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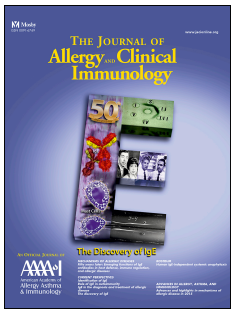

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Extracellular eosinophilic traps in association with *Staphylococcus aureus* at the site of epithelial barrier defects in severe airway inflammation

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Abstract (250 words)

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a Th2-biased eosinophilic inflammation. Eosinophils have been shown to generate so-called extracellular eosinophilic traps (EETs) under similar pathological conditions.

Objective: Our aim was to investigate a possible link between EET formation and the presence of *Staphylococcus aureus*, an organism frequently colonizing the upper airways, at the human mucosal site of the disease.

Methods: Tissue slides were investigated for the presence of EETs and *S. aureus*, using immunofluorescent staining and PNA-fish assay respectively. An *ex vivo* human mucosal disease tissue model was used for artificial infection with *S. aureus*. Cell markers were analyzed using immunohistochemistry, luminex Multiplex assay, ELISA, PCR, immunobloting and linked to the presence of EETs.

Results: About 8.8 ± 4.8 % of the infiltrating eosinophils exhibited EETs in patient's nasal polyp tissues. The formation of EETs was associated with increased IL-5 ($p < 0.05$) and periostin ($p < 0.05$) tissue levels, and colonization with *S. aureus* ($p < 0.05$). Using an *ex vivo* human mucosal disease tissue model, EET formation was induced (4.2 ± 0.9 fold) upon exposure to *S. aureus*, but not to *S. epidermidis*. Eosinophils were shown to migrate ($p < 0.01$) towards *S. aureus* and entrap the bacteria both inside and outside the mucosal tissue. Blocking NAPDH oxidase activity, led to a complete inhibition ($p < 0.05$) of EET formation by *S. aureus.*

Conclusion: Eosinophils are likely to be specifically recruited to and form EETs at sites of airway epithelial damage to protect the host from infections with *S. aureus* and possibly other microorganisms in CRSwNP.

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51 **Capsule Summary**

52 Eosinophils in nasal polyp tissue are likely to be recruited to sites of airway epithelial damage to
53 protect the host against *S. aureus* infections by forming EETs.

54

55 **Key Messages**

56 - EETs are formed in nasal polyp tissue, mainly at subepithelial sites with epithelial barrier
57 defects, and are associated with increased IL-5 tissue levels and *S. aureus colonization*.
58 - EET formation is induced in nasal polyp tissue, upon exposure to *S. aureus*, but not to *S.*
59 *epidermidis*.
60 - Eosinophils migrate towards *S. aureus* and entrap the bacteria both inside and outside the
61 mucosal tissue.
62 - Reactive oxygen species are involved in this rapid process.

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76 **List of abbreviations**

77 AD : atopic dermatitis

78 C5a : Complement factor 5a

79 CRSwNP : Chronic rhinosinusitis with nasal polyps

80 DAPI : 4',6-diamidino-2-phenylindole

81 DNA : Deoxyribonucleic acid

82 DPI : diphenyleneiodionium

83 *E. coli : Escherichia Coli*

84 EETs: extracellular eosinophilic traps

85 IFN : interferon

86 IL-5 : Interleukin

87 IT : inferior turbinate

88 lfTSLP : long form TSLP

89 LPS : lipopolysachariden

90 MBP : Major Basic Protein

91 MGG : May-Grünwald-Giemsa staining

92 ROS : reactive oxygen species

93 *S. aureus : Staphylococcus aureus*

94 *S. epidermidis : Staphylococcus epidermidis*

95 sfTSLP : short form TSLP

96 TCM : Tissue culture medium

97 Th2 : T-helper 2

98 TSLP : Thymic stromal lymphopoietin

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1. Introduction

Asthma, allergy, aspirin hypersensitivity and chronic rhinosinusitis with nasal polyps (CRSwNP) have all been characterized as Th2-biased eosinophilic inflammations and often represent comorbid diseases. [1-3] Nasal polyp tissue of Caucasian patients usually shows high levels of interleukin IL-5 and eosinophil-related chemokines.[4] Together, these cytokines seem to orchestrate the chemotaxis, activation and survival of eosinophils.[1,5,6] Tissue eosinophilia increases the likelihood of recurrent disease and comorbid asthma in CRSwNP patients, indicating that eosinophils play a central role in the pathology of CRSwNP.[7] However, their role in airway disease and their contribution to the inflammation is not fully understood.

Eosinophils have cytotoxic functions and are involved in both the innate and adaptive immune responses; they have been correlated with epithelial damage via the release of major basic protein (MBP).[8] In addition, activated eosinophils can contribute to antibacterial defense by releasing mitochondrial DNA in association with granule proteins.[9,10] *In vitro*, these so-called eosinophil extracellular traps (EETs) are able to bind and kill bacteria like *S. aureus*, *S. epidermidis* and *E. coli*.[10,11] Here we hypothesized that EETs are present and are activated in nasal polyp tissue by *S. aureus.* As a consequence, these findings could provide further insights and understanding of the underlying causative mechanisms that lead to eosinophilic nasal polyps.

In vitro co-culture with eosinophils was sufficient to evoke EETs in the case of *S. aureus*, an additional stimulus with thymic stromal lymphopoietin (TSLP) was required for *S. epidermidis* to generate traps *in vitro,* suggesting differing responses of eosinophils according to the type of bacteria.[10,11] Enzymatic digestion of extracellular DNA obviates the capacity of EETs for killing bacteria. This indicates that, despite the fact that DNA itself has no antibacterial effect, EETs need both intact DNA and granule proteins to orchestrate this function.[12] The generation of EETs is considered to be an active process, not associated with cell death.[10,12] *In vitro,* EET formation is stimulated by priming eosinophils with IL-5, and interferon (IFN)-ɣ or adhesion molecules followed by activation with complement factor 5a (C5a), LPS, TSLP, and eotaxin.[11,12]

129 In addition to its association with eosinophilic inflammation, CRSwNP is also associated with a strong
130 colonization by *S. aureus*.[13] Interestingly, eosinophils were recently reported to generate EETs
131 immediately after co-culture with *S. aureus in vitro*.[11] This occurred without additional stimuli and as
132 the result of both direct and indirect interactions. Under these circumstances, EETs were found to
133 entrap and inhibit the growth of *S. aureus*.[11] The observation that extracellular nucleases are found in
134 various pathogenic bacteria including *S. aureus,* support the hypothesis of a pathophysiological
135 relevance for extracellular DNA traps.[14]
136 In recent years, EETs have been linked to various infectious and noninfectious diseases, including
137 inflammatory skin and intestinal diseases. Unsurprisingly, EET formation is often observed in allergic
138 diseases, such as bronchial asthma, contact dermatitis, atopic dermatitis (AD) and allergic drug
139 reactions.[9,15-17] In eosinophilic esophagitis and bullous pemphigoid, an increased presence of EETs is
140 observed at sites of epithelial barrier defects.[17] Therefore, EET formation could be an important
141 mechanism for protecting against infections, but might simultaneously cause damage to the
142 surrounding tissue, further compromising the epithelial barrier.[12] As epithelial barrier defects are
143 more and more recognized as a key factor in various diseases, it is likely that EET formation may
144 contribute to the pathophysiology of chronic airway diseases.
145 Altogether, these findings make eosinophils, EETs and their possible interactions with *S. aureus* an
146 interesting field of study in chronic airway diseases. Data from our group have shown that CRSwNP is
147 associated with eosinophilic infiltration, increased IL-5 and eotaxin [3], but also IL-33 and TSLP levels
148 (unpublished data) and a consistent colonization with *S. aureus*. The presence of EETs was shown in
149 endobronchial biopsies from asthma patients and in secretions of eosinophilic chronic rhinosinusitis
150 patients.[9,18] However, the dynamics of their activation and the factors regulating their release have
151 not been clarified in human diseased mucosal tissue. We aimed to understand the relevant
152 mechanism behind this activation in the context of clinical patient samples.
153
154

155 **2. Materials and Methods**

156 **2.1. Sample collection**

157 Nasal polyp tissue samples from 15 patients undergoing endoscopic sinus surgery for chronic
158 rhinosinusitis with nasal polyps (CRSwNP) and inferior turbinates (IT) from healthy individuals (5
159 patients undergoing septal surgery because of anatomical deviations) were collected. For the
160 isolation of peripheral blood eosinophils, whole blood was collected from healthy volunteers.
161 Written informed consent was obtained from all patients prior to enrollment in the study. Depending
162 on the experiment, the tissues were either used immediately, snap frozen and/or embedded in
163 paraffin. The study was approved by the local Ethics Committee (2015/0883) and the regulatory
164 authorities of Belgium. None of the patients received intranasal, oral and/or intramuscular
165 corticosteroids within the 4 weeks before surgery. For female subjects, pregnancy or lactation was
166 excluded. Patient characteristics are summarized in Table 1.

167

168 **2.2. Isolation of peripheral eosinophils and migration assay.**

169 Blood eosinophils were purified using the granulocyte fraction after whole blood Ficoll-paque
170 centrifugation from healthy donors using the CD16 depletion kit (Miltenyi Biotec) according to the
171 manufacturer's instructions. The purity of isolated eosinophils was determined as > 95% by May-
172 Grünwald-Giemsa (MGG) staining and light microscopy.
173 For the migration assay, eosinophils were primed for 20 minutes with 50 ng/ml IL-5 (PeproTech) and
174 then allowed to migrate through 5 µm pore size poly (vinylpyrrolidone)-free polycarbonate filters
175 (VWR International) for 90 minutes at 37°C. The lower compartment contained RPMI 1640 with 5%
176 Bovine Calf Serum (Life Technologies) and was considered as control tissue culture medium (TCM).
177 TCM containing 10 ng/ml eotaxin (PeproTech) was used as a positive control. Three days before the
178 experiment primary epithelial cells, isolated from nasal polyp tissue as described previously [19], were

179 seeded (BEGM medium, Lonza) in the basolateral compartment. Before the migration assay, the
180 medium was changed to TCM medium with or without $2x10^7$ CFU *S. aureus*.
181 After the migration assay, the membrane was subjected to MGG staining and its lower side was
182 evaluated for the number of migrated eosinophils. The chemotactic index was calculated by dividing
183 the number of cells migrating under the experimental condition by the number of cells migrating
184 under TCM condition.
185

186 **2.3. Immunofluorescent staining**

187 The tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After rehydration of the
188 tissue slides (5 µm), they were subjected to different staining procedures.

189 **A. EET staining**

190 EETs were visualized in paraffin-embedded tissue slides (5 µm) by means of indirect
191 immunofluorescence followed by counterstaining for DNA. After blocking and incubation with the
192 polyclonal anti-major basic protein (MBP) (Monosan) antibody (1:50), the slides were incubated with
193 a fluorescein isothiocyanate-conjugated secondary antibody (1:400) (Life Technologies) and the DNA
194 was stained by incubation with propidium iodide (1 µg/ml) (Sigma Aldrich). Subsequently the slides
195 were analyzed with a confocal laser-scanning microscope (Leica MicroSystems). Since the EETs are
196 difficult to detect in fixed tissues, staining was performed on three different tissues for each patient.
197 For each patient and each piece of tissue, 5 fields were selected in the studied regions (stroma,
198 subepithelial or at epithelial defects). In those fields, the amount of EETs was counted and
199 normalized for the amount eosinophils. EETs were expressed as % of eosinophils generating EETs
200 throughout the manuscript.

201 **B. Immunohistochemistry staining**

202 After blocking, the tissue slides were incubated with the primary antibody. Primary antibodies for
203 MBP (Monosan) and Caspase-3 (1:200) (Cell Signaling Technology) were used for the staining of

204 eosinophils and apoptotic cells respectively. The immunohistochemistry stain was further performed
205 using a REAL Detection System, Alkaline Phosphatase/RED kit (Dako) according to the manufacturer's
206 instructions.
207

208 **2.4. *S. aureus* staining**

209 Paraffin sections of nasal polyp tissue (for CRSwNP patients) or IT (for normal control patients) were
210 rehydrated and incubated for 30 minutes with the *S. aureus* probe using PAN-fish kit (AdvanDx
211 Company) at 55°C. After incubation, the unbound residual probe was removed by incubation with
212 wash solution (AdvanDx Company) for 30 minutes at 55°C. After washing, the slides were
213 counterstained with propidium iodide (1 µg/ml) (Sigma Aldrich) for 10 minutes at room temperature.
214 Finally the slides were mounted and examined using a fluorescence microscope. Patients and
215 controls were screened in triplicate for *S. aureus* and received a score as follows: '0' no colonization;
216 '1' planktonic colonization, not more than two bacteria in close proximity; '2' planktonic colonization,
217 more than two bacteria in close proximity; '3' colonization with biofilm formation.

218 To stain both *S. aureus* and EETs, the procedure was performed as described in 2.3. The slides were
219 washed with PBS and subjected to the immunofluorescent staining as described above with following
220 changes: a red fluorescent protein -conjugated secondary antibody (1:400) (Life Technologies) was
221 used and the DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies).

