

Original article

Staphylococcus aureus in nasal lavage and biopsy of patients with chronic rhinosinusitis

Background: *Staphylococcus aureus* may play a relevant etiologic role in chronic rhinosinusitis (CRS) and may explain the T_{H2} shift observed in CRS with nasal polyps (CRSNP⁺). Naturally occurring *S. aureus* small colony variants (SASCV) escape immune surveillance, antibiotic treatment and microbiologic routine diagnostic techniques. The frequency of *S. aureus* and SASCV in CRS patients and *S. aureus*-related effects on the local immune response should be prospectively investigated.

Methods: Nasal lavages and mucosal biopsies of CRS patients were examined with bacterial culture suitable for detecting SASCV, real time PCR and fluorescence *in situ* hybridization. To assess the effects of *S. aureus* positivity, interleukin-5 (IL-5), interferon- γ , total immunoglobulin E (IgE), eotaxin, granulocyte-colony stimulating factor, and eosinophil cationic protein in nasal lavages were determined and gene transcription analysis of nasal biopsies from *S. aureus* positive and negative CRSNP⁺ patients was performed.

Results: Thirty-one CRSNP⁺ patients, 13 CRS patients without polyps, and 21 control patients were evaluated. *Staphylococcus aureus* was detected by any method in 25 patients (39%). *Staphylococcus aureus* detection rates did not differ between the three disease groups ($P = 0.3$). *Staphylococcus aureus* small colony variants were not found. In nasal lavages, IL-5 and total IgE levels were higher in CRSNP⁺ patients than in CRSNP⁻ patients or controls ($P < 0.05$). *Staphylococcus aureus* positivity did not influence biomarker concentrations in nasal lavages. Genes for T_{H2} cytokines were not differentially transcribed.

Conclusions: We could not observe a higher prevalence of *S. aureus* in CRS patients with or without nasal polyps than in controls. We could not substantiate that *S. aureus* intensifies the T_{H2} shift in CRSNP⁺ patients. *Staphylococcus aureus* small colony variants were not detected in any sample.

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Recent studies suggest that *Staphylococcus aureus* is a significant factor in the development of chronic rhinosinusitis (CRS). In particular, the immunomodulatory effects of *S. aureus* superantigens have been proposed as an elemental cause of the T_{H2} shift in CRS with nasal polyps (CRSNP⁺) (1–3). Reported *S. aureus* detection rates in CRS patients range from 15% to 70% (4). Possible reasons for this wide range include type and severity of disease, method and location of sampling, and different microbiological detection techniques. Moreover, naturally occurring *S. aureus* variants may escape routine microbiological detection. In two recent studies, intracellular *S. aureus* was detected in CRS patients and it was supposed that these intracellular isolates were *S. aureus* small colony variants (SASCV) (5, 6). *Staphylococcus aureus* small colony variants are adapted to intracellular survival and persistence, protecting them from immune surveillance and antibiotic treatment. Detection of SASCV requires specific culture techniques (7).

In this prospective study, the prevalence of *S. aureus* in CRS patients was investigated using different microbiological and immunological methods including *S. aureus* bacterial culture suitable to detect *S. aureus* and SASCV, two different real-time polymerase chain reaction (PCR) assays for detection of *S. aureus*-DNA (8, 9), and fluorescence *in situ* hybridization (FISH) (10). To assess detection rates in CRS subgroups, CRSNP⁺ patients and CRS patients without nasal polyps (CRSNP⁻) were compared. Patients surgically treated for nasal structural abnormalities without rhinosinusitis served as a control. Two sampling techniques were employed, i.e. nasal lavage and intraoperative mucosal tissue biopsies from the anterior ethmoid and inferior turbinate, respectively. Finally, biomarkers in nasal lavages including interferon- γ (IFN- γ), interleukin-5 (IL-5), total immunoglobulin E (IgE), granulocyte-colony stimulating factor (G-CSF), eotaxin, and eosinophil cationic protein (ECP) (11), and gene expression analysis of 1232 genes (12)

served to investigate a possible *S. aureus* related influence on CRS physiopathology.

Materials and methods

Study population

Patients treated in the Department of Otorhinolaryngology, Head and Neck Surgery of the University Hospital of Ulm between May 2006 and May 2007 were consecutively screened. Patients complying with the CRS-definition of the European Academy of Allergy and Clinical Immunology (13) aged 18–70 years were eligible. A modified Lund and Mackay CT score (14) of at least 15 was required for inclusion. Chronic rhinosinusitis patients with bilateral nasal polyps and a Malm-score (15) of at least 3 were categorized as CRSNP⁺ whereas CRSNP⁻ patients required a bilateral Malm-score of 0, i.e. no polyps visible at nasal endoscopy. Chronic rhinosinusitis patients with Malm-scores of 1 and 2 were not eligible, to exclude patients in whom the CRS subtype could not be clearly defined. Further reasons for exclusion were treatment with antibiotics as well as treatment with local and oral steroids within the previous 4 weeks, acute CRS exacerbations, current immunotherapy or aspirin desensitization, cystic fibrosis, or unilateral sinus disease. Pregnant or lactating women were also excluded. Various additional parameters were recorded including atopy defined as a positive skin prick test to common inhalant allergens, self-reported or clinically diagnosed acetyl salicyl acid (ASA)-intolerance, and bronchial asthma. Patients admitted for surgical treatment of nasal structural abnormalities were eligible as controls if their history did not suggest sinus pathologies and they showed no signs of mucosal inflammation at nasal endoscopy. The study was approved by the Institutional Review Board of the University of Ulm (No. 243/2005).

