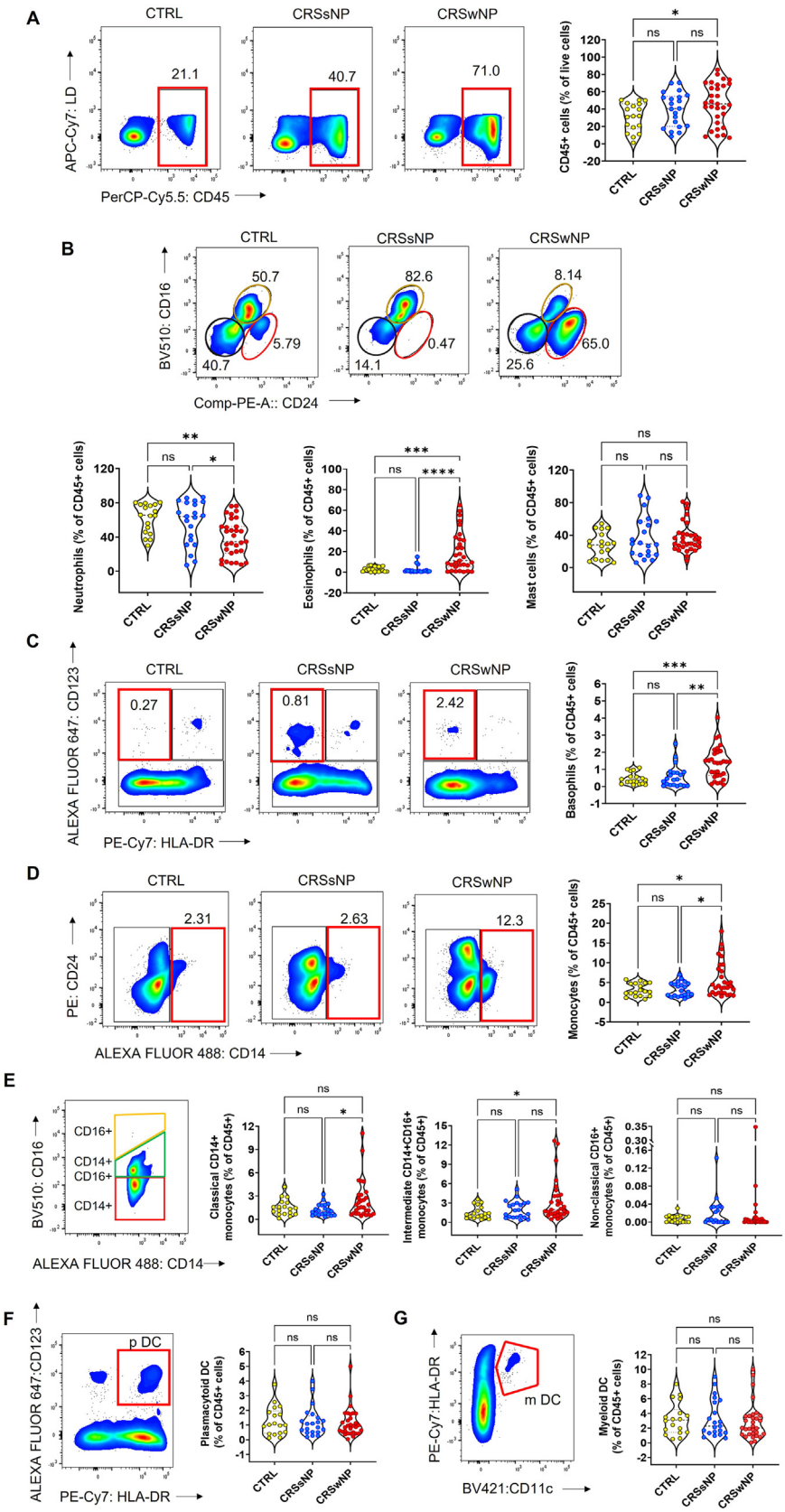

Fig. 1. The frequency of innate immune cells in sinonasal tissue of CRS patients and non-CRS controls. Frequency values and gating for each cell type are given in representative flow cytometry plots. The violin plots are displayed as median (dashed line) plus lower and upper quartiles (dotted lines) for CRSwNP (n = 31), CRSsNP (n = 21), and non-CRS control (CTRL) tissue samples (n = 18). The frequency of CD45+ cells **(A)**, Eosinophils (red line), neutrophils (yellow line) and mast cells (black line) **(B),** basophils **(C),** monocytes **(D),** monocyte subsets including CD14+ classical (red), CD14+CD16+ intermediate (green) and CD16+ non-classical (yellow) **(E),** plasmacytoid dendritic cells (p DC) and myeloid dendritic cells (m DC) **(F).** One-way ANOVA followed by Tukey's multiple comparisons test was used. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

fluorescent intensities were normalised to values for the ATCC 25923 strain and reported as normalised fluorescence intensity.