222 **2.5. *Staphylococcus* exposure experiments**

223 *S. aureus* (kind gift of Prof. Dr. von Eiff, Münster, Germany) and *S. epidermidis* (ATCC 14990) were
224 grown overnight in tris-buffered saline (TSB) and diluted in RPMI:DMEM (Dulbecco's Modified Eagle
225 medium) (1:1) (Life Technologies) to $2x10^7$ CFU/ml. Patient tissues were kept submerged in 2 ml of
226 the bacteria solution or in tissue culture medium and maintained for 2 hours at 37°C. Tissues
227 submerged in RPMI:DMEM medium, tissue control medium (TCM), was considered as control tissue.
228 Subsequently, the tissues were placed on a stainless steel grid and incubated at air-liquid interface

229 for 30 minutes, 1 and 2 hours. For the inhibition experiment, the tissue cubes were pre-incubated
230 (submerged) with 10 µM of the NADPH oxidase inhibitor diphenyleneiodionium chloride (DPI) in
231 RPMI:DMEM for 1 hour at 37°C before proceeding as described above. After incubation, the tissues
232 were immediately fixed in 4 % paraformaldehyde and/or snap frozen in liquid nitrogen.

233 **2.6. Reactive oxygen species measurements**

234 The tissues were immediately fixed in 4% paraformaldehyde and embedded in paraffin. Upon
235 rehydration, tissue slides were incubated with 5 µM dihydroethidium (Sigma Aldrich) for 30 minutes.
236 After washing steps with PBS, the slides were mounted with Vectashield anti-fade mounting medium
237 (Vector Laboratories). The slides were evaluated under a confocal fluorescent microscope and
238 pictures were taken while keeping the parameters (12 µs exposure time, 13% laser intensity)
239 constant. For each experiment (n=3), 10 fields in the subeptihelial region (< 100 µm distance from
240 epithelium) per condition were selected randomly, and assessed/measured by 2 (blinded)
241 individuals. The fluorescent mean intensity was quantified and normalized for surface area by
242 measuring the integrated fluorescent density using ImageJ analysis software.

243

244 **2.7. Immunoblot analysis for TSLP**

245 Protein lysates from patient tissues were made in RIPA buffer containing protease inhibitors (Sigma)
246 as described previously.[20] The concentration of protein in the lysates was determined using a protein
247 assay (Bio-Rad) with bovine serum albumin as a protein standard. The lysates were mixed 1:1 tricine
248 sample buffer (Bio-Rad) with 2% β-mercaptoethanol. Thirty micrograms of protein lysate was loaded
249 on a tris-tricine 16.5% precast gel (Bio-rad). Proteins were transferred to nitrocellulose membrane
250 and incubated in 5% bovine serum albumin overnight. Long form TSLP (lfTSLP) and short form TSLP
251 (sfTSLP) protein were detected using anti-TSLP antibody (1:1000)(Abcam). The secondary antibody
252 used was anti-rabbit Ig donkey whole Ab-HRP linked (1:6000) (NA934, GEAkta). Reactive bands were
253 visualized using SuperSignal West Femto maximum sensitivity substrate (ThermoFisher Scientific).

254

255 **2.8. Real-Time PCR**

256 Snap frozen tissue samples (± 30 mg) were disrupted and thawed directly into lysis solution (QIAGEN)
257 followed by homogenization using a QIAshredder homogenizer (QIAGEN). Subsequently, RNA was
258 isolated using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. RNA quality
259 was assessed with an Experion Automated Electrophoresis System (Bio-Rad Laboratories, Belgium)
260 and cDNA was synthesized from 1 µg of RNA using the iScript Advanced cDNA Synthesis Kit for RT-
261 qPCR (Bio-Rad). Quantitative real-time PCR was used to quantify mRNA levels of TSLP. Amplification
262 reactions were performed on a Light Cycler LC480 System (Roche) by using a specific PrimePCR Assay
263 (Bio-Rad). qPCR reactions contain 5 ng cDNA (total RNA equivalent), 0.25 µl PrimePCR primermix
264 (Bio-Rad), 2.5 µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a final volume of 5 µl. PCR
265 protocol consisted of 1 cycle at 95°C for 2 minutes followed by 44 cycles at 95°C for 5 seconds, at
266 60°C for 30 seconds and at 72°C for 1 second, and a dissociation curve analysis from 60°C to 95°C.
267 After a validation with geNorm (Biogazelle, Belgium), two reference genes, elongation factor 1 (EF-1)
268 and succinate dehydrogenase complex flavoprotein subunit A (SDHA), were used to normalize for
269 transcription and amplification variations among samples. Primer sequences are shown in Table S2.
270 The normalized relative quantities (NRQs) were calculated with the qBase+ software (Biogazelle,
271 Belgium) and the final gene expression results are expressed as the logarithm of NRQs per 5 ng
272 cDNA.

273

274 **2.9. Cytokine measurements**

275 The frozen tissues were weighed, homogenized and centrifuged as described previously.[20] The
276 samples were then assayed for IL-5, TSLP and periostin using commercially available ELISA kits from
277 R&D Systems (Minneapolis, Minnesota, USA) and for ECP and IgE using the UniCAP method (Thermo
278 Fisher Scientific) with the appropriate dilution factor and according to kit instructions. The samples
279 were measured following the instructions of the manufacturer, on a Bio-Plex 200 Array Reader (Bio-
280 Rad, Hercules, CA, USA).

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281 **2.10. Statistical analysis**

282 Statistical analysis was performed using the SPSS version 23 software program. For between-group
283 comparisons (Fig. 3A-C, Fig. 5C, Fig. 7AB, Fig. S1 A-D, Fig. S3 A-B, Fig. S4 A-D) the Mann-Withney U
284 test was used. For the comparison of related samples (comparison of EET formation related to tissue
285 localization, Fig. 4 E, Fig. 6 A-C, H), data were analyzed with a Friedman test, followed with Dunns
286 post test. Linear correlation analysis (Fig. S3 C-D) was performed with a Spearman correlation test.
287 Significances were expressed as *$p<0.05$,**$p<0.01$, ***$p<0.001$, p-values less than or equal to 0.05
288 were considered as statistically significant.

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299 **3. Results**

300 **Subepithelial eosinophils in nasal mucosa of patients with CRSwNP**

301 CRSwNP patients selected for the study (Table 1) had significantly elevated tissue levels of IgE, IL-5
302 and ECP (Fig. S1 A-C) compared to the normal controls. Significant number of eosinophils (Fig. S1D
303 and Fig. S1 E-F, $p < 0.001$), and to a much lesser extent neutrophils (Fig. S1D, $p < 0.05$) were elevated
304 in the tissues of the diseased population.

305 No infiltration or alignment of eosinophils was observed in the IT control population (n=5, Table S1
306 and Fig. 1A). In the tissue of the control population, eosinophils were rarely observed, even in the
307 allergic individuals. An increased presence and alignment of eosinophils underneath both intact (Fig.
308 1B) and barrier defective epithelium (Fig. 1D-E) was observed in all CRSwNP patients Table S1, n=15).
309 In some patients however, the alignment was more pronounced than in others. Regions with
310 completely denuded epithelium (Fig. 1E) were always associated with subepithelial infiltrates of
311 eosinophils. In the latter case, the subepithelial region could range from large and diffuse depositions
312 of MBP or free granules with very few to no intact eosinophils showing clear tissue damage (Fig. 1E),
313 to infiltrations of large numbers of intact eosinophils, depending on the patient and the localization
314 within the polyp. Regions with visually deviant epithelium (e.g. single cell layers, missing epithelial
315 cells) were often associated with larger and denser zones of subepithelial eosinophils Fig. 1C).

316 **Increased presence of EETs in subepithelial regions**

317 Although there were substantial differences between patients, we observed EETs in all of the 15
318 patients analyzed. Considering all patients, the mean fraction of EET-releasing eosinophils in the
319 subepithelial region (selected as region < 100 µm distance from epithelium) was 8.8 ± 4.8 % of the
320 infiltrating eosinophils, but ranged from < 2 % to 15 % of the eosinophils contributing to EET
321 formation (Fig. 2A). The proportion of EET-releasing eosinophils was differently distributed
322 throughout the polyp. Deep in the stroma, eosinophils were found mainly intact (Fig. 2B) (and

323 sometimes degranulated) and EET formation was very rare (0.2 ± 1.1% of the eosinophils);
324 subepithelially (< 100 µm distance from epithelium) denser eosinophilic infiltrates (Fig. 2C) were
325 located wherein eosinophils were found intact, or degranulating, releasing single EETs (Fig. 2D)
326 and/or clusters of eosinophils releasing EETs (Fig. 2E). The long and thin EET structures often seemed
327 to align underneath the epithelium, and sometimes even seemed to connect the EET releasing
328 eosinophils with other cells including other eosinophils. In most of the specimens we observed more
329 clusters of eosinophils releasing EETs than single EET releasing cells. No EET- releasing eosinophils
330 were found in the control ITs. No correlation was found between the extent of EET formation and
331 presence of atopy or asthma.

332 **Subepithelial regions with enhanced EET formation are not correlated with the presence of**
333 **apoptotic epithelial cells.**

334 In the subepithelial region (< 100 µm distance from epithelium), the proportion of eosinophils
335 generating EETs was in general higher than in the stroma. The subepithelial regions with visual
336 deviant epithelium showed a significant increase of EETs (3.1 ± 1.9 fold, $p < 0.01$), compared to
337 subepithelial regions with intact epithelium. These abnormal epithelial regions with increased
338 subepithelial eosinophilic infiltrates (Fig. S2 A) were not characterized by apoptotic (epithelial) cells
339 (Fig. S2 B), as indicated by an IHC stain for caspase-3. We also observed no abnormal nuclear
340 morphology in infiltrating eosinophils, indicating that cell lysis did not occur under the conditions of
341 EET formation (Fig. S2 B, inset B').

342 **EET formation is associated with elevated IL-5 and periostin levels, and with *S. aureus* infection**

343 CRSwNP patients with higher numbers of infiltrating eosinophils had significantly higher (relative)
344 amounts of EET formation ($p < 0.05$, Fig. S3 A). Patients with EETs were characterized by significantly
345 higher IL-5 levels in the tissue (Fig. 3A). Periostin levels were significantly increased in CRSwNP
346 patients, compared to controls ($p < 0.01$, Fig. S3 B) and were positively correlated with IL-5 levels ($p <$
347 0.01, $R^2=0.665$, Fig. S3 C). In addition, patients with more EETs had significantly increased periostin

348 levels in the tissue (Fig. 3B). Interestingly, no correlation between EET formation and TSLP protein
349 concentration (Fig. S3 D) was found in the tissue.

350 Because of the well-known association between *S. aureus* colonization and IL-5, all patients (n=15)
351 were evaluated and received a score (Table 2) for the *S. aureus* infection status as indicated in the
352 Materials and Methods section. The group of CRSwNP patients with the highest percentages (10% < x
353 < 20%, Fig. 3C) of eosinophils generating EETs, had a significant higher score for *S. aureus*
354 colonization than the patients with the lowest (< 5%, Fig. 3C, $p \leq 0.05$) and intermediate EET levels (<
355 10 %, Fig. 3C, $p \leq 0.01$).

356 Based on findings described above, five patients with high EET formation and *S. aureus* colonization
357 were selected and screened for co-localization of *S. aureus* and EETs in the tissue. In 3 of the 5
358 patients, *S. aureus* was found entrapped in an EET in the tissue (Fig. 3D). Other EETs were often
359 observed in the neighborhood of *S. aureus* bacteria.

360 ***In vitro* exposure of *S. aureus,* but not *S. epidermidis,* leads to increased EET formation in tissue**
361 **fragments**

362 After one hour exposure of CRSwNP tissue fragments to *S. aureus* in air-liquid culture, EET formation
363 was markedly enhanced (3.5 ± 1.2 fold increase, Fig. 4B and Fig. 4E) with up to 60% of eosinophils
364 (local effect) contributing to EETs as compared to the TCM control (Fig. 4A). The traps formed were
365 connected with each other as well as with other cells. After 2 hours exposure in air-liquid culture, we
366 noticed the increased presence of eosinophils releasing EETs at sites where *S. aureus* was observed
367 (4.2 ± 0.9 fold increase, Fig. 4C and Fig. 4E). The EET formation was concentrated in the subepithelial
368 regions (Fig. 4C) and, more specifically, at sites with epithelial defects and near *S. aureus*. These
369 findings were in strong contrast to the results of similar exposure experiments with *S. epidermidis*
370 (Fig. 4D) where no increased EET formation (Fig. 4E) could be observed at any time point.

371 **Eosinophils migrate towards and entrap *S. aureus* in an *ex vivo* diseased human mucosal tissue**
372 **model**

373 In the *ex vivo* human tissue model, after 2 hours air-liquid exposure, large numbers of eosinophils
374 were concentrated (compared to the general distribution of eosinophils in the tissue) at the sites of
375 epithelial defects, having sometimes even migrated out of the tissue. The eosinophils trapped *S.*
376 *aureus* at sites of epithelial defects (Fig. 5A), but also at sites with an intact, but single cell-layered
377 epithelium (Fig. 5B). Active migration of eosinophils towards *S. aureus* was confirmed by an *in vitro*
378 migration assay (Fig. 5C). In addition, the migration towards *S. aureus* was enhanced by the
379 combined presence of *S. aureus* and epithelial cells and had a tendency to further increase further
380 with longer co-incubation times (4 hours).

381 Isolated cells from nasal secretions of CRSwNP patients were shown to be extremely reactive with
382 massive EET formation upon *ex vivo* challenge with *S. aureus* for 15 minutes without the need for
383 priming. The bacteria were found entrapped in the massive EET network (Fig. 5D).

384 ***In vitro* generation of EETs in response to *S. aureus* is associated with elevated production of**
385 **reactive oxygen species**

386 Measurements of reactive oxygen species (ROS) in the tissue revealed a significant increase in ROS
387 production after exposure to *S. aureus*. The production of ROS was significantly enhanced after 30
388 minutes (Fig. 6A) and one hour exposure to *S. aureus* as compared either to the TCM control or to a
389 comparable exposure to *S. epidermidis* (Fig. 6B). After 2 hours, no further significant differences in
390 ROS production between the different *in vitro* conditions were observed (Fig. 6C). The role of ROS
391 and NADPH oxidase was confirmed by pretreatment of the tissue with DPI (Fig. 6D-G and Fig. 6H)
392 which was able to block totally (Fig. 6G and Fig. 6H) the extensive EET formation caused by exposure
393 to *S. aureus* (Fig. 6E).