Sampling technique

Before surgery, nasal lavages were obtained as described elsewhere (16). Briefly, each nostril was flushed with 5 ml of isotonic saline solution. After 10 s, the patient blew the solution in a sterile plastic tube, which was immediately transferred for microbiological examination. An aliquot was used for nasal biomarker determinations. During surgery, biopsies from the anterior ethmoid were obtained in CRS patients and from the inferior turbinate close to the middle meatus in control patients after disinfection of the nasal vestibules. Immediately after harvest, tissue samples were put on ice and were then appropriately processed for microbiological cultures, real-time PCR, FISH, and gene expression analysis.

Bacterial culture

Within 4 h after sampling, 10 µl of the native nasal lavage specimen as well as a 1 : 100 dilution of the nasal lavage in 0.9% sterile saline were plated. Biopsy specimens were minced and suspended in sterile 0.9% saline. One drop of the tissue suspension was plated semi-quantitatively. The plates used were Columbia sheep blood agar, Schaedler agar (both Heipha, Heidelberg, Germany) and *S. aureus*-ID (SAID) agar (BioMérieux, Marcy l'Etoile, France). All plates were incubated at 36°C for 5 days at room air plus 5% CO₂, SAID agar plates were incubated at room air without CO₂ and examined every day. Small, nonpigmented, and only slightly or nonhemolytic colonies which grew after 24–72 h were susceptible for SASCV. Colonies suspicious of *S. aureus* or SASCV were further investigated by Gram stain, catalase test, and Pastorex[®]Staph-Plus (Bio-Rad, Munich, Germany) detecting clumping factor, protein A,

and capsular polysaccharides of *S. aureus*. If the test results were uncertain, tube coagulase test was performed and examined after 4 and 24 h, respectively.

A spiking experiment with SASCV was performed to prove that SCACV can be recovered from mucosal specimens and that we are able to detect SASCV. Therefore, samples of nasal polyp tissue were incubated with serial dilutions of a 10⁸ colony forming unit (CFU)/ml stock solution containing the SASCV strain SCV-R (kindly provided by B. Kahl, Muenster, Germany) and cultured for 2 h at 36°C and 5% CO₂. Sterile saline served as negative control. After incubation, the infected tissue was centrifuged and the supernatant was plated on Columbia agar for the detection of extracellular bacteria. The tissue was then washed with sterile 0.9% saline followed by the addition of 400 µl saline. The tissue was minced and the suspension was plated on Columbia blood agar in duplicate (200 µl per plate). Plates were incubated overnight at 36°C under 5% CO₂ and colonies were counted over the following days. To calculate SASCV recovery rates, the infected samples and 200 µl of each serial dilution were cultured in parallel and the number of CFU was compared.

S. aureus-specific LightCycler PCR

Biopsy specimens were minced and dissolved in 0.9% sterile saline. DNA was isolated using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the recommendations of the manufacturer with the following exceptions: for disruption of the Gram-positive cell wall, 20 µl proteinase K (Qiagen), 18 µl lysozyme (50 mg/ml; Roche Diagnostics, Mannheim, Germany), and 18 µl mutanolysin (5000 U/ml; Sigma, Munich, Germany) were added to 180 µl ATL buffer and the AL buffer was incubated at 95°C instead of 70°C. The DNA was eluted in 50 µl AE buffer (all Qiagen) and stored at –20°C until measurement. The PCR was performed on a LightCycler[®] (Roche Diagnostics, Mannheim, Germany) using the primers Sa442-F and Sa442-R for the *Sa442* gene target sequence, and nuc274R and nuc274F for the *nuc* gene target sequence. For amplicon detection, fluorescence resonance energy transfer probes Sa442-HP-1 and Sa442-HP-2, and nuc274FL and nuc2747LC, respectively, were used (TibMolBiol, Germany) (8, 9). The amplification mixtures consisted of 6 µl of PCR grade A. dest., 4 µl of LightCycler[®] FastStart DNA Master^{Plus}HybProbe reaction mix (Roche Diagnostics), 2 µl of each probe (1.5 µM), 1 µl of each primer (10 pmol/µl). Five microlitre of template DNA were added to 15 µl of the amplification mixture to a final volume of 20 µl. The PCR assays included an initial 10 min denaturation step at 95°C (Sa442-PCR) or 50°C (nuc-PCR) followed by 50 cycles of repeated denaturation (Sa442: 0 s at 95°C, nuc: 10 s at 95°C), annealing (Sa442: 15 s at 54°C, nuc: 10 s at 50°C) and polymerization (Sa442: 10 s at 72°C, nuc: 15 s at 72°C) followed by melting curve analysis. The Sa442 PCR assay had a sensitivity of 5–50 CFU per PCR reaction. The nuc274 PCR assay had a sensitivity of 1 CFU per PCR reaction (data not shown). A positive control (10 pg DNA of *S. aureus* ATCC 29213) and a negative control (purified PCR-grade water; Roche Diagnostics) were included in each PCR run.