1.5. DNA extraction and whole-genome sequencing of S. aureus clinical isolates

DNA extraction and whole-genome sequencing (WGS) were performed by SA Pathology, Adelaide, Australia. In brief, *S. aureus* isolates were grown on nutrient agar plates (Sigma-Aldrich) at 37 °C for 24 h. Genomic DNA was then extracted using the MN NucleoSpin®Microbial DNA Extraction Kit (Machery-Nagel GmbH and Co.KG, Duren, Germany). Sequencing libraries were prepared using a modified protocol for the Nextera XT DNA library preparation kit (Illumina Inc., San Diego, Ca, USA). Briefly, genomic DNA was fragmented, followed by the amplification of Nextera XT indices (Illumina Inc.) to the DNA fragments using a low-cycle PCR reaction. Subsequently, the amplicon library was manually purified and normalised. Sequencing was performed on the Illumina NextSeq 550 platform with NextSeq 500/550 Mid-Output kit v 2.5 (Illumina Inc.), yielding paired-end 150 bp reads. Quality control on sequencing reads was conducted using FastQC (v 0.11.9) [25].

1.6. Genomic analysis

S. aureus genomes were assembled using Unicycler v 0.4.8 [26] and annotated with Prokka, v 1.14.6 [27]. Assemblies were quality controlled using QUAST, v 5.0.2 [28]. All genomes were multilocus sequence typing (MLST) typed and grouped into clonal complexes using MLST [29] and tseemann/mlst at Github (https://github.com/tseemann/mlst). Antimicrobial resistance and virulence genes were identified by screening all isolate contigs through the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/) and Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/) using ABRicate, v 1.0.1 (https://github.com/tseemann/abricate).

1.7. Statistical analysis

Statistical calculations were performed using Graph Pad Prism v 9.0.0, (121) (GraphPad Software, San Diego CA, USA). Statistical differences among groups were evaluated using one-way ANOVA or Kruskal-Wallis test depending on the normality of the variables' distribution. The Independent Samples t-test or Man-Whitney was used to assess the statistical differences between the means of two groups. The Pearson or Spearman's rank correlation coefficient was used for the correlation analysis where r-value: 0.00–0.19 = very weak, 0.20–0.39 = weak, 0.40–0.59 = moderate, 0.60–0.79 = strong, 0.80–1.0 = very strong. The results were considered statistically significant when the *p*-value was <0.05. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

2. Results

2.1. Demographic data

A total of 72 participants were recruited in this study. They included CRSwNP patients (n = 32) with a mean age of 50.06 years (range 21–76) and 23/7 male/female ratio (71.87% male); CRSsNP patients (n = 21) with a mean age of 39.38 years (range 21–70) and 12/7 male/female ratio (57.14% male); and non-CRS controls (n = 19) with a mean age of 56.32 years (range 20–89) and 12/7 male/female ratio (63.16% male). In CRSwNP and CRSsNP, about 38% of patients had asthma [(12/32) and (8/21), respectively], while only 15.79% (3/19) of controls had asthma. 15.62% (5/32) of patients with CRSwNP had aspirin sensitivity, while it was only reported in 4.76% (1/21) of CRSsNP patients and none of the non-CRS controls. The demographic data and clinical characteristics of patients are detailed in Table S3. CRS severity scores were higher in CRSwNP than in CRSsNP and control subjects and are illustrated in Fig. S2.

2.2. CRSwNP patients are characterised by increased cell frequencies of eosinophils and basophils and decreased frequencies of neutrophils