394 **Periostin and TSLP levels were not significantly increased after *in vitro* exposure to *S. aureus***

395 Periostin (Fig. 7A) and TSLP levels (Fig. 7B) were not significantly elevated in tissue homogenates
396 after exposure to *S. aureus*, as determined by ELISA. Immunoblot analysis (Fig. 7C) showed that the
397 short form TSLP (sfTSLP) was undetectable, while the expression of long form TSLP (lfTSLP) was not
398 significantly altered between the different conditions. At the mRNA level, lfTSLP was elevated after 2
399 hours exposure to both *S. aureus* and *S. epidermidis* (Fig. 7D), although in the latter case the increase
400 was not statistically significant. ELISA measurements of supernatant showed only detectable
401 (secreted) TSLP levels after 2 hours exposure to *S. aureus* (data not shown), but in none of the earlier
402 time points was TSLP detectable in the supernatants.

403

404 **4. Discussion**

405 The generation of EETs has been linked to various pathologies, including inflammatory skin and
406 intestinal diseases. Unsurprisingly, EET formation was also observed in allergic diseases, such as
407 bronchial asthma, contact dermatitis, atopic dermatitis (AD) and allergic drug reactions.[9,15-17] Our aim
408 was to investigate the presence of EETs in CRSwNP tissues. Due to the eosinophilic environment and
409 the increased levels of IL-5, eotaxin and TSLP, we believed this was an ideal context for the
410 generation of EETs. Our data have now shown that EETs are also formed in the nasal polyp tissues.
411 An increased presence of eosinophils and EETs was observed at subepithelial sites, especially at sites
412 with epithelial defects. These data are in line with similar observations described in eosinophilic
413 esophagitis and bullous pemphigoid.[21] Our research in patient samples showed that about 9% of the
414 eosinophils present contribute to EET formation. It is very likely that EET formation *in vivo* is much
415 more frequent as EETs are generated in a very short time frame, and possibly rapidly degraded.

416 The relationship between *S. aureus* and elevated levels of IL-5 and CRSwNP has been recognized for
417 years.[5,13] The antibacterial properties of EETs and their formation as a response to *S. aureus in vitro*,
418 made it interesting to study this relationship in the context of clinical tissue samples from CRSwNP
419 patients. Our data have now shown that EET formation is associated with increased IL-5 levels, but

420 also with *S. aureus* colonization in patient's tissues. In addition, we show for the first time direct
421 proof of *S. aureus* entrapment by EETs in patient's tissues. Furthermore, we were able to show the
422 induction of EET formation after exposure to *S. aureus*, but not to *S. epidermidis* in tissues of CRSwNP
423 patients. The presence of DNA traps in secretions of eosinophilic chronic rhinosinusitis patients and
424 their role in increasing the viscosity of these secretions has already been reported.[18] Our experiments
425 now demonstrate that eosinophils migrate towards *S. aureus* and entrap the organism both inside
426 and outside the human diseased mucosal tissue. Eosinophils from CRSwNP patient tissues showed a
427 massive reactivity towards *S. aureus* exposure with extensive EET formation. This suggests that the
428 reaction in the patient and at the diseased location could be far more extensive than currently is
429 recognized from *in vitro* exposure experiments using peripheral eosinophils. In contrast to the
430 situation in diseased tissue, *in vitro* exposure also needed prior priming for EET formation. These data
431 also imply that a direct contact between the bacteria and the eosinophil is likely to initially trigger
432 EET formation. This could also explain the increased EET formation at sites of epithelial defects.

433 Our results imply that eosinophils are likely to be specifically recruited to and generate EETs at sites
434 of epithelial damage, possibly to protect the host from infections. In nasal polyps, several defects in
435 the mucosal defense have been shown, including increased levels of M2 macrophages, a decrease in
436 β-defensin 2 and 3, defective epithelial tight junctions, a compromised expression of Toll-like
437 receptor 9, etc.[22-25] In this situation, eosinophil-mediated immune responses, like EET formation,
438 might be crucial for maintaining the barrier function after inflammation-associated epithelial cell
439 damage, protecting the host from an uncontrolled invasion of bacteria.

440 This hypothesis is further supported by, (i) the increased presence and alignment of eosinophils at
441 subepithelial sites, (ii) the observed migration and entrapment of *S. aureus* in the *in vitro* exposure
442 model and (iii) the *in vitro* migration assay confirming an active migration of eosinophils. As the
443 cytotoxicity of eosinophilic granule proteins is well known, our observations could also point to a
444 more prominent role of eosinophils in damaging or preventing the repair of the epithelial barrier in

445 CRSwNP.[21,26,27] Whether the observed EETs are a causal effect of damage to the epithelial barrier, or
446 (partly) the source of epithelial damage, will be the subject of future investigations.

447 Recently the subepithelial deposition and association between periostin and eosinophilic
448 inflammation was shown in CRSwNP.[28] This made periostin an interesting candidate to investigate in
449 association to EET formation. Indeed, we found significant elevated periostin levels in patients with
450 more EETs. Despite this fact, no increase in periostin was found after experimental exposure to *S.*
451 *aureus*. This can possibly be attributed to the short time frame wherein we studied the trap
452 formation. In addition, periostin could facilitate the infiltration of eosinophils in the tissue, as
453 reported for allergic lungs and eosinophilic esophagitis.[29] Therefore, periostin could be increased as a
454 consequence of the Th2 context without being directly involved in EET formation.

455 Interestingly, the induced generation of the EETs was observed within a narrow time frame (1 hour).
456 With these observations, questions arise about the trigger of EETs release in the tissue after
457 exposure. Our experiments have clearly shown that, upon stimulation with *S. aureus*, ROS levels are
458 increased within the same time frame. Previously, it had been demonstrated that inhibition of
459 NADPH oxidase activity, responsible for ROS production, also inhibited EET formation by eosinophils
460 *in vitro*.[10,11] Our data have shown that inhibition of NADPH oxidase was also able to block ROS
461 production and EET formation in human mucosal tissue, implying an important role of NADPH
462 oxidase activity in EET formation following *S. aureus* challenge *in vivo.*

463 To our knowledge, a similar role for eosinophilic granule proteins has not been reported. We feel
464 that solving this question is beyond the scope of our manuscript and would imply a separate EET-
465 based study. However, would like to thank the reviewer for highlighting this as we feel this could be a
466 valuable hypothesis for our future research.

467 IL-5 and TSLP are both well-known triggers of EET formation *in vitro*.[10,11,16] Therefore IL-5 and TSLP
468 were investigated as potential triggers for EET formation in CRSwNP tissues. Unsurprisingly,
469 increased IL-5 levels were indeed associated with more EETs. Interestingly however, TSLP levels were

470 not markedly enhanced in the tissue as determined by ELISA. This was in contrast to our expectations
471 since TSLP is known to be elevated after epithelial damage, was correlated with EET release in active
472 eosinophilic esophagitis and had been reported to directly trigger EET formation.[11,21,31]. Previously,
473 EETs were described as capable of entrapping and killing *S. aureus* and *S. epidermidis in vitro*. In
474 contrast to *S. aureus*, direct contact with *S. epidermidis* was not sufficient for EET release and was
475 dependent on the addition of TSLP to execute this effect.[11] This could explain why EETs were mainly
476 concentrated at sites of epithelial defects (direct contact with S. aureus) and why *S. epidermidis* was
477 unable to induce EET formation in the experimental human mucosal exposure experiments, as TSLP
478 levels were not elevated in the time frame studied. PCR data however, showed an increase in TSLP
479 production after two hours. Therefore, it is possible that at later time points, TSLP could still play a
480 role in EET formation.

481 Altogether these data imply that the tissue context, the nature of the trigger and the interplay
482 between these two, play an important role in determining the intensity of EET formation. It is likely
483 that the initiation of EET formation is triggered by direct contact between *S. aureus* and eosinophils
484 and that, as a consequence of secondary signaling events, EET formation in later stadia is further
485 supported by increased IL-5, TSLP or other triggers at later time points.

486 In neutrophils it was shown that, besides ROS, also the granule proteins neutrophil elastase and
487 myeloperoxidase had a regulatory role in the formation of NETs. [30] Eosinophilic granule proteins
488 could possibly exert a similar function in EET formation but this is subject of future research.

489 Although EET formation seems to have a beneficial role by targeting S. aureus, the targeting of
490 eosinophils in eosinophils-associated diseases is highly effective. This paradox raises questions about
491 cause and consequences in the pathophysiologic role of epithelial barrier defects, germs such as S.
492 aureus, and the type-2 inflammatory reaction.

493 In conclusion, we describe here for the first time the formation of EETs in nasal polyp tissue; their
494 localization, dynamics and relationship with the presence of *S. aureus*, suggesting a role for EETs in

epithelial barrier defects. ROS could be identified as essential requirement for EET formation by eosinophils. These findings yield new insights into the possible role of EETs in human airways, and link *S. aureus* to eosinophilic airway disease such as CRSwNP and co-mordid asthma.

5. Acknowledgements

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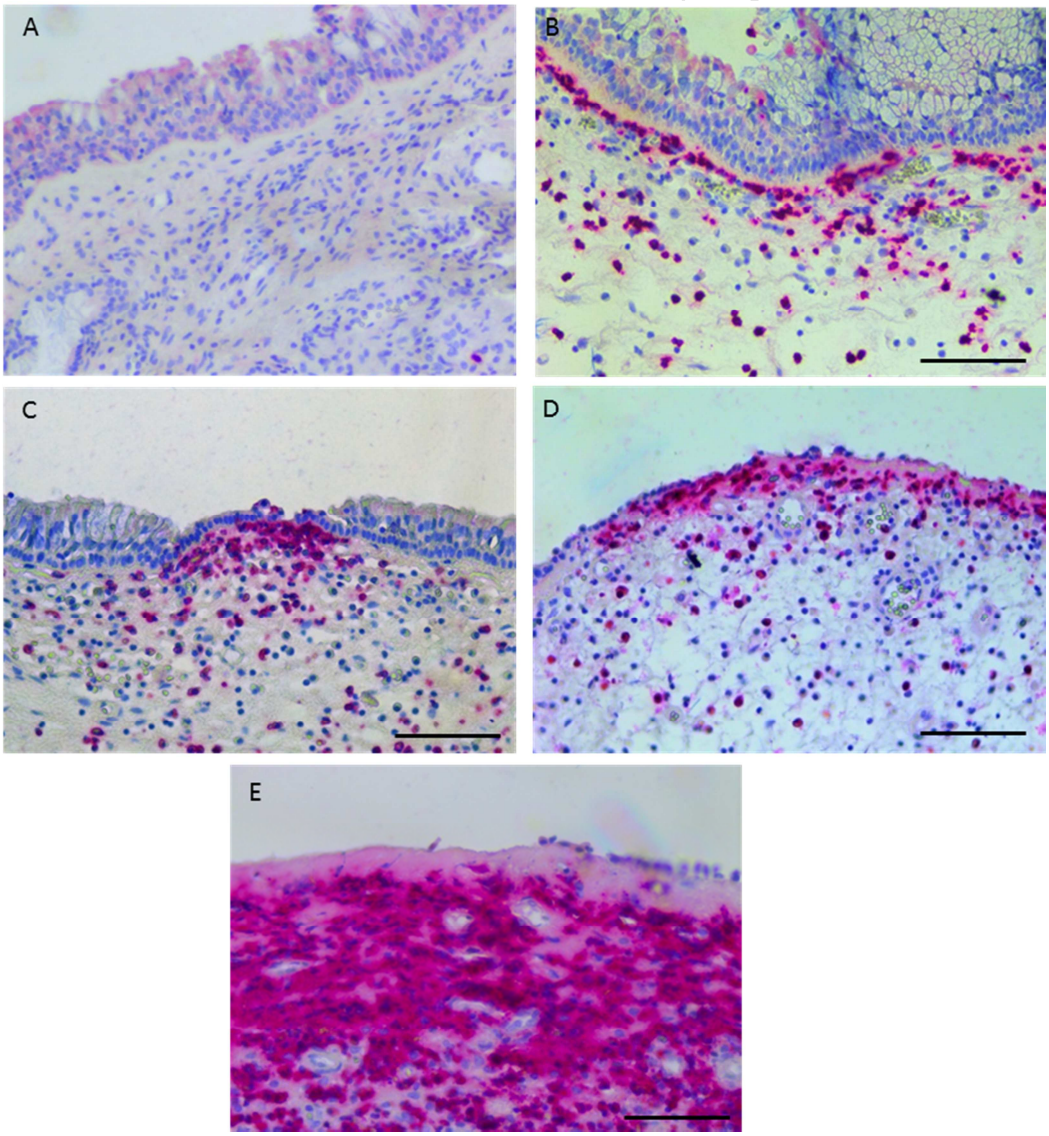
524 **6. Figures**

		CRSwNP	Controls
Total cases		15	5
Gender	M/F	10/5	3/2
Age	Median (range)	35 (21-92)	33 (29-42)
Ethnicity		Caucasian	Caucasian
Allergy	-/+	2/12	2/2
	missing cases	n=1	n=1
Asthma	-/+	7/7	1/4
	missing cases	n=1	n=0
Treatment asthma	-/+	4/3	2/2
IgE	Mean ± SD	1448.64 ± 1935.70	62.03 ± 77.95
SAE-IgE	Mean ± SD	6.85 ± 7.41	BDL