Fluorescence *in situ* hybridization

Paraffin embedded tissue sections were deparaffinized in xylene (J.T.Baker, Deventer, Holland) for 10 min followed by 3 min in 100% ethanol. After hydration in a descending ethanol series, the samples were incubated for 15 min in 1× phosphate buffer saline at 36°C (PAA Laboratories, Austria), air dried and treated with 50 µl of proteinase K (25 µg/µl; Sigma) for 15 min in a humid chamber.

Following dehydration in ascending ethanol series, the slides were air dried and FISH was performed. A hybridization chamber (Gene Frame® 65 µl; Thermo Life Sciences, Germany) was placed on the slide and the specimens were covered with a FITC-marked *S. aureus*-specific peptide nucleic acid (PNA) probe (AdvanDx, MA, USA) according to the manufacturer's instructions. The slides were wrapped with parafilm to avoid evaporation of the probes and incubated at 56°C overnight. The following day, slides were washed in washing buffer (AdvanDx) for 30 min at 56°C. The stained slides were air dried and rapidly embedded in VECTASHIELD® mounting medium with 4',6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and immediately examined under a fluorescence microscope (Zeiss Axioplan II, Jena, Germany).

Biomarker concentrations in nasal lavages

Nasal lavages were stored at -20°C before examination. Human IFN-γ, human IL-5, G-CSF, and eotaxin were determined with Quantikine® Immunoassay kits (R&D Systems, Wiesbaden, Germany) following the manufacturer's instructions. However, plates were incubated with standard, sample or control (calibrator diluent; R&D systems) overnight instead of the denoted 2 h. Eosinophil cationic protein and total IgE levels in nasal secretions were measured using UniCAP 100 fluorescence enzyme immunoassays (Phadia, Freiburg, Germany) according to the manufacturer's recommendations. The limits of detection were 2 µg/l for ECP and 2 kU/l for total IgE, 3 ng/l for IL-5 and 8 ng/l for IFNγ, 20 pg/ml for G-CSF, and 5 pg/ml for eotaxin.

Gene transcription analysis

Gene transcription of three *S. aureus* negative CRSNP⁺ patients was compared with three *S. aureus* positive CRSNP⁺ patients. Briefly, 70 mer-cDNA oligonucleotides (Operon Biotechnologies, Koeln, Germany) for 1232 human genes were spotted on Ultra Gaps II coated slides (Corning, Schiphol-Rijk, The Netherlands) using an OmniGrid 100™ (Genemachines, San Carlos, CA, USA). The biopsies were disrupted using the Fast Prep FP 120 (MP Biomedicals, Eschwege, Germany) and RNA was extracted using RNeasy™ Mini-Kit (Qiagen). Spiking controls added to the total RNA consisted of synthetic mRNA-oligonucleotides of 10 different Arabidopsis and Sinorhizobium genes (Operon) synthesized on an ABI 394 synthesizer (Applied Biosystems, Foster City, CA, USA; PURIMEX, Staufenberg, Germany). Reverse RNA transcription was performed with Superscript-2 (Invitrogen, Karlsruhe, Germany) and cDNA labeling with the 3DNA Array 350 Kit™ (Genisphere, Hatfield, PA, USA): 5 µg total RNA from *S. aureus*-positive CRSNP⁺ patients were reversely transcribed with an oligo dT primer carrying a cDNA capture sequence for the fluorescent dye cyanine 5 (Cy5) and 5 µg total RNA from *S. aureus*-negative CRSNP⁺ patients with a capture sequence for Cy3. Hybridization was performed overnight (16 h) at 57°C in a hybridization chamber. The 3DNA capture reagent was hybridized for 3 h at 59°C with addition of anti-fade-reagent. The microarray slides were scanned with a dual-laser microarray scanner (GenePix 4000 B) and analyzed with the GENE PIX PRO 4.1™ software (both Axon Instruments, Foster City, CA, USA). To identify differentially transcribed genes on one microarray, a nonparametric algorithm was employed (12). Only genes differentially transcribed in at least two of the three arrays were considered relevant. Genes were denoted according to the HUGO nomenclature (17) and up-regulated or down-regulated genes over-represented within Gene Ontology categories were identified with the web-based programme WebGestalt (<http://bioinfo.vanderbilt.edu/webgestalt/>) (18).