FACS analysis of fresh sinonasal polyp or mucosal samples from CRSwNP (n = 31), CRSsNP (n = 21), and non-CRS control subjects (n = 18) showed a significant increase in the frequency of CD45+ cells, as the common leukocyte antigen, in CRSwNP compared to controls ($p = 0.0434$) (Fig. 1A). A dramatic increase was found in the frequency of eosinophils in CRSwNP patients' tissue compared to CRSsNP ($p < 0.0001$) and controls ($p = 0.0001$). In contrast, there was a significant decrease in neutrophils frequencies in CRSwNP compared to CRSsNP ($p = 0.0140$) and controls ($p = 0.0022$). No statistically significant differences were observed in mast cell frequencies among the patient cohorts ($p > 0.05$) (Fig. 1B). Furthermore, the frequency of basophils was elevated in CRSwNP patient tissue compared to CRSsNP ($p = 0.0013$) and controls ($p = 0.0003$) (Fig. 1C). Monocyte cell frequencies also showed a significant increase in CRswNP compared to CRSsNP ($p = 0.0238$) and controls ($p = 0.0224$) (Fig. 1D). The quantification of monocyte subsets, including classical (CD14+), intermediate (CD14+ CD16+), and non-classical (CD16+), exhibited enhanced CD14+ and CD14+ CD16+ cell frequencies in CRSwNP compared to CRSsNP or controls ($p < 0.05$) while there were no significant differences in the frequencies of CD16+ cells among the patient cohorts ($p > 0.05$) (Fig. 1E). Similarly, no significant differences were observed in plasmacytoid- and myeloid dendritic cell (pDC and mDC, respectively) frequencies among CRSwNP, CRSsNP, and controls (Fig. 1F). Eosinophils to neutrophils (EOS/Neu) ratio analysis showed a significantly higher EOS/Neu ratio in CRSwNP patients compared to CRSsNP ($p < 0.0001$) and controls ($p = 0.0009$) (Fig. 2). The correlation analysis between innate immune cells showed a very strong inverse correlation between neutrophils and mast cell frequencies ($r = -0.81$, $p < 0.0001$). Likewise, we found a strong negative correlation between neutrophils and eosinophils frequencies ($r = -0.51$, $p < 0.0001$). In contrast, there were moderate positive correlations between eosinophils and basophils ($r = 0.46$, $p < 0.0001$), monocytes and basophils ($r = 0.59$, $p < 0.0001$),

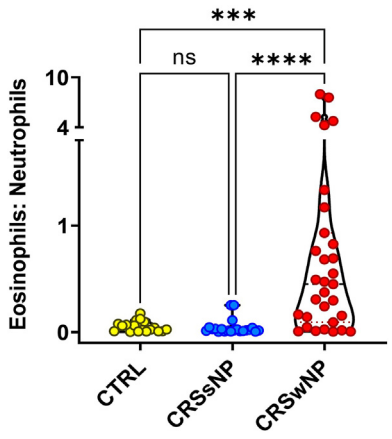

Fig. 2. Eosinophil to neutrophil (EOS/Neu) ratio analysis between CRSwNP (red dots, n = 31), CRSsNP (blue dots, n = 21), and non-CRS control (CTRL) tissue samples (yellow dots, n = 18). Kruskal-Wallis test followed by Dunn's multiple comparisons test was used. ***$p < 0.001$ and ****$p < 0.0001$.