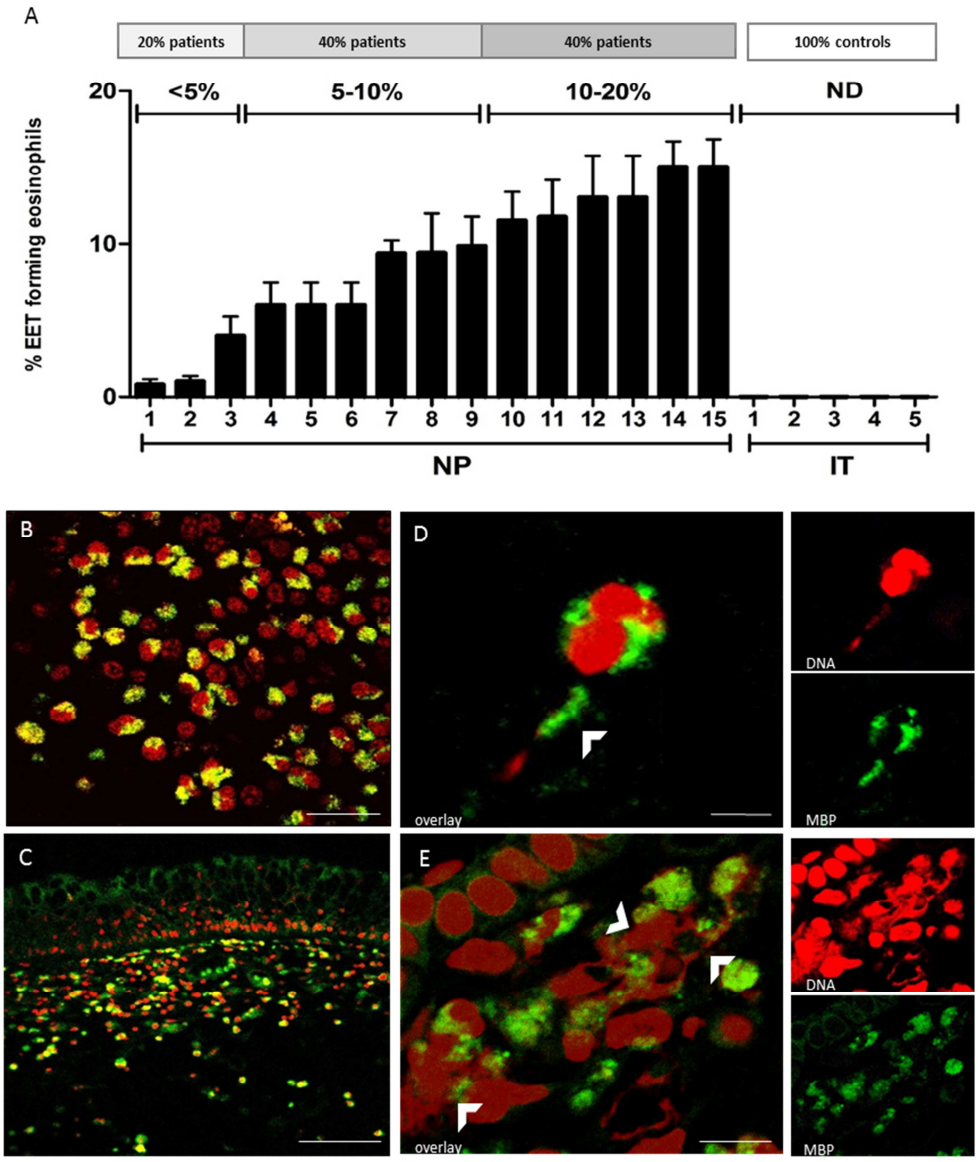
525

Table 1. Patient characterization. Chronic rhinosinusitis with nasal polyps (CRSwNP), Male (M), Female (F), present (+), not present (-), below detection limit (BDL), standard deviation (SD), immunoglobulin E (IgE), *S. aureus* specific immunoglobulin E (SAE-IgE). Allergy was defined as 'present' when the patient has a positive skin prick test for at least one of the allergens commonly tested in our region.



549

550 **Fig. 1 Eosinophils in nasal mucosa tissue.** (A-E) Immunohistochemical stain for MBP (red) in the
551 subepithelial region of inferior turbinate from a control patient (A) and polyp tissue from different
552 patients (B-E) showing the localization of eosinophils and different intensities of subepithelial
553 localization. Nuclei (blue) are counterstained with hematoxylin. (Scale bar = 100 µm)



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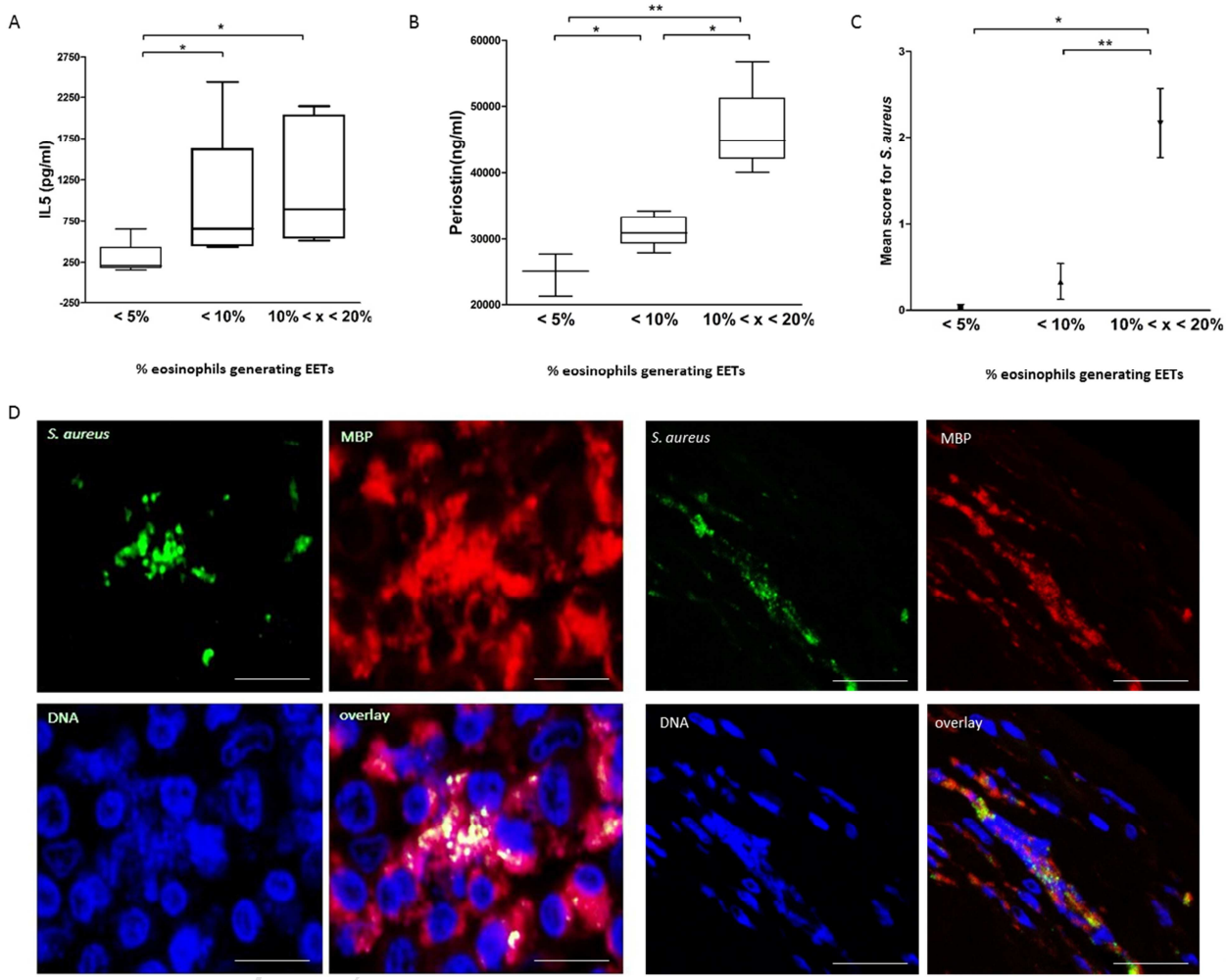
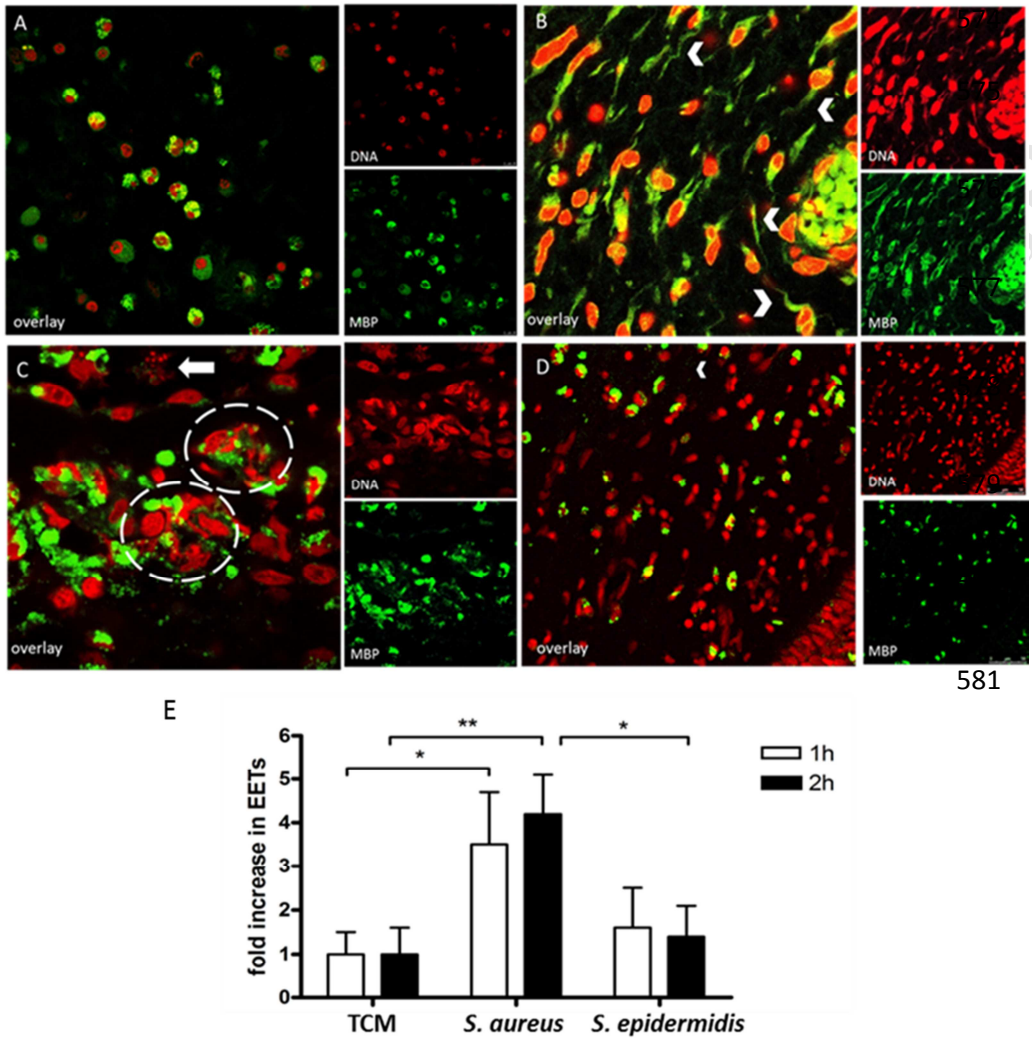
555 **Fig. 2 Eosinophils and their DNA traps in nasal polyp tissue.** (A) Quantification of EETs in the
556 subepithelial region of patients (NP 1-15) and control (IT 1-5) tissues, EETs were expressed as % of
557 eosinophils generating EETs relative to the total amount of present eosinophils. (B-E)

558 Immunofluorescent staining of MBP (green) and DNA (red). (B) Intact eosinophils in the stroma (scale
559 bar = 25 µm), (C) increased subepithelial eosinophilic aggregation (scale bar = 50 µm), (D) a single
560 eosinophil is shown generating an EET (indicated by an arrowhead) (scale bar = 5 µm) (E) a cluster of
561 eosinophils generating EETs (indicated by arrowheads). (Scale bar = 10 µm).