Data analysis

Frequencies of categorical data were tabulated and evaluated with Fisher's exact, chi-square or Fisher-Freeman-Halton test. As a measure of concordance, Cohen's kappa was employed. A patient was considered *S. aureus*-positive, if *S. aureus* was found in lavages and/or biopsy specimens with at least one of the three detection techniques used. For ordinal data, medians and interquartile ranges were calculated and tested with the Kruskal Wallis or Mann-Whitney test. Out of nasal biomarker concentrations, means and standard errors of mean were calculated. Comparisons were performed with two factorial ANOVA (diagnosis with three levels and *S. aureus* positivity with two levels) without interaction. Equality of variances was tested with Levene's test. Calculation was performed with SYSTAT 12.0 (Systat Software Inc., San Jose, CA, USA) or STATXACT 8 (Cytel, Cambridge, MA, USA).

Results

Study population

Of 148 patients screened for this study, 95 were eligible. Reasons for noninclusion were nasal steroid use within the last 4 weeks ($n = 19$), too low CT-score ($n = 16$), polyp score of 1 or 2 ($n = 10$), or other reasons ($n = 8$). From the 95 eligible patients, insufficient tissue was obtained in 24 patients, four tissue samples were used in another study, tissue of one patient was used for spiking controls and one tissue sample was contaminated. From the remaining 65 patients (Fig. 1) 31 suffered from CRSNP⁺, 13 from CRSNP⁻, and 21 patients (control) had undergone surgery for nasal structural abnormalities without rhinosinusitis (Table 1).

Bacterial cultures

In the spiking experiment, 10⁵ CFU of SASCV were recovered in tissue and 10⁵ CFU in the supernatant. Cultured isolates from the dilution controls as well as from the spiked tissue specimens were phenotypically confirmed as SASCV. In 21 of 65 study participants, *S. aureus* was cultured in either lavage and/or tissue biopsies (32%). The lowest *S. aureus* detection rate was observed in patients with CRSNP⁻ (Table 2A, $P = 0.3$). In 42 participants, both nasal lavage and tissue biopsy were culture-negative, and in 14 patients both were positive. In seven patients, *S. aureus* was only detected in lavage, and in none of the participants was tissue culture positive and lavage negative (Table 2B). *Staphylococcus aureus* small colony variants were not detected in any specimen.

S. aureus-specific LightCycler PCR

For PCR analysis, 52 biopsy samples were available including samples from 20 controls, 11 CRSNP⁻, and 21 CRSNP⁺ patients. PCR was defined as positive, if in at least one of the two PCR-techniques, an *S. aureus* specific signal was detected. Of the 52 biopsies, 35 were *S. aureus* negative for both culture and PCR, and 10 were positive

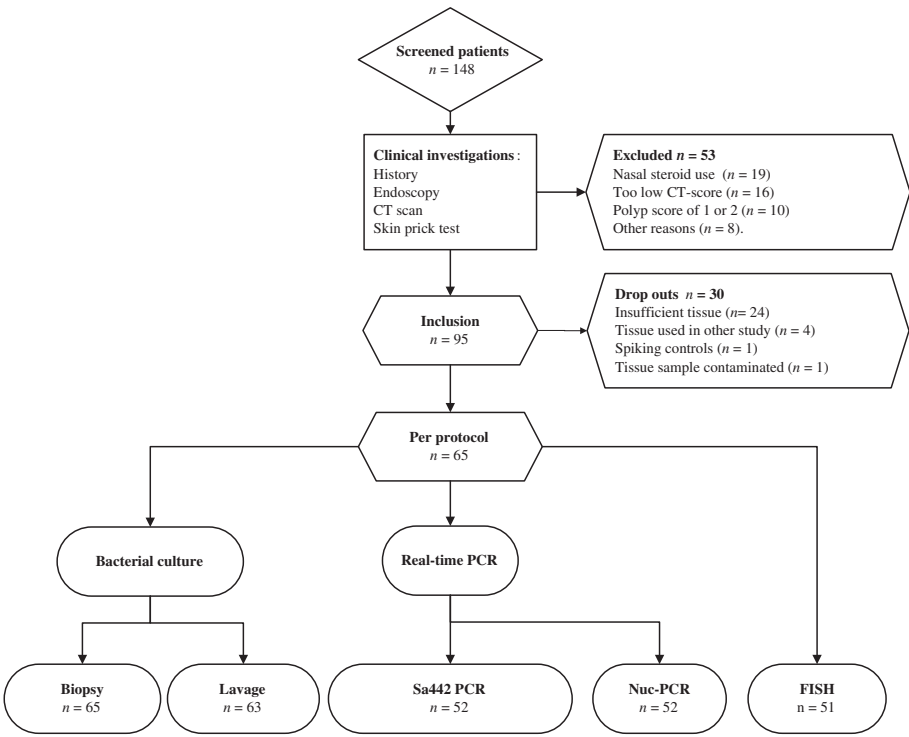


Figure 1. Study flow chart.

Table 1. Demographic and disease characteristics of the study population

	Control	CRSNP ⁻	CRSNP ⁺	P-value
n	21	13	31	
Age*	34 ± 10	49 ± 14	44 ± 11	0.001
Male/female	16/5	9/4	20/11	0.6
Smokers	7	4	6	0.5
Asthma	2	2	11	0.06
ASA-intolerance	0	0	2	0.2
Atopy	5	4	7	0.8
Polyp-score†	0 (0–0)	0 (0–0)	4 (4–5)	<0.001
CT-Score†	5 (0–6.5)	18 (17–19)	26 (19.5–34.5)	<0.001

*Years, mean ± SD.