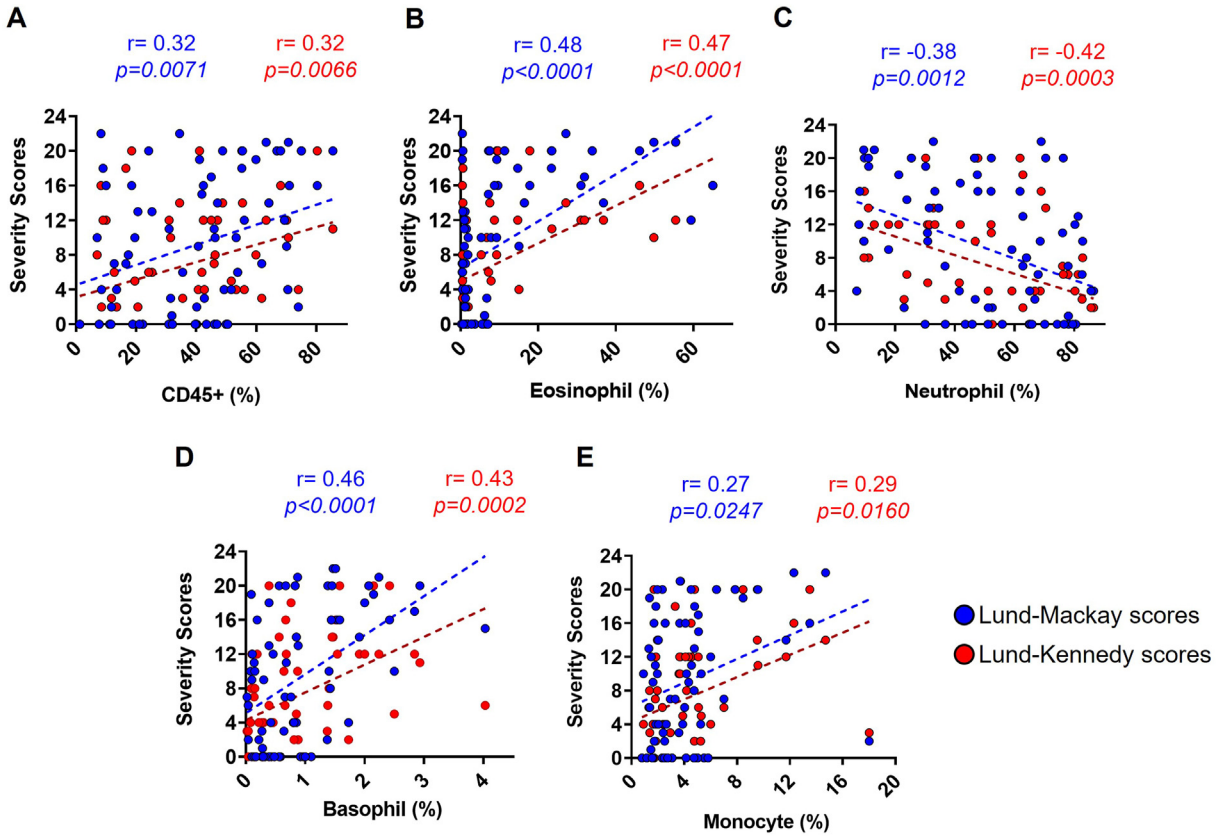

Fig. 3. The correlation analysis between CRS severity scores and the frequency of innate immune cells in the sinonasal tissue from CRS patients and controls (n = 70). Each dot represents the result for an individual patient for the Lund-Mackay radiologic score (blue dots) and Lund-Kennedy endoscopic score (red dots) with the corresponding innate immune cells frequencies. CD45+ cells **(A)**, eosinophils **(B)**, neutrophils **(C)**, basophils **(D)** and monocytes **(E)**. The Spearman's rank correlation coefficient was used.

monocytes and pDc (r = 0.56, $p < 0.0001$), monocytes and mDC (r = 0.52, $p < 0.0001$), and basophils and pDC (r = 0.42, $p = 0.0003$). Furthermore, the correlation analysis by CRS status showed that correlations for neutrophils versus mast cells, monocytes versus basophils, and basophils versus pDC were stronger for control and CRSsNP samples compared to CRSwNP samples, whilst for neutrophils versus eosinophils, strong inverse correlations were seen for CRSwNP tissue samples but not for CRSsNP and control samples (Fig. S3).

2.3. CRS disease severity scores correlate positively with eosinophil and basophil frequencies and inversely with neutrophil frequencies

The correlation analysis of patients' Lund-Mackay, Lund-Kennedy, and SNOT22 severity scores with the frequency of sinonasal tissues' innate immune cells was carried out. Weak to moderate positive correlations were found between the Lund-Mackay and Lund-Kennedy scores and CD45+, eosinophils, basophils, and monocytes frequencies. In contrast, a moderate inverse correlation was observed between the frequency of tissue neutrophils and Lund-Mackay and Lund-Kennedy severity scores. There was no correlation between the patient's subjective SNOT22 scores and the innate immune cell frequencies (Fig. 3).

2.4. S. aureus is cultured from sinonasal cavities in both CRS patients and controls

29/72 (~41%) *S. aureus* isolates were identified from CRS patients and non-CRS controls. The proportion of *S. aureus* among the patients varied with 6/19 (31.58%) and 23/53 (43.40%) isolates derived from controls and CRS patients, respectively. The proportion of CRSwNP, CRSsNP, and controls in *S. aureus*+ and *S. aureus*-

	Samples (n)	*S. aureus*+	*S. aureus*-
CTRL	19	6	13
CRSsNP	21	9	12
CRSwNP	32	14	18
Total	**72**	**29**	**43**

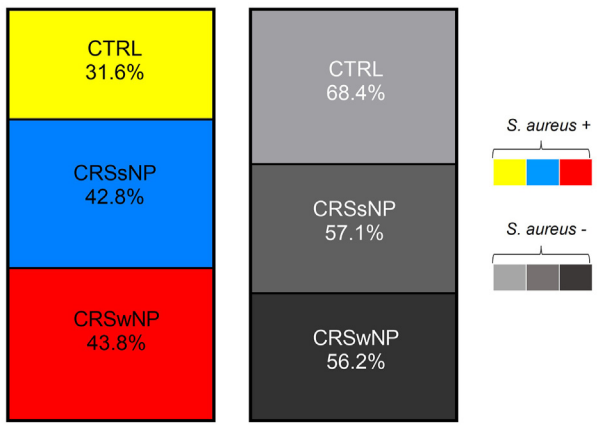

Fig. 4. The number and distribution of *S. aureus* in CRSwNP, CRSsNP, and non-CRS control patients. *S. aureus* + refers to patients who had *S. aureus* in their nasal swabs, while *S. aureus*-refers to patients who did not have *S. aureus* in their nasal swabs.