562
563 **Fig. 3 Relevant percentage of eosinophils forming EETs and IL-5, periostin and *S. aureus***
564 **colonization.** Tissue levels of IL-5 and periostin were measured (CRSwNP patients, n=15) by means of
565 ELISA. *S. aureus* colonization was assessed for all patients using a PNA-Fish technique. These data
566 were studied for bacteria association with EETs. (A) IL-5 levels were elevated in patients where higher
567 fractions of eosinophils generating EETs were observed. (B) Periostin levels where increased where
568 higher fractions of eosinophils generating EETs were detected. (C) In patients, increased EET
569 formation significantly correlated with higher scores for *S. aureus* colonization. Data were analyzed
570 by a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001. (D) Co-

571 localization of *S. aureus* and EETs. Immunofluorescent staining for MBP (red) and DNA (blue)
572 combined with a PNA-Fish stain for *S. aureus (Green).* (left picture scale bar = 25 µm, right picture
573 scale bar = 50 µm)



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586 **Fig. 4 EETs in nasal polyp tissue after *in vitro* exposure to *S. aureus* or *S. epidermidis*.**
587 Immunofluorescent stain for MBP (green) and DNA (red). (A) Intact eosinophils after 2 hours air-
588 liquid exposure to tissue culture medium (control) (Scale bar = 25 µm); (B) increased EET formation
589 (arrowheads indicate some, but not all, of the EETs) after 1 hour air-liquid exposure to *S. aureus*
590 (Scale bar = 25 µm); (C) increased subepithelial formation of EETs (dashed line) in the proximity of *S.*
591 *aureus* (arrow) (scale bar = 25 µm); (D) mainly intact eosinophils after 2 hours air-liquid exposure to
592 *S. epidermidis*. (Scale bar = 50 µm); (E) Increase of EET formation in the subepithelial region after

exposure to *S. aureus* (Sa) and *S. epidermidis* (Se) compared to tissue culture medium (TCM) in nasal polyp tissue (n=3). Data were analyzed with a Friedman test, followed with Dunns post test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001

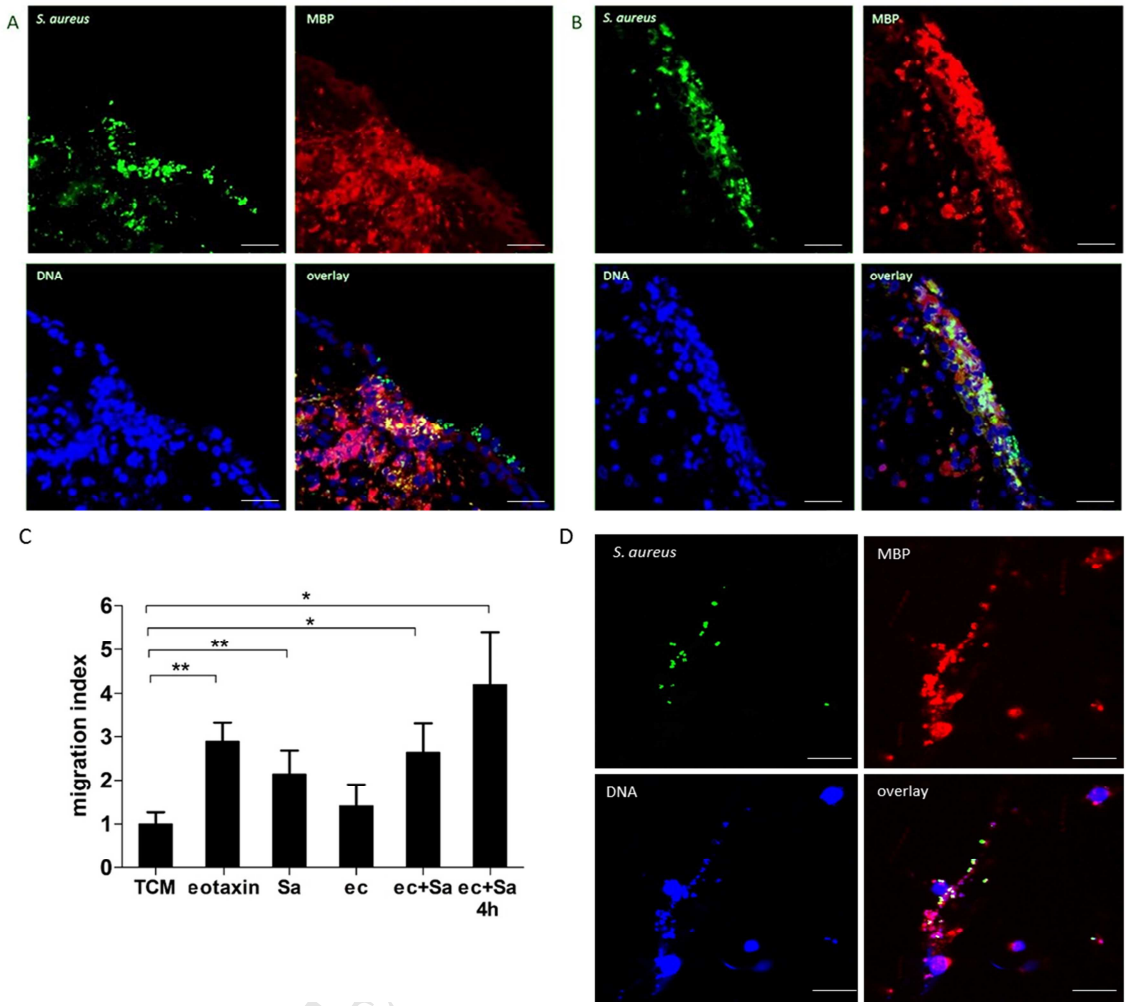

Fig. 5 Transepithelial migration and massive EET formation of eosinophils to entrap *S. aureus*. Immunofluorescent staining of MBP (red) and DNA (blue), combined with PNA-Fish stain for *S. aureus* (green) in tissue sections after 2 hours exposure to *S. aureus*. (A) Indicates the migration of eosinophils and EETs at sites of epithelial defects where direct contact with *S. aureus* is observed. (Scale bar = 25 µm) (B) Indicates transepithelial migration of a large fraction of eosinophils and the trapping of *S. aureus* in a large EET. (Scale bar = 25 µm) (C) *In vitro* migration assay of eosinophils in response to different stimuli: tissue culture medium (TCM), eotaxin, *S. aureus* (Sa), epithelial cells (ec), epithelial cells with *S. aureus* (ec+Sa), and epithelial cells with *S. aureus* after 4 hours of co-

incubation (ec+Sa 4h). Data were analyzed with a Mann-Whitney U test, n=3, significances are expressed as *p<0.05,**p<0.01, ***p<0.001. (D) Eosinophils from a CRSwNP patient showing EET formation after challenge with *S. aureus in vitro*. (Scale bar = 25 µm)

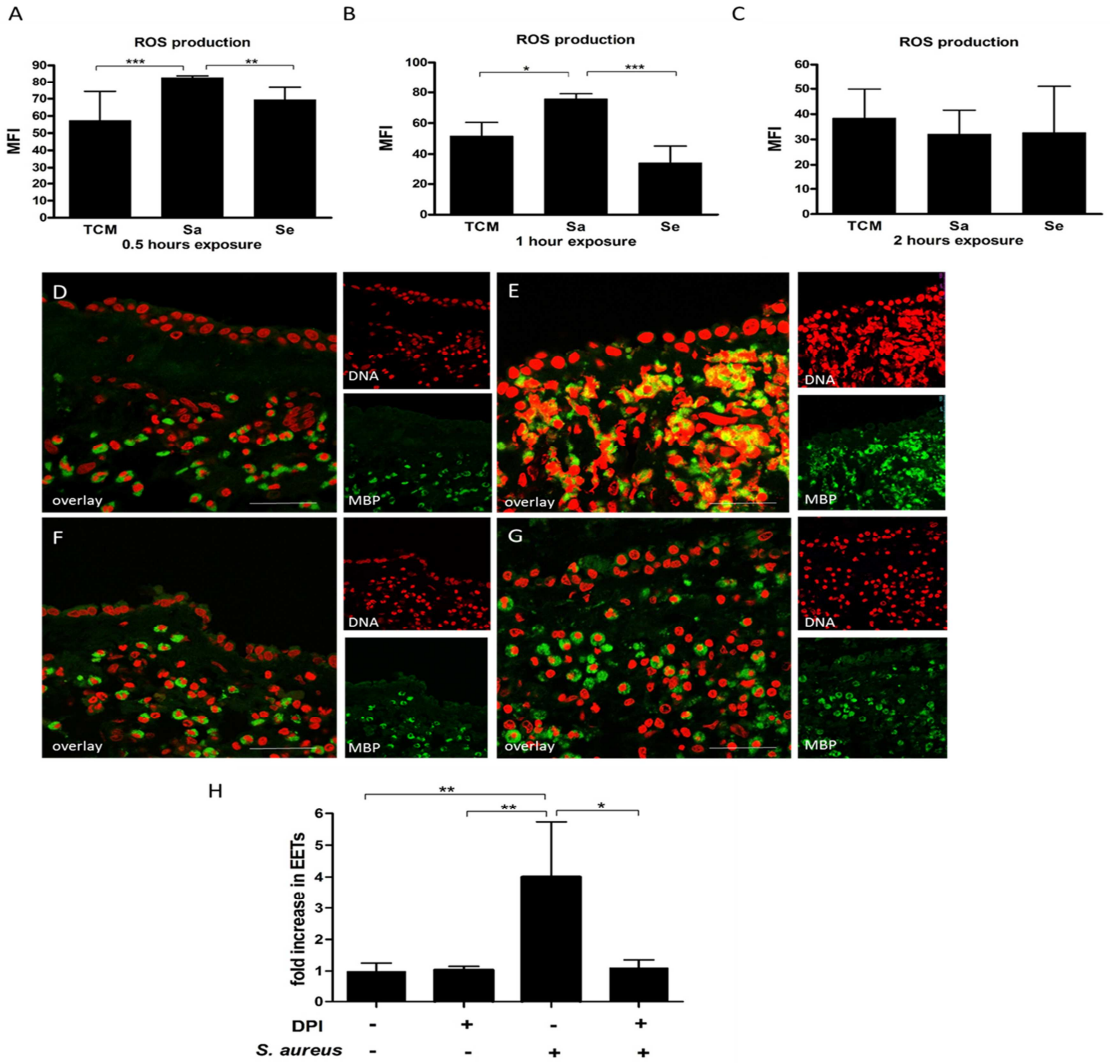

Fig. 6 Reactive oxygen species formation after exposure to *S. aureus*, and inhibition of EET formation by DPI. (A-C) Production of ROS in the tissues after 0.5, 1 and 2 hours *in vitro* exposure to *S. aureus* (Sa) or *S. epidermidis* (Se) (n=3). (D-G) Immunofluorescent stain for MBP (green) and DNA (red). (D) intact eosinophils after 1 hours air-liquid exposure to tissue culture medium (control); (E) increased EET formation after 1 hour air-liquid exposure to *S. aureus*; (F) intact eosinophils after 1 hour DPI treatment and 1 h air liquid exposure to tissue culture medium (control); (G) mainly intact eosinophils after 1 hour DPI treatment and 1 hour air-liquid exposure to *S. aureus*; (scale bar = 100

616 µm) (H) Increase of EET formation after exposure to *S. aureus* and inhibition by DPI treatment in
617 nasal polyp tissue (n=3). Data were analyzed with a Friedman test, followed with Dunns post test,
618 significances are expressed as *p<0.05,**p<0.01, ***p<0.001

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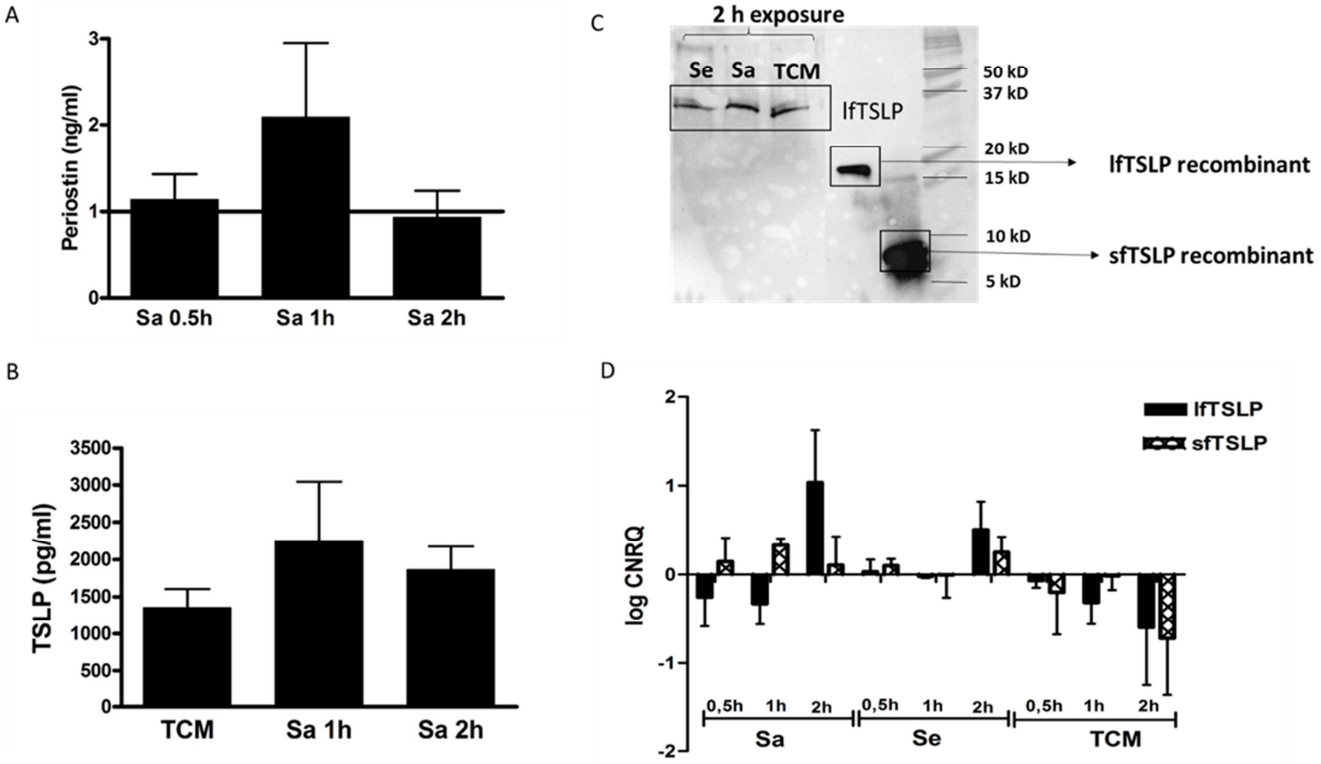

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621 **Fig. 7 Periostin and TSLP levels after *in vitro* exposure to *S. aureus*.** (A) Periostin levels in tissue
622 homogenates were not significantly elevated after exposure to *S. aureus*. Black line represents the
623 control value (TCM) (n=4). (B) TSLP levels, determined by ELISA, in tissue homogenates were not
624 significantly elevated after exposure to *S. aureus* (n=4). (C) Western blot analysis showing no
625 difference in lfTSLP expression in tissue homogenates after exposure to either *S. aureus*. or *S.*
626 *epidermidis*. In addition the western blot indicates that sfTSLP was not detected. (D) PCR analysis in
627 tissue homogenates indicates an increase in lfTSLP after 2 hours exposure to *S. aureus* (n=3).