†Median and interquartile range.

Table 2. Frequencies of *Staphylococcus aureus* detection: (A) In at least one of the employed sampling and detection techniques (Fisher–Freeman–Halton, $P = 0.38$); (B) In cultures of nasal lavages and tissue biopsies (Cohen’s $\kappa = 0.73$, $P < 0.001$)

	Control	CRSNP ⁻	CRSNP ⁺	Total
<i>S. aureus</i> negative	11	10	19	40
<i>S. aureus</i> positive	10	3	12	25
Total	21	13	31	65

	Lavage negative	Lavage positive	Total
Tissue culture negative	42	7	49
Tissue culture positive	0	14	14
Total	42	21	63

for both. In five biopsies, *S. aureus* was detected with PCR only and in two in culture only. The results of the two PCRs used in this trial were discordant in 11 of 52 biopsies (Cohen’s $\kappa = 0.29$, $P = 0.074$). Considering tissue culture as the reference method, Nuc-PCR had a sensitivity of 58% and a specificity of 93%, and Sa442-PCR had a sensitivity of 58% and a specificity of 95%.

Fluorescence *in situ* hybridization

In biopsies of 51 patients, both culture and FISH results were evaluated. One FISH result was positive in a culture negative biopsy; all other FISH examinations were negative including 10 tissue culture positive specimens.

Biomarker concentrations in nasal lavages

Interleukin-5 concentrations were higher in patients with CRSNP⁺ (3.72 ± 1.15 ng/l) than in patients with CRSNP⁻ (2.69 ± 1.17 ng/l) and in controls (2.24 ± 1.15 ng/l, $P = 0.03$). Total IgE levels were also higher in CRSNP⁺ (8.13 ± 1.12 kU/l) than in CRSNP⁻ (5.75 ± 1.05 kU/l) and controls (5.5 ± 1.02 kU/l, $P = 0.02$). No relevant differences between the three diagnostic groups were observed in the concentrations of ECP and IFN- γ ($P > 0.15$, Fig. 2, left panel). The G-CSF concentrations were not significantly different in controls (324 ± 1357 pg/ml) compared to CRSNP⁺ (66.5 ± 754 pg/ml, $P = 0.10$) and CRSNP⁻ ($57 \pm$

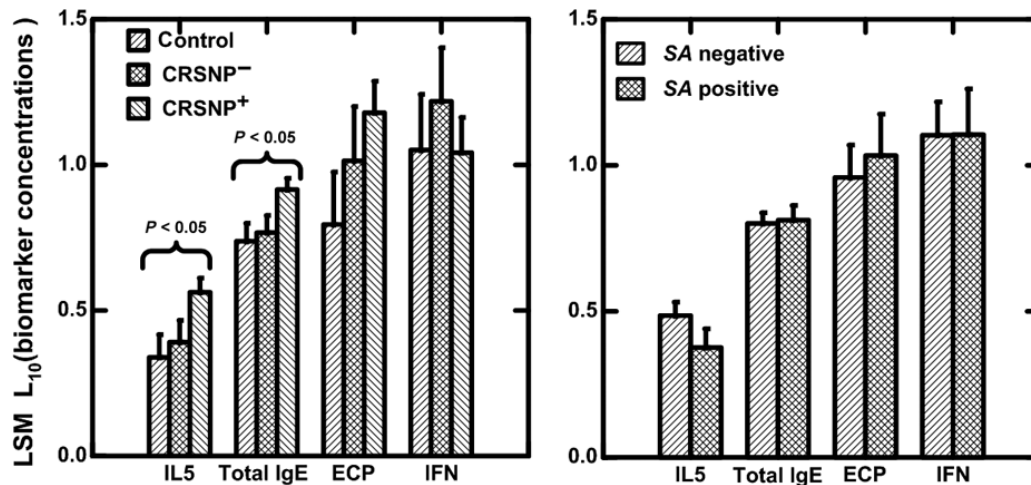


Figure 2. Biomarker concentrations in nasal lavages in patients with CRS and controls (left panel), and in *Staphylococcus aureus* positive and *S. aureus* negative patients independent of the disease entity (right panel); LSM, least significant means; common logarithm of biomarker concentrations for IL-5 (ng/l), IFN- γ (ng/l), ECP (mg/l) and total IgE (kU/l).

210 pg/ml, $P = 0.14$) patients. Eotaxin was detectable in the same concentration in CRS patients and in control patients (controls: 13 ± 14.3 , CRSNP⁺: 8 ± 16.0 , CRSNP⁻: 11 ± 11.4 pg/ml). *Staphylococcus aureus* detection did not influence the concentration of biomarkers in nasal lavages (all $P > 0.15$, see also Fig. 2, right panel).