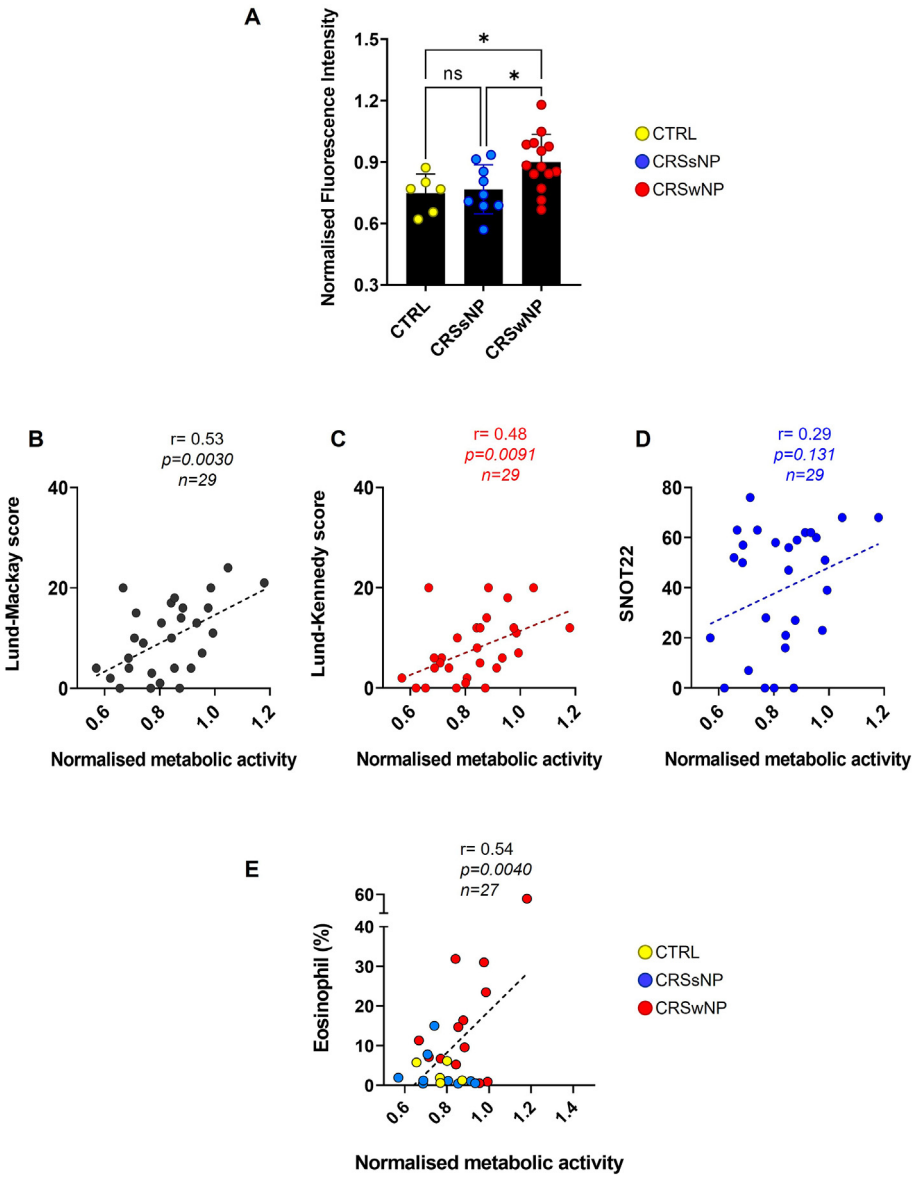

Fig. 5. The *in vitro*-grown *S. aureus* biofilm metabolic activity after 3 h incubation with alamarBlue for non-CRS controls (CTRL, yellow dots, n = 6), CRSsNP (blue dots, n = 9), and CRSwNP (red dots, n = 14). *S. aureus* metabolic activities were normalised to values for ATCC 25923 **(A)**. The correlation between Lund-Mackay (black dots), Lund-Kennedy (red dots), and SNOT22 (blue dots) severity scores and the *in vitro*-grown *S. aureus* biofilm metabolic activity after 3 h incubation with alamarBlue (n = 29) **(B–D)**. The correlation between the *in vitro*-grown *S. aureus* biofilm metabolic activity after 3 h incubation with alamarBlue and the frequency of eosinophils in the sinonasal tissue from CRS patients and controls (n = 27) **(E)**. The boxplots are displayed as mean ± SD. One-way ANOVA followed by Tukey's multiple comparison test was used **(A)**. The Pearson's rank correlation coefficient was performed for the correlation analysis **(B–E)**. Each dot represents individual patients **(A–E)**. *$p < 0.05$.

groups were also displayed (Fig. 4). Detailed characteristics of *S. aureus* isolates are given in Table S4.