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633 **7. Supplemental information**

Primer	sequence
SDHA-FW	TGGGAACAAGAGGGCATCTG
SDHA-Rev	CCACCACTGCATCAAATTCATG
EF1-FW	CTGAACCATCCAGGCCAAAT
EF1-Rev	GCCGTGTGGCAATCCAAT
TSLP short form FW	CGTAAACTTTGCCGCCTATGA
TSLP short term Rev	TTCTTCATTGCCTGAGTAGCATTTAT
long form TSLP - FW	GGGCTGGTGTTAACTTACGACTTCA
long form TSLP - Rev	ACTCGGTACTTTTGGTCCCACTCA

634 **Table S1. PCR primer sequences**

635

Investigated feature		CRSwNP (n/N)	Controls (n/N)
Degranulated eosinophils	< 1% eosinophils/not present	5/15	5/5
	< 10 % eosinophils	6/15	0/5
	< 20 % eosinophils	4/15	0/5
***S. aureus* presence**	Not present	7/15	4/5
	Planktonic < 2 spots	4/15	1/5
	Planktonic > 2 spots no biofilm	1/15	0/5
	Biofilm	3/15	0/5

636 **Table S2. Semi-quantitative evaluation of investigated parameters including degranulation and**

637 **colonization of *S. aureus*.**

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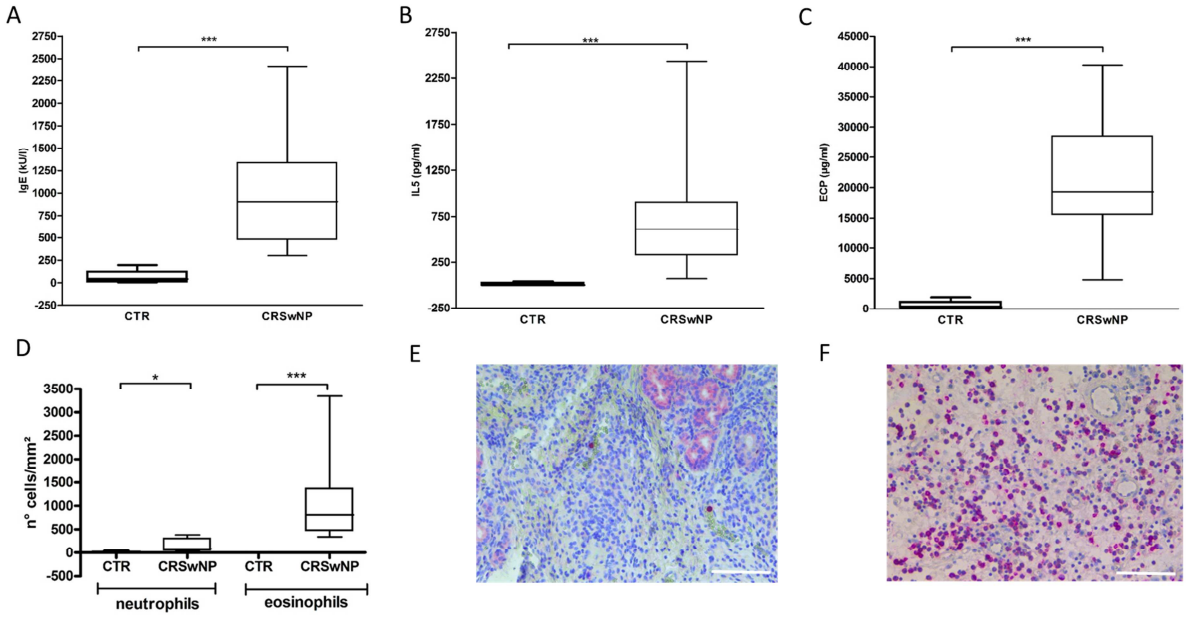

Fig. S1 Characterization of the study population. Tissue levels of IgE (A), IL-5 (B), and ECP (C) were measured in all patients (CRSwNP, n=15) and controls (CTR, n=5) by means of ELISA and were found to be elevated in the CRSwNP patients. Eosinophils and neutrophils (D) were counted in tissue of controls and CRSwNP patients. Tissue slides stained for major basic protein in controls (E) and CRSwNP (F) showing the general distribution of eosinophils throughout the tissue (Scale bar = 100 µm). Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001

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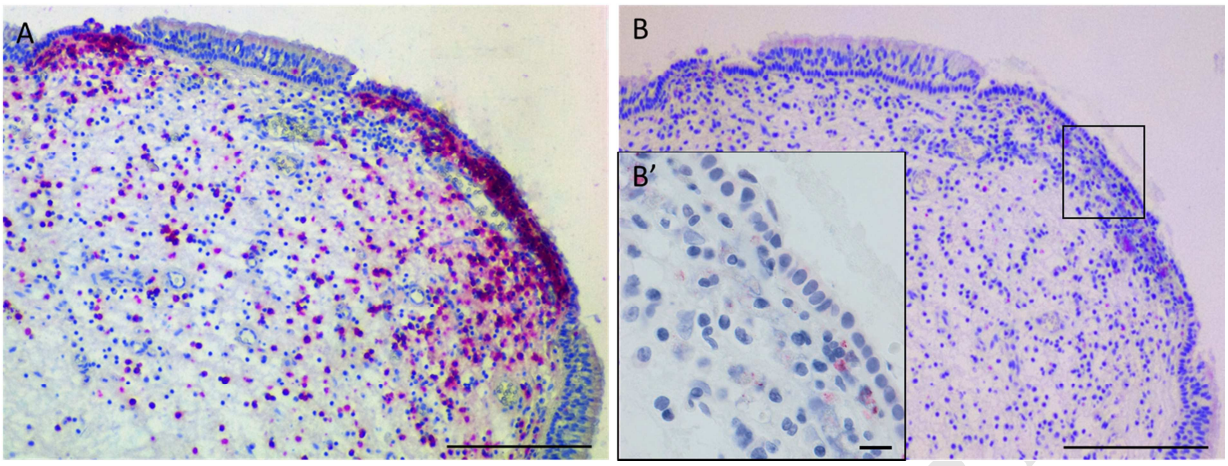

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651 **Fig. S2 Subepithelial eosinophils and damaged epithelium.** *Immunohistochemistry was performed*
652 *on subsequent tissue slides and the same region was selected for pictures A-B. The protein of interest*
653 *is stained in red/purple, nuclei are stained with hematoxylin (blue).* (A) An immunohistochemical stain
654 for MBP (red) shows the localization of eosinophils in polyp tissue. (Scale bar = 200 µm); (B) An
655 immunohistochemical stain for caspase-3 (red) demonstrates very few apoptotic cells in the tissue.
656 Inset image (B') shows no evidence for cell lysis. (B: Scale bar = 200 µm, B': scale bar = 10 µm)

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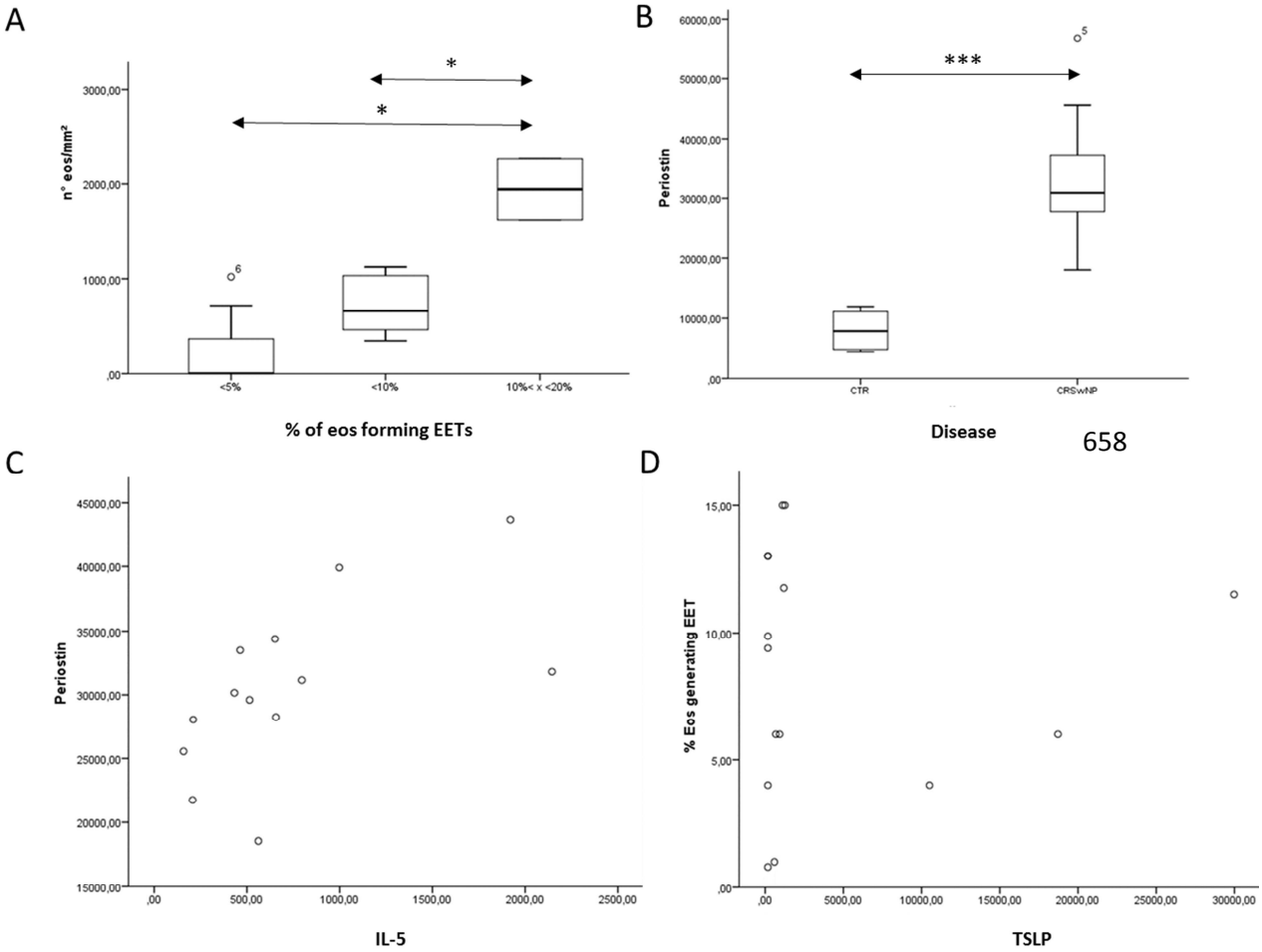

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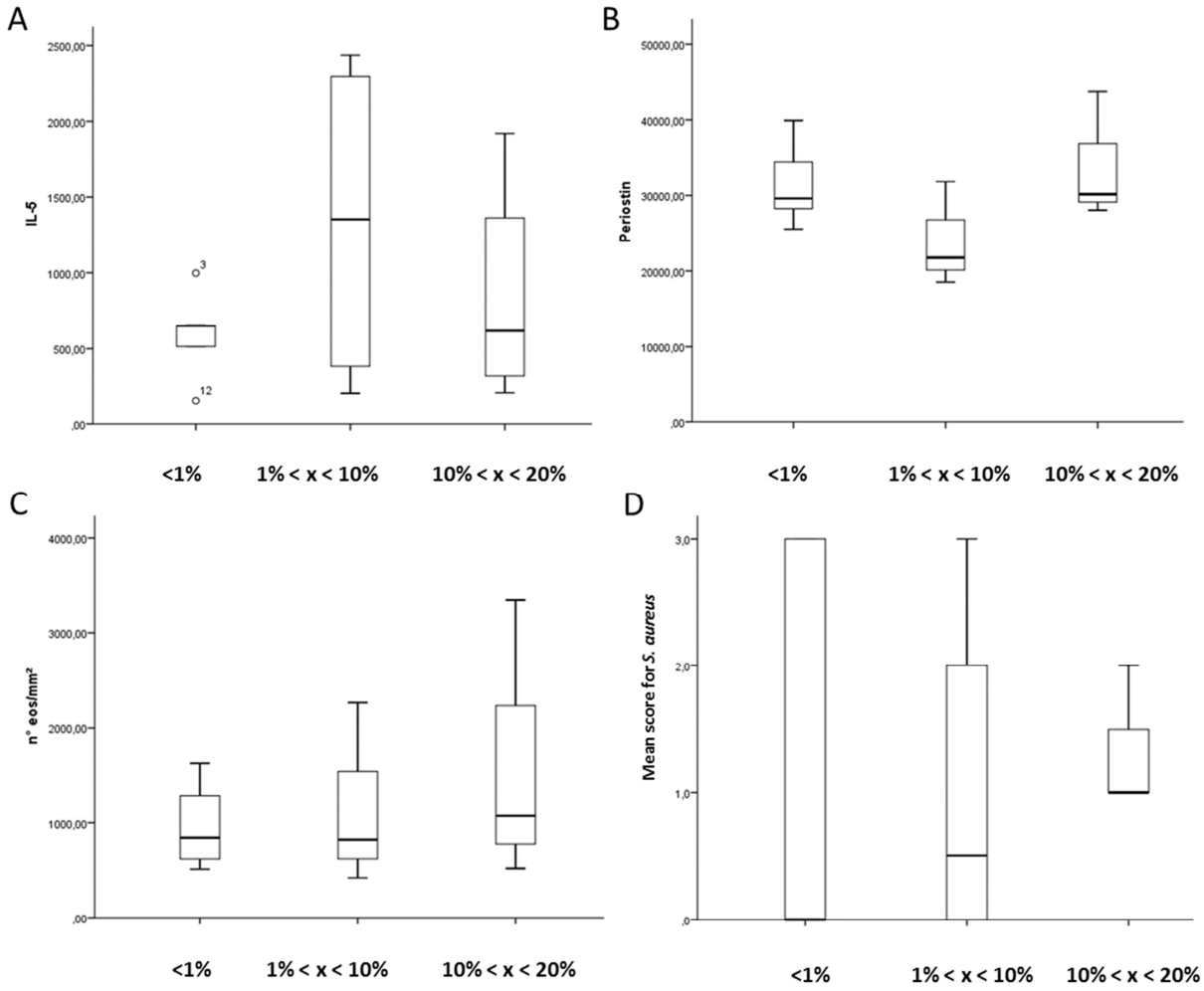
Fig. S3 (A) Relation between EET formation and number of eosinophils in the tissues. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001 (B) Periostin levels in CRSwNP patients and controls. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001 (C) Scatterplot of periostin and IL-5 levels in CRSwNP patients with a significant correlation (p=0.013, R^2=0.665), as determined by Spearman Correlation. (D) Scatterplot of TSLP and % of eosinophils forming EETs with no correlation as determined by Spearman Correlation.