Gene transcription analysis

RNA of biopsy specimens of three *S. aureus* positive CRSNP⁺ patients was compared with biopsy specimens

of three *S. aureus* negative CRSNP⁺ patients. Of 1232 examined genes, 29 were up-regulated and 31 were down-regulated in *S. aureus*-positive patients. In the Gene Ontology domain 'biological process', up-regulated genes were significantly overrepresented in the category 'antigen processing and presentation' and in the domain molecular function in the category 'MHC class I receptor activity' (Fig. 3). Down-regulated genes were overrepresented in the GO-categories, 'response to unfolded protein', and 'glutathione transferase activity'. The differentially transcribed genes can be seen in Table 3.

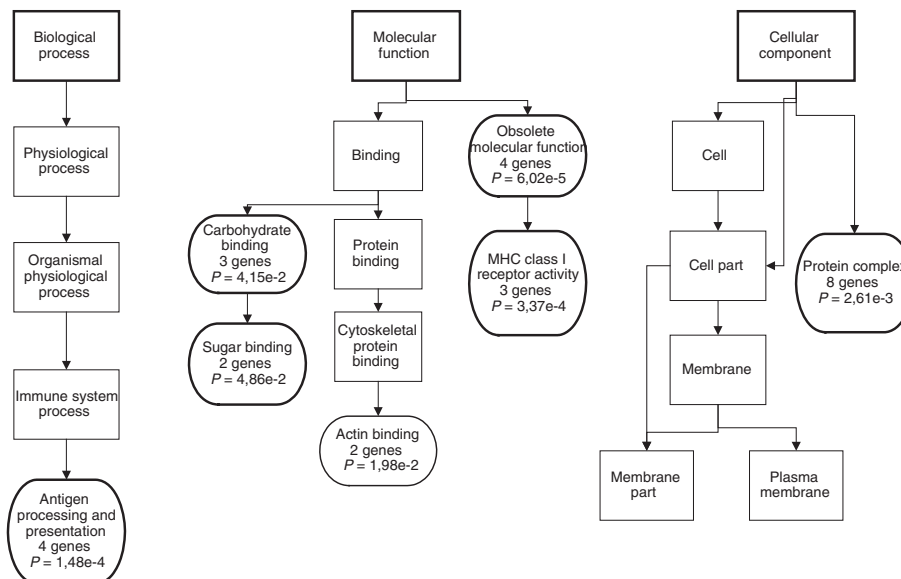


Figure 3. Directed acyclic graph of the Gene Ontology main categories biological process, molecular function and cellular component (levels 1–5). Bold boxes represent Gene Ontology categories enriched with up-regulated genes in *Staphylococcus aureus*-positive CRSNP⁺-patients, the number of up-regulated genes and the hypergeometric probability of observing this number of up-regulated genes within this category. Graph generated with 'WebGestalt' (<http://bioinfo.vanderbilt.edu/webgestalt/gsadag.php>).

Table 3. List of differentially regulated genes

Gene name	Symbol	log ₂ -fold change
<i>Up-regulated genes</i>		
Fc fragment of IgG binding protein	FCGBP	3.476
Major histocompatibility complex, class II, DP alpha 1	HLA-DPA1	1.848
Ribosomal protein L32	RPL32	1.762
Chemokine (C-C motif) ligand 13	CCL13	1.629
Major histocompatibility complex, class I, A	HLA-A	1.61
Thymosin, beta 4, X-linked	TMSB4X	1.567
Ribosomal protein L37a	RPL37A	1.561
Major histocompatibility complex, class I, F	HLA-F	1.549
Scavenger receptor class B, member 1	SCARB1	1.401
Cystatin C (amyloid angiopathy and cerebral hemorrhage)	CST3	1.397
Tumor protein, translationally-controlled 1	TPT1	1.332
Lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	1.199
Profilin 1	PFN1	1.16
Eukaryotic translation elongation factor 1 gamma	EEF1G	1.016
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	0.974
Integrin, beta 5	ITGB5	0.954
Reticulocalbin 1, EF-hand calcium binding domain	RCN1	0.925
FK506 binding protein 1A, 12 kDa	FKBP1A	0.895
Major histocompatibility complex, class I, E	HLA-E	0.869
Cyclin I	CCNI	0.81
Insulin-like growth factor binding protein 4	IGFBP4	0.8
Membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)	MS4A2	0.784
Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	FGFR1	0.768
Chemokine (C-X-C motif) ligand 1 pseudogene	CXCL1P	0.727
Calnexin	CANX	0.703
S100 calcium binding protein A9	S100A9	0.63
Histamine N-methyltransferase	HNMT	0.618
<i>Down-regulated genes</i>		
Retinoic acid receptor responder (tazarotene induced) 3	RARRES3	-0.374
TNFRSF1A-associated via death domain	TRADD	-0.377
V-akt murine thymoma viral oncogene homolog 1	AKT1	-0.381
Sorcin	SRI	-0.453
Cold shock domain protein A	CSDA	-0.489
Growth hormone releasing hormone	GHRH	-0.495
Proteasome (prosome, macropain) subunit, beta type, 3	PSMB3	-0.537
Cysteine-rich protein 1 (intestinal)	CRIP1	-0.544
Neuroepithelial cell transforming gene 1	NET1	-0.548
Integral membrane protein 2C	ITM2C	-0.552
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	PSMD2	-0.606
Glutathione S-transferase M4	GSTM4	-0.628
Keratin 7	KRT7	-0.631
Calpain, small subunit 1	CAPNS1	-0.703
N-acetyltransferase 1 (arylamine N-acetyltransferase)	NAT1	-0.774
Programmed cell death 4 (neoplastic transformation inhibitor)	PDCD4	-0.807
Keratin 17	KRT17	-0.856
Heat shock 10 kDa protein 1 (chaperonin 10)	HSPE1	-0.876
Superoxide dismutase 2, mitochondrial	SOD2	-0.878
Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	CDKN2B	-0.895
High-mobility group box 2	HMGB2	-0.916
CD68 molecule	CD68	-0.975
Glutathione S-transferase M2 (muscle)	GSTM2	-1.027
Glutathione S-transferase M1	GSTM1	-1.131
DnaJ (Hsp40) homolog, subfamily A, member 1	DNAJA1	-1.135
Keratin 4	KRT4	-1.236
Heat shock 105 kDa/110 kDa protein 1	HSPH1	-1.454
Glutathione S-transferase A2	GSTA2	-1.613
Mucin 4, cell surface associated	MUC4	-1.619
Anti-Mullerian hormone receptor, type II	AMHR2	-1.652