2.5. CRSwNP-*derived* S. aureus *biofilms are more metabolically active than those from CRSsNP patients in correlation with the disease severity and the frequency of eosinophils*

The quantification of *S. aureus* metabolic activity was carried out at different time points after incubation with alamarBlue. The comparison of *S. aureus* metabolic activity among our patient cohorts after 3 h incubation with alamarBlue showed significantly higher metabolic activity in CRSwNP-derived clinical isolates than those from CRSsNP patients ($p = 0.0478$) and controls ($p = 0.0476$) (Fig. 5A). Additionally, the correlation analysis between the *in vitro* grown *S. aureus* biofilm metabolic activity and the patients' Lund-Mackay, Lund-Kennedy, and SNOT22 severity scores exhibited moderate positive correlations (Fig. 5B–D). Furthermore, among the innate immune cells, eosinophils were positively correlated with *S. aureus* metabolic activity (Fig. 5E).

2.6. *Mast cell and dendritic cell frequencies are higher in patients who carry* S. aureus *isolates encoding* lukF.PV, sea, fnbB, *and* essC *genes, while neutrophils and eosinophil frequencies are lower in patients who carry isolates encoding* fnbB *and* vWbp *genes*

Whole-genome sequencing (WGS) was conducted on all 29 *S. aureus* isolates from CRSwNP (n = 14), CRSsNP (n = 9), and non-CRS control (n = 6) patients. Antimicrobial resistance (n = 18) and virulence (n = 80) genes were detected by screening all isolate contigs through the Comprehensive Antibiotic Resistance Database