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671 **Fig. S4** (A) Relation between IL-5 and percentage of degranulated eosinophils. (B) Relation between
672 periostin and percentage of degranulated eosinophils. (C) Relation between number of eosinophils
673 and percentage of degranulated eosinophils. (D) Relation between *S. aureus* colonization and
674 percentage of degranulated eosinophils.

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		CRSwNP	Controls
Total cases		15	5
Gender	M/F	10/5	3/2
Age	Median (range)	35 (21-92)	33 (29-42)
Ethnicity		Caucasian	Caucasian
Allergy	-/+	2/12	2/2
	missing cases	n=1	n=1
Asthma	-/+	7/7	1/4
	missing cases	n=1	n=0
Treatment asthma	-/+	4/3	2/2
IgE	Mean ± SD	1448.64 ± 1935.70	62.03 ± 77.95
SAE-IgE	Mean ± SD	6.85 ± 7.41	BDL

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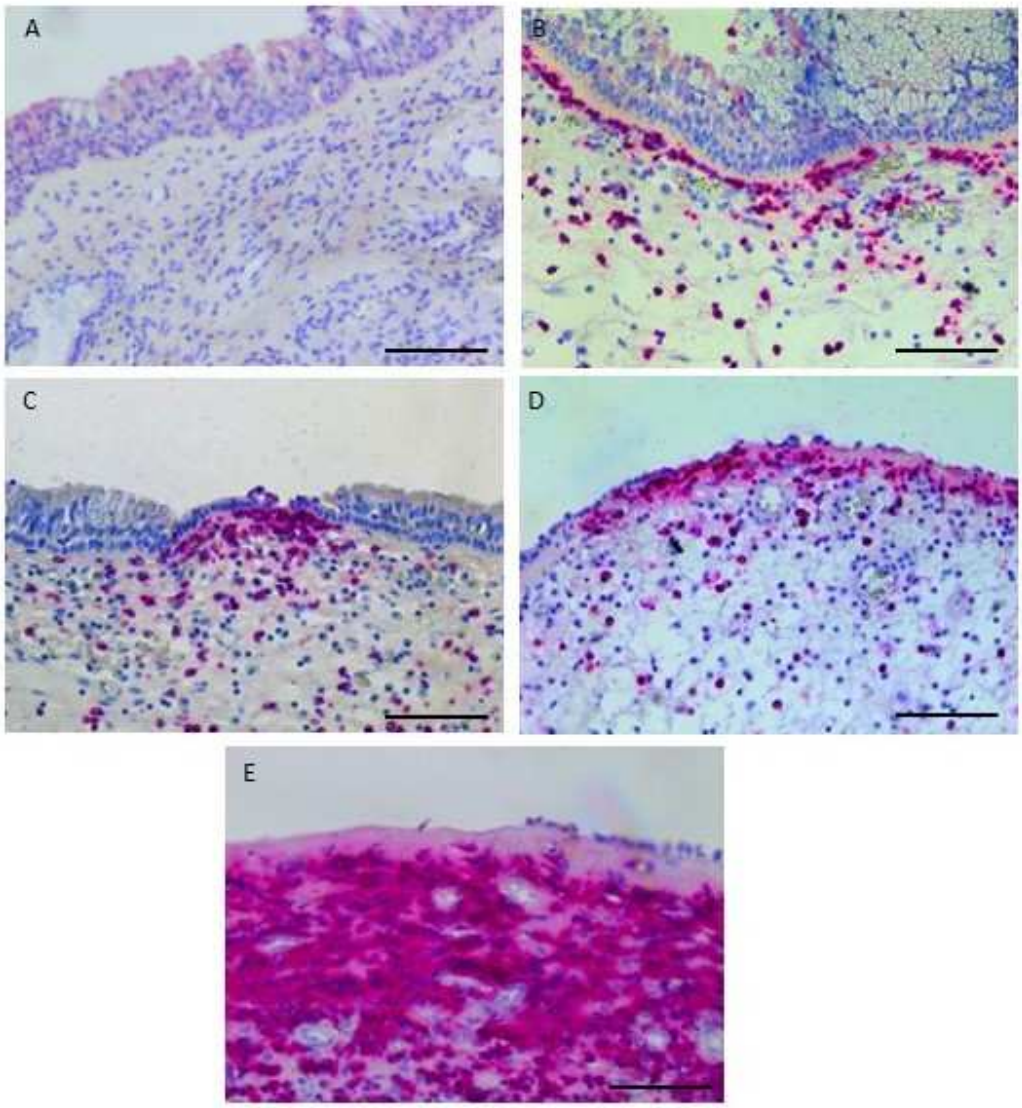

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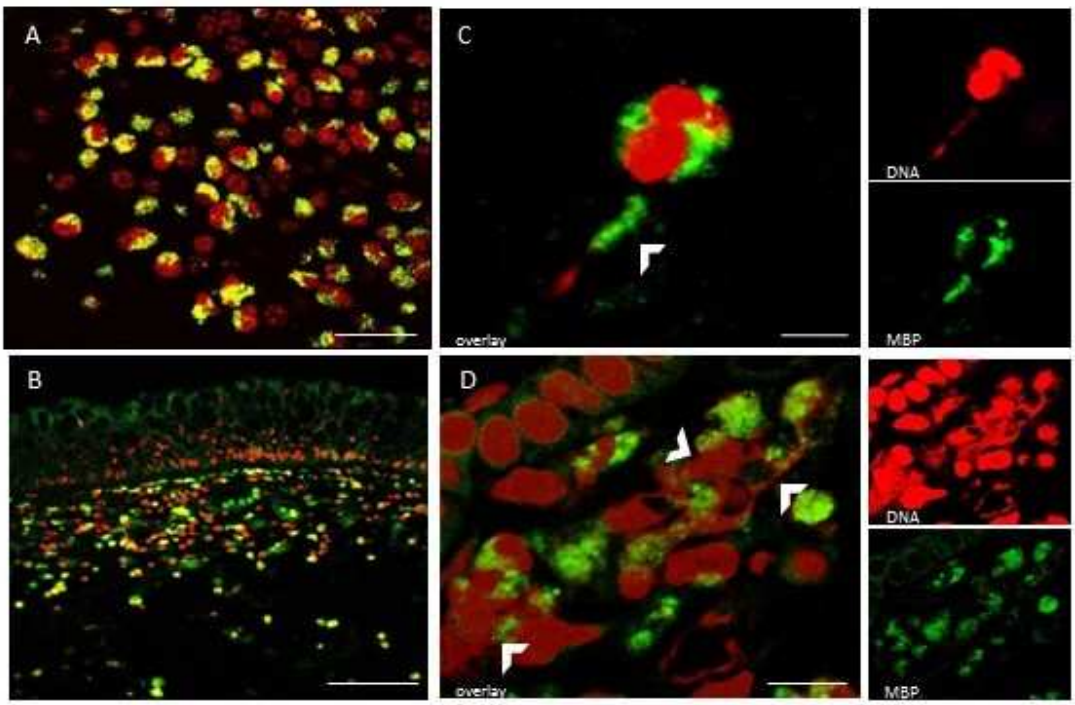
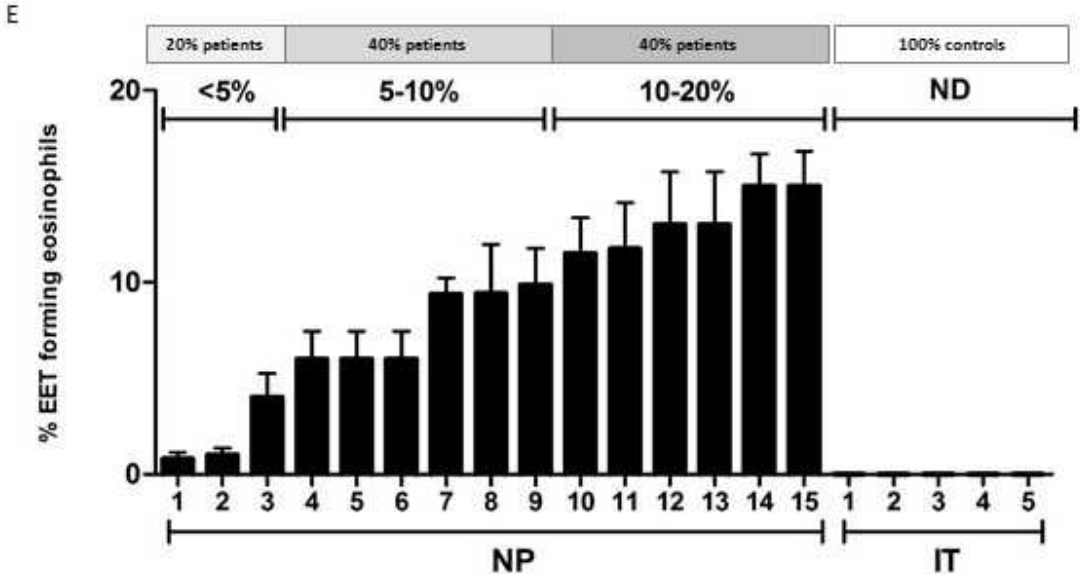
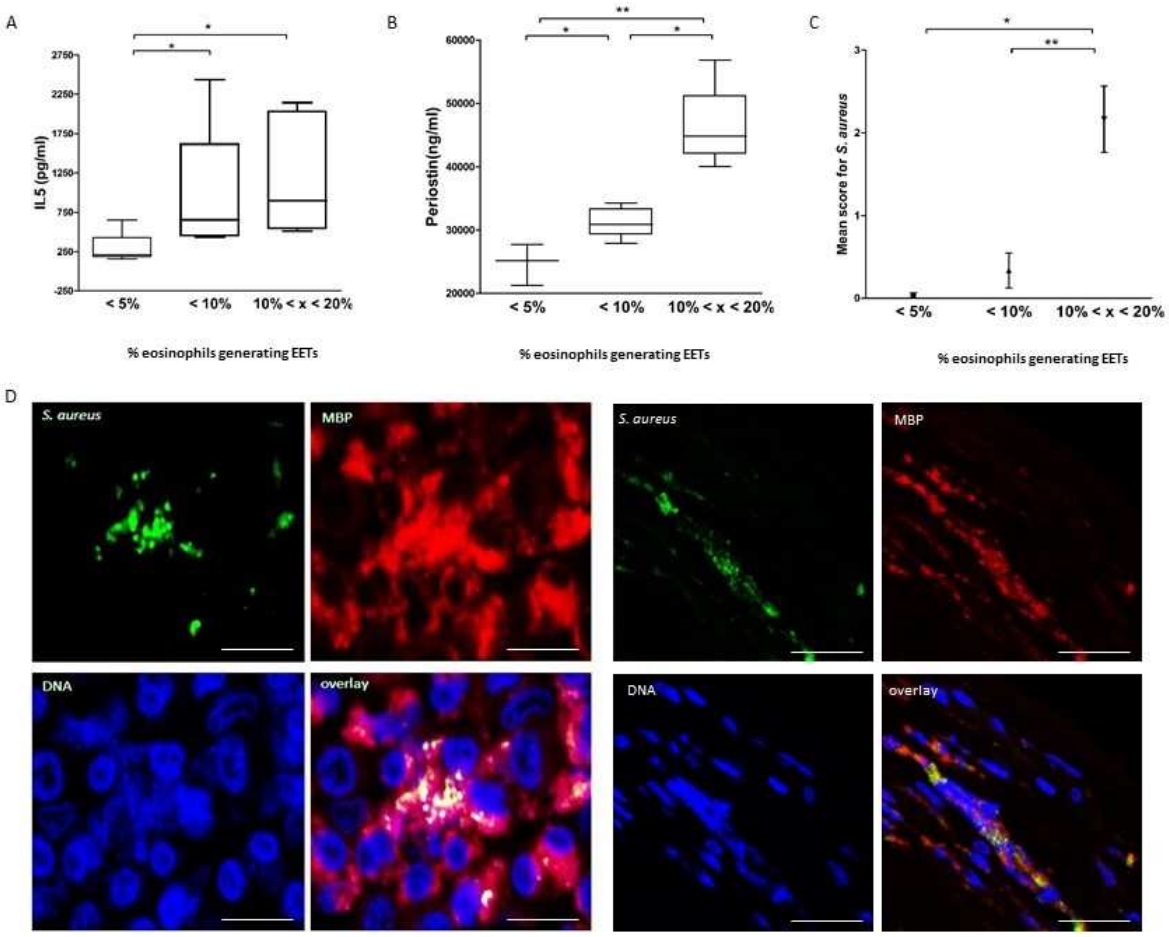