Discussion

Chronic rhinosinusitis is a significant and increasing health problem, which results in a large financial burden on society and considerable impairment of quality of life of affected persons (19). Despite its high prevalence, the etiology of CRS remains unclear (13). However, recent investigations suggest a significant etiological role of *S. aureus* (2). Therefore, we investigated the prevalence of *S. aureus* in patients with CRSNP⁻, CRSNP⁺, and in patients undergoing surgery for nasal structural deformities without CRS (controls). Moreover, we were interested if and how *S. aureus* may influence the immune response in CRS.

To assess *S. aureus* prevalence in CRS, two sampling techniques were employed: nasal lavage and mucosa biopsies from the ethmoid. Hereby, we aimed to obtain an optimum *S. aureus* yield and to ensure appropriate sampling directly from the diseased sinuses. Moreover, we wondered whether noninvasive nasal lavages could be used as reliable indicators of *S. aureus* in the middle meatus and anterior ethmoid. Compared to swab specimens, nasal lavages are representative for almost all areas in the nose and thus yield an overview of the bacteriology of the nose (20). Furthermore, nasal lavage can be plated semiquantitatively thus giving a hint on the number of bacteria in the nose. In patients with *S. aureus*-positive biopsies, all lavages were also *S. aureus* positive (Table 2B) suggesting that lavages are reasonably sensitive. Lavage positive and biopsy negative results in seven patients are considered to reflect nasal vestibule contamination only, which may bias the results of nasal swabs (21).

The multi-technique approach for detection of *S. aureus* and SASCV improved sensitivity and allowed a direct comparison of the three methods employed. Bacterial culture was the most sensitive single method of detecting *S. aureus* in biopsy specimens of nasal mucosa. As SASCV grows much slower than the normal *S. aureus* phenotype, we extended incubation to 5 days and used plates recommended for identification of SASCV (7). The results of the spiking experiments confirmed that the culture techniques were suitable to identify the SASCV. Moreover, the spiking experiments showed that the SASCV phenotype did not switch back to the normal phenotype in the milieu of nasal mucosa. This would preclude the detection of SASCV, which would appear as normal *S. aureus*. As we did not find any SASCV in the clinical specimens either in bacterial culture or in FISH, our data do not support the hypothesis that SASCV plays a major role in the pathogenesis of CRS.

Sensitivities of PCR assays for different *S. aureus* target genes vary. We compared *S. aureus* detection rates of two PCR assays with cultures of the same tissue biopsies. If *S. aureus* culture is chosen as the reference method, sensitivities of Sa442-PCR and nuc-PCR (both 58%) were comparably low with specificities greater than 90%. The results of Sa442-PCR and nuc-PCR were discordant in 11

of 52 patients. If both PCR-techniques are combined counting all biopsies positive in which at least one of the PCR assays detected *S. aureus*, sensitivity changed to 83% and specificity to 88%. The advantages of PCR are that it is a fast method that detects DNA of living as well as dead bacteria. The five patients with negative biopsy cultures and positive PCR may represent nonviable *S. aureus* and were therefore considered *S. aureus* positives. Nevertheless, the low sensitivities of the PCR assays may be explained by the difficult extraction of DNA from the bacteria because of the Gram-positive murein-rich cell wall and the smaller sample aliquot used for PCR compared to the sample used for culture.

Using FISH as the third diagnostic technique, we aimed to find a fast method to detect intracellular *S. aureus* in biopsy specimens. Preliminary tests showed that SASCV could be labeled better with PNA probes than with DNA probes. PNA probes exhibit better penetration of bacterial cell walls because of their hydrophobic character (10). We also tested different incubation times and methods to suppress tissue autofluorescence (data not shown). Best results were obtained when the samples were incubated overnight after proteinase K treatment. We were not able to suppress the autofluorescence of the tissue completely, and possibly because of the varying density of the tissue in the paraffin sections, it was difficult to detect bacteria by FISH. The sensitivity of FISH under our assay conditions was low. As there is limited experience in FISH for *S. aureus* in paraffin sections, further studies should be investigated with high focus on evaluating the best assay conditions.