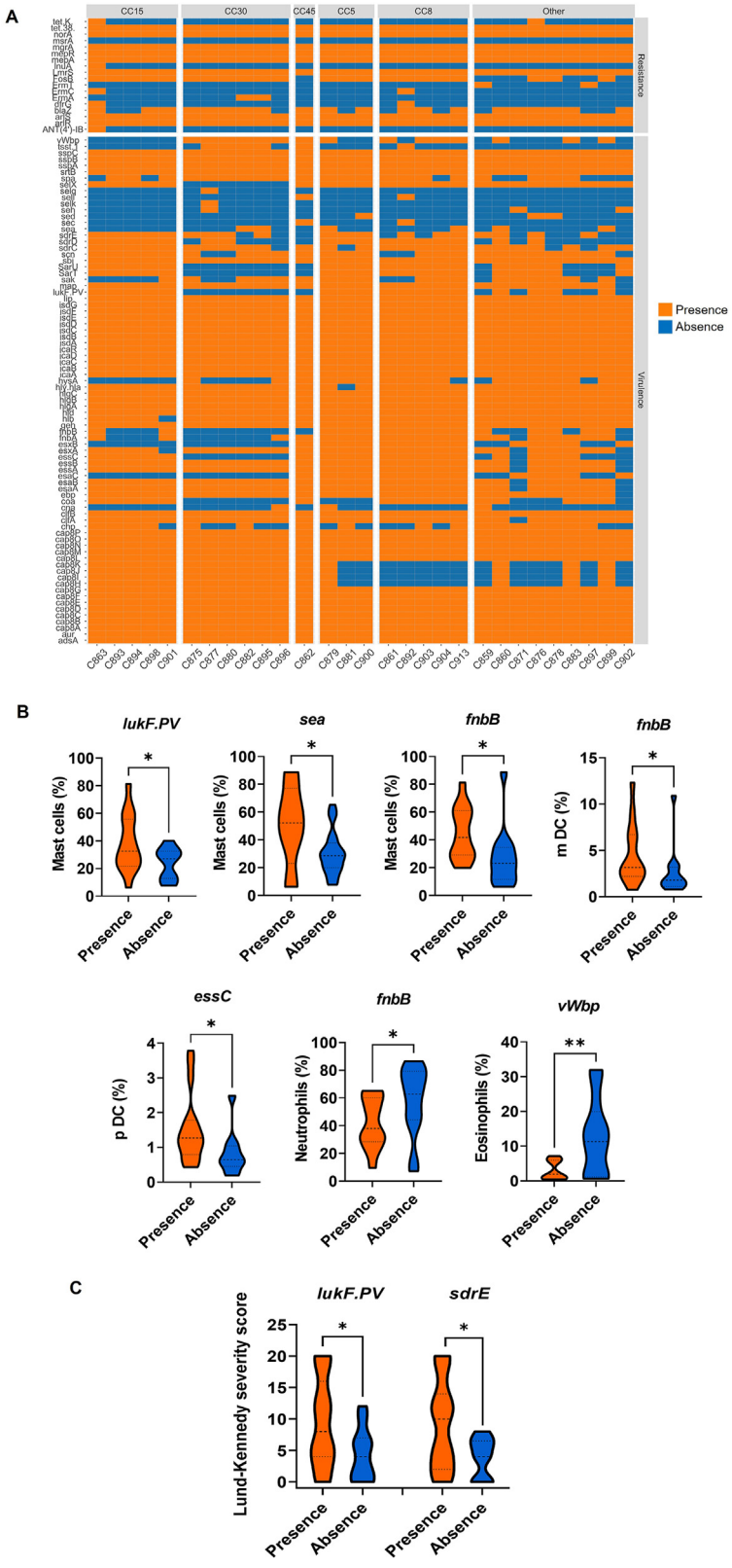

Fig. 6. The presence/absence profile of 18 *S. aureus* genes encoding for antimicrobial resistance and 80 genes encoding for virulence factors. The rows refer to genes, and the columns refer to *S. aureus* clinical isolates (n = 29). The orange colour = gene presence, while the blue colour = gene absence. CC15, CC30, CC45, CC5, CC8 are the clonal complexes to which most clinical isolates belonged. The "Other" group includes isolates without a defined Clonal Complex **(A)**. The frequency of the innate immune cells in sinonasal tissue from CRS patients and non-CRS controls in relation to the presence/absence of patients' *S. aureus* virulence genes **(B)**. The Lund-Kennedy disease severity score from CRS patients and non-CRS controls in relation to the presence/absence of patients' *S. aureus* virulence genes **(C)**. The violin plots are displayed as median (dashed line) plus lower and upper quartiles (dotted lines) **(B and C)**. A Mann-Whitney test was used to compare the two groups except for the *lukF.PV* and *sea* genes in **B** and **C**, in which an independent sample t-test was used. Data shown in **B** and **C** represent pooled data from all samples. *$p < 0.05$ and **$p < 0.01$.

(CARD) and Virulence Factor Database (VFDB). All genomes of *S. aureus* isolates were MLST typed and grouped into five Clonal Complexes (CC15, CC30, CC45, CC5, and CC8), comprising about ~69% of the isolates. Nine isolates with unknown clonal complexes were classified under "other." The presence/absence profile of the antimicrobial resistance and virulence genes in different clonal complexes was investigated (Fig. 6A). The correlation analysis between the virulence genes and innate immune cell frequencies showed higher mast cell frequencies in tissue samples harbouring the isolates carrying the *lukF.PV* ($p = 0.049$), *sea* ($p = 0.020$) and *fnbB* ($p = 0.019$) genes. Similarly, a higher frequency of myeloid- and plasmacytoid dendritic cells was found in patients' sinonasal tissues with *S. aureus* isolates carrying *fnbB* ($p = 0.0301$) and *essC* ($p = 0.0270$) genes, respectively. In contrast, the frequency of neutrophils and eosinophils was significantly lower in patients' sinonasal tissues with *S. aureus* isolates carrying the *fnbB* ($p = 0.0496$) and *vWbp* ($p = 0.0086$) genes, respectively (Fig. 6B).

2.7. *Patients with* S. aureus *encoding* lukF.PV *and* sdrE *genes have higher disease severity score*

The correlation analysis between *S. aureus* virulence genes and CRS severity scores showed higher Lund-Kennedy severity scores in patients harbouring the isolates carrying the *lukF.PV* ($p = 0.046$) and *sdrE* ($p = 0.043$) genes (Fig. 6C).

3. Discussion

Research investigating the contribution of *S. aureus* to CRS pathogenesis lacks the characterisation of strain-dependent variation in *S. aureus* biofilm properties in relation to inflammation and disease severity [30,31]. Here, we first characterised the frequency of innate immune cells in sinonasal tissue samples of CRS patients and non-CRS controls in relation to CRS severity. We found eosinophils, basophils, and monocytes in positive correlation but neutrophils in negative correlation with CRS disease severity scores. We also grew the patient-derived *S. aureus* into biofilms *in vitro* and studied their biofilm metabolic activity in relation to disease severity and innate immune response. We found that *S. aureus* biofilms from CRSwNP patients were metabolically more active than those from CRSsNP and non-CRS controls and positively correlated with disease severity and frequency of eosinophils.

This study provided a detailed FACS analysis of innate immune cells, and demonstrated significant immunophenotype changes of innate immune cells in CRSwNP with a substantial increase in eosinophils, basophils, and monocytes frequencies. Interestingly, those immune cell frequencies correlated positively with Lund-Mackay and Lund-Kennedy severity scores. However, neutrophils decreased in CRSwNP patients in an inverse correlation with disease severity. This finding, together with the higher eosinophils to neutrophils ratio observed in CRSwNP, indicates the presence of an ongoing immune response dominated by type-2 inflammation in those patients. This is supported by previous studies showing that a high degree of tissue eosinophilia in CRSwNP tissue samples is a hallmark of severe disease and increases the likelihood of recurrent disease and comorbid asthma in those patients [4,32,33].