A
B
C
D
overlay
DNA
MBP
overlay
DNA
MBP


E
20% patients
40% patients
40% patients
100% controls
<5%
5-10%
10-20%
ND
% EET forming eosinophils
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 1 2 3 4 5
NP
IT


A
IL5 (pg/ml)
2750
2250
1750
1250
750
250
-250
< 5%
< 10%
10% < x < 20%
% eosinophils generating EETs
B
Periostin(ng/ml)
60000
50000
40000
30000
20000
< 5%
< 10%
10% < x < 20%
% eosinophils generating EETs
C
Mean score for S. aureus
3
2
1
0
< 5%
< 10%
10% < x < 20%
% eosinophils generating EETs
D
S. aureus
MBP
DNA
overlay

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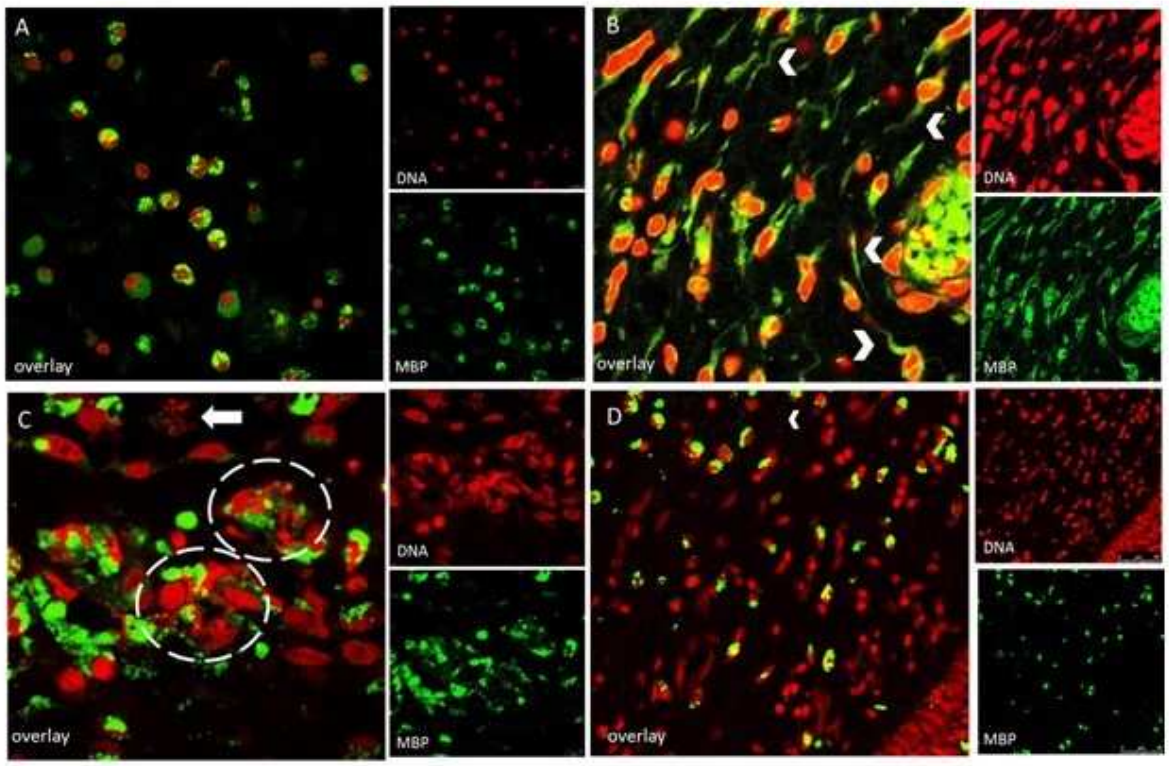
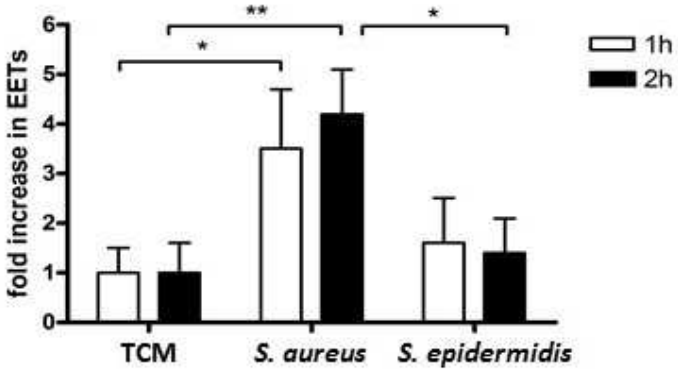
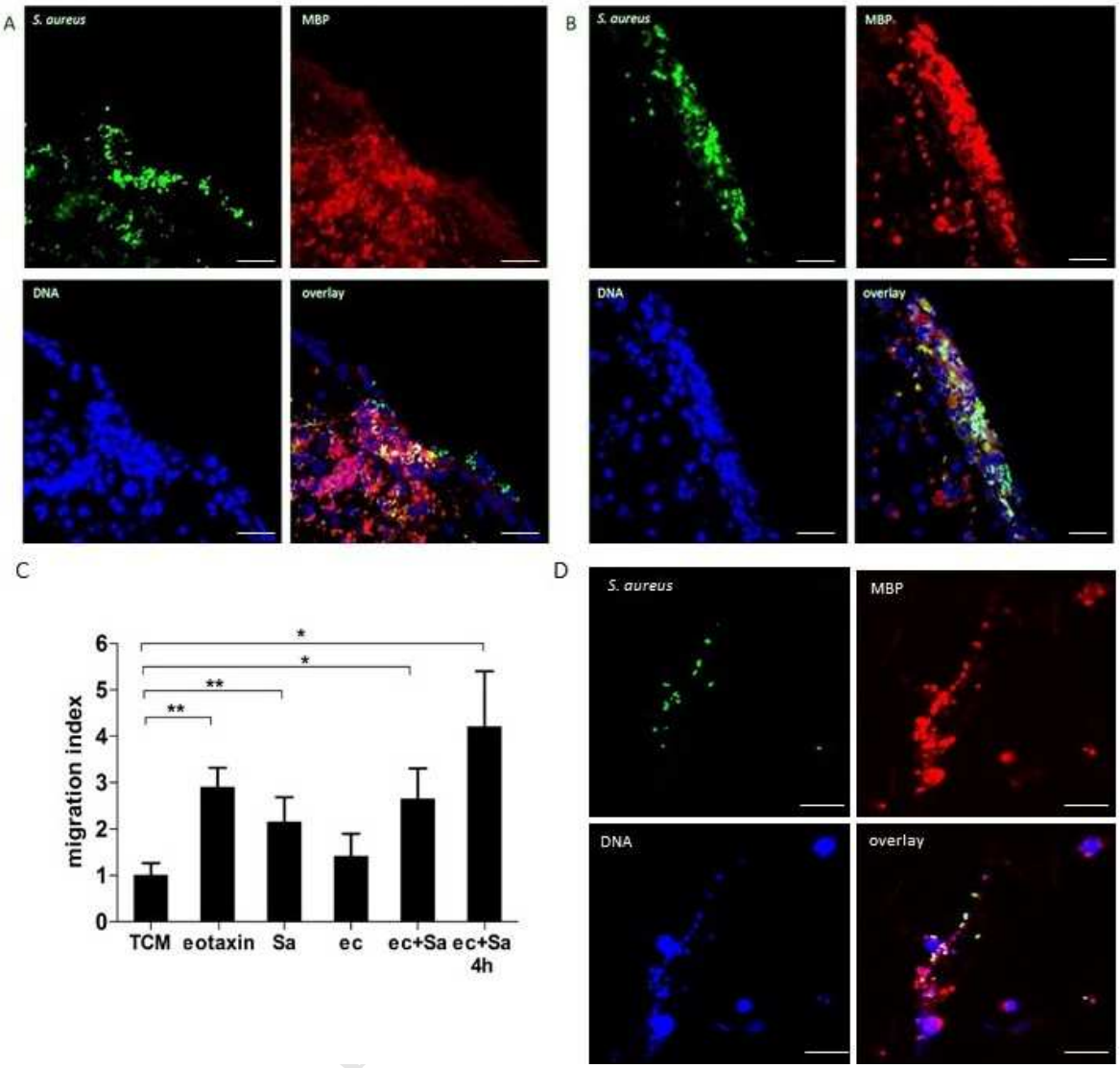

A
overlay
DNA
MBP
B
overlay
DNA
MBP
C
overlay
DNA
MBP
D
overlay
DNA
MBP


fold increase in EETs
0
1
2
3
4
5
6
*
**
*
1h
2h
TCM
S. aureus
S. epidermidis


A
S. aureus
MBP
DNA
overlay
B
S. aureus
MBP
DNA
overlay
C
migration index
0
1
2
3
4
5
6
*
*
**
**
TCM
eotaxin
Sa
ec
ec+Sa
ec+Sa 4h
D
S. aureus
MBP
DNA
overlay

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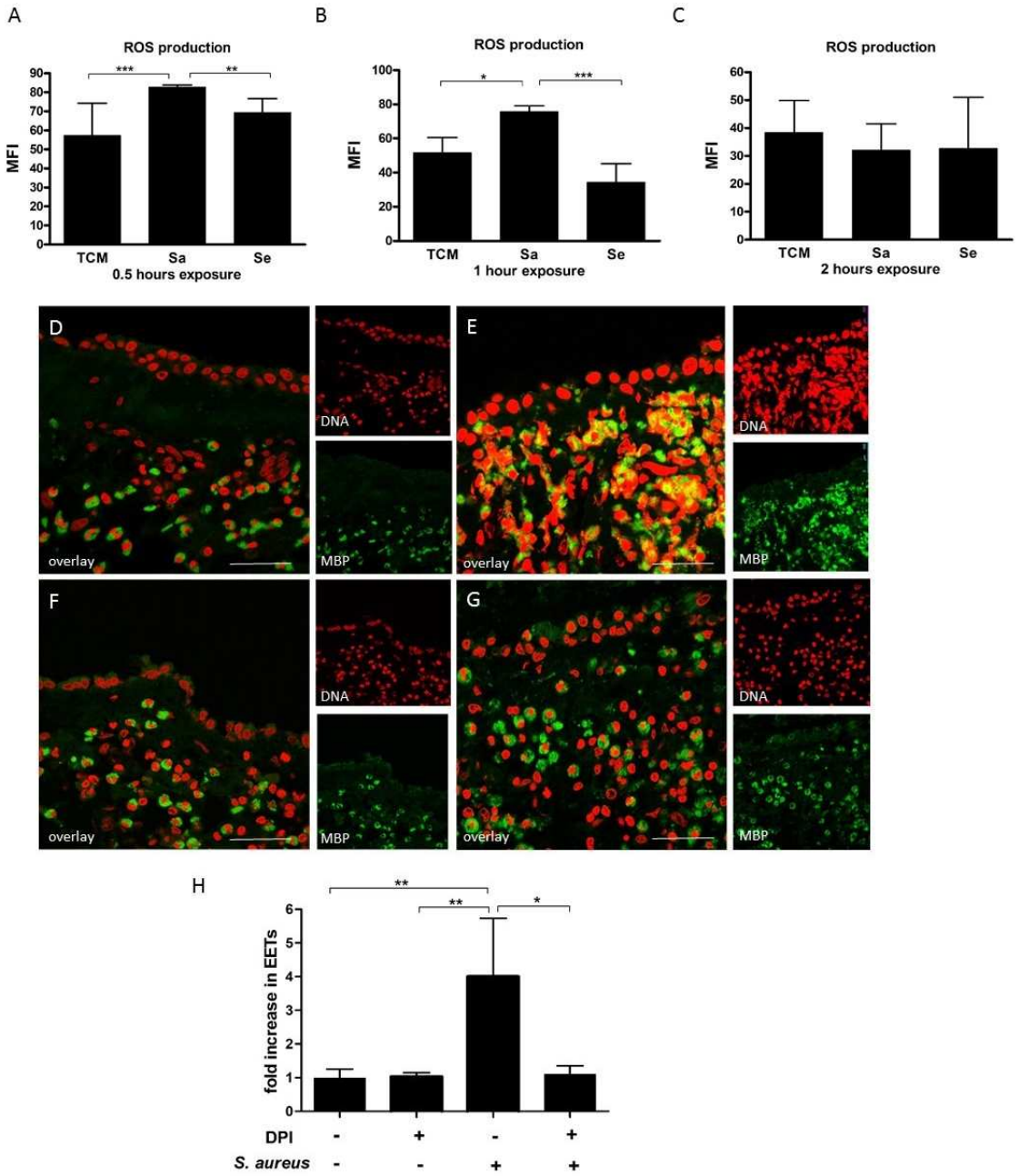
A
ROS production
MFI
TCM
Sa
Se
0.5 hours exposure
B
ROS production
MFI
TCM
Sa
Se
1 hour exposure
C
ROS production
MFI
TCM
Sa
Se
2 hours exposure
D
E
F
G
DNA
MBP
overlay
H
fold increase in EETs
DPI
S. aureus