Combining two sampling and three detection techniques suitable also for SASCV, we could verify the presence of nasal *S. aureus* in our study population with high sensitivity. The primary question of this study was, whether differences in the prevalence of *S. aureus* between CRSNP⁺ patients, CRSNP⁻ patients and controls could be observed. *Staphylococcus aureus* was detected in less than 50% of the study population. This is compatible with current data on nasal *S. aureus* carrier state in hospitalized patients (22). Differences between disease groups were not significant (Freeman–Fisher–Halton test $P = 0.38$, Table 2). *Staphylococcus aureus* detection rates were also not influenced by gender ($P = 0.8$), atopic state ($P = 0.6$), or presence of asthma ($P = 0.3$). The results did not differ relevantly if only *S. aureus* detection rates in biopsy specimens were considered.

We further investigated possible modulatory effects of *S. aureus* on the local immune response (2), in particular whether *S. aureus* contributes to the T_H2 shift observed in CRSNP⁺. For this purpose, we measured several biomarkers in nasal lavages including IL-5, IFN- γ , ECP, Eotaxin, G-CSF, and total IgE. Consistent with a previous study (11), IL-5 and total IgE levels were higher in CRSNP⁺ than in CRSNP⁻ patients and controls (Fig. 2, left panel). Nasal lavage concentrations of the investigated biomarkers were not influenced by *S. aureus*

detection (all $P > 0.13$). Again, these results did not differ relevantly if only *S. aureus* detection rates in biopsy specimens were considered. In our study population, levels of ECP, eotaxin and IFN- γ did not differ between the three diagnostic groups, which contrasts to data collected by others (1, 13). These differences may be explained by the use of different samples, as we investigated nasal lavage and not tissue homogenisates like Bachert et al. (1).

Effects of *S. aureus* positivity in CRSNP⁺ patients were more broadly assessed employing gene transcription analysis with a custom made oligonucleotide chip covering 1232 genes (12). Approximately, 2% of the genes on this array were up-regulated and 2% were down-regulated. For biological interpretation of gene transcription data, the Gene Ontology Consortium provides a fundamental framework (23). Current computational tools, such as the web-based system 'WebGestalt' allow identification of overrepresentation of up-regulated or down-regulated genes within the various Gene Ontology categories (18). In *S. aureus* positive CRSNP⁺ patients, up-regulated genes were overrepresented in the category antigen processing and presentation, mainly via MHC class I molecules. Up-regulation of MHC class I molecules was also reported in *S. aureus* infected human umbilical vein endothelial cells (24). As *S. aureus* is primarily an extracellular pathogen, one would rather expect MHC class II genes to be up-regulated in *S. aureus* positive specimens. MHC I molecules carry pathogens occurring in the cytoplasm, e.g. viruses or bacteria surviving within the cell. Recent studies indicate that also *S. aureus* with normal phenotype may enter the cytoplasm of various cell types including epithelia by fibronectin bridging between the bacterial fibronectin binding proteins and the receptor $\alpha 5\beta 1$ -integrin (25). This

could explain the observed up-regulation of MHC class I molecules in *S. aureus* infected tissues. It would have been interesting to compare differences in gene regulation between controls, CRSNP⁺ and CRSNP⁻, but unfortunately in CRSNP⁻ and control patients, there was not enough tissue available for all examinations.

Moreilhon et al. (26) exposed MM-39 tracheal gland epithelial cells to live *S. aureus* and its culture supernatants. They observed up-regulation of various genes involved in inflammatory response including IL-1 β , IL-6, and IL-8. Our gene transcription analyses showed, that in *S. aureus* positive CRSNP⁺ patients, transcription of these proinflammatory genes was not up-regulated when compared to that in *S. aureus* negative CRSNP⁺ patients. It may be that these inflammatory genes were already activated in CRSNP⁺ patients as a consequence of the inflammatory process, and the additional presence of *S. aureus* in the inflamed tissues could not further enhance their gene transcription. This could also apply to the various T_{H2} type cytokine genes present on the array including IL-4, IL-5, IL-13, and GM-CSF (see repository), which were also not up-regulated in *S. aureus* positive patients. In other words, as on the protein level, no *S. aureus*-related shift of the immune response towards T_{H2} was observed on the transcriptional level.

In conclusion, conventional culture techniques were sufficient for *S. aureus* detection. However, the sensitivity can be increased if both Sa442-PCR and nuc-PCR are employed. An unusually high prevalence of *S. aureus* in CRS patients of both subtypes, with and without nasal polyps, could not be observed in our study population. Moreover, we could not substantiate that *S. aureus* intensifies the T_{H2} skew of the immune response in CRSNP⁺ patients. *Staphylococcus aureus* small colony variants was not detected in any sample.

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