Mast cells are activated by local IgE and secrete substantial quantities of cytokines, especially IL-5 and IL-13, which facilitate type-2 eosinophilic inflammation in CRSwNP patients [12]. Although the frequency of mast cells was high in our CRS patients, we found no significant changes among our patient cohorts using FACS analysis of CD16 versus CD24 cells surface markers. This is contrary to previous studies showing increased mast cells frequencies in patients' nasal polyps compared to control; however, in those studies, different methods (PCR and ELISA) or flow cytometry markers were used [34–36]. Higher frequency of eosinophils in CRSwNP patients, in addition to the presence of a very strong inverse correlation between mast cells and neutrophils, indirectly implies the involvement of mast cells in CRS pathogenesis. Elevated basophil cell frequencies in CRSwNP patients compared to controls and positive correlation between these cells and eosinophils agree with a study conducted by Mahdavinia [37].

S. aureus contributes to the formation of nasal polyps [4], and its mucosal biofilms have been shown to play an important role in recalcitrant CRS [15,16]. However, *S. aureus* biofilms are also found in healthy and less severe CRS patients, questioning the significance of *S. aureus* biofilms in CRS pathogenesis [38]. Here, we reported that CRSwNP-derived *in vitro*-grown *S. aureus* biofilms were more metabolically active than those from CRSsNP and non-CRS controls in positive correlations with the frequency of eosinophils. This supports the hypothesis that more metabolically active biofilms are more immunogenic, leading to more severe disease. This was further supported by observing positive correlations between *S. aureus* biofilm metabolic activity and CRS objective and subjective severity scores. *S. aureus,* as a highly successful pathogenic bacterium, produces an extensive repertoire of surface components and secreted products, such as toxins and enzymes, which contribute to the pathogenesis of infection [39,40] by promoting tissue adhesion, immune evasion, and host cell damage [20]. Here, we observed a higher frequency of mast cells in patients' tissue harbouring isolates carrying *lukF.PV* (encoding Panton-Valentine Leukocidin), *sea* (encoding enterotoxin A) and *fnbB* (encoding fibronectin-binding protein B) genes. Interestingly, neutrophil frequencies were significantly lower in patients' tissue carrying isolates encoding the *fnbB* gene. Although the interaction between mast cells and Panton-Valentine Leukocidin, as an important pore-forming toxin, has not previously been reported, their interaction with other pore-forming toxins of *S. aureus* such as α-Hemolysin (Hla) has been reported in which *S. aureus* internalisation by mast cells is enhanced by α-Hemolysin and leads to the release of extracellular traps, antimicrobial compounds, and pro-inflammatory cytokines [41–43]. Furthermore, the cytotoxic effect of Panton-Valentine Leukocidin on human neutrophils is already well established [44]. Our finding is also supported by a recent study demonstrating that *S. aureus* internalisation into mast cells is enhanced by staphylococcal enterotoxin [45]. Additionally, fibronectin-binding proteins are closely associated with the pathogenicity of staphylococci since their adherence to the extracellular matrix is a crucial step in the formation of biofilm and the invasion of host cells [46,47], and deletion of FnBPs in mice have strongly attenuated the ability of *S. aureus* to colonise the kidney and to cause fatal sepsis [48]. The correlation of Lund-Kennedy CRS severity scores with the carriage of some virulence genes, including *lukF.PV* and *sdrE* imply that the isolates containing those genes produce various potentially highly immunogenic proteins that can cause severe disease.

Overall, the correlation between the metabolic activity of *S. aureus* biofilms, eosinophils, as type-2 inflammatory markers, and CRS severity suggests a potential pathogenic link between those factors, which could lead to developing more tailored treatments for recalcitrant disease. Further investigation is required to determine differential gene expression and to see how *S. aureus* biofilms and virulence genes interact with the host, as well as whether and how they influence immunophenotypic changes in innate immune cells.

Statement of ethics

Ethics approval for the collection, storage, and use of clinical isolates and patient samples from CRS and non-CRS control

patients was granted by The Queen Elizabeth Hospital (TQEH) Human Research Ethics Committee, South Australia (HREC/15/TQEH/132), and all patients had signed written informed consent.

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Author contributions

S.V. and G.S. conceived the study. G.S. and S.V. contributed to the experimental design. G.S. conducted the experiments. R.N. and G.H. assisted with isolates' biofilm quantification. G.S. and G.B. performed data analysis and bioinformatic analyses. G.S. and S.V. drafted the manuscript. G.S. made the illustration. S.V., A.J.P., P.J.W., and C.C. provided supervision. All authors reviewed the manuscript and approved the submission of this work.

Data availability

The assembled *S. aureus* genomes have been deposited in the NCBI BioProject database under accession code RJNA914892. Supplementary data are provided for this paper. Further enquiries can be directed to the corresponding author.

Declaration of competing interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105213.

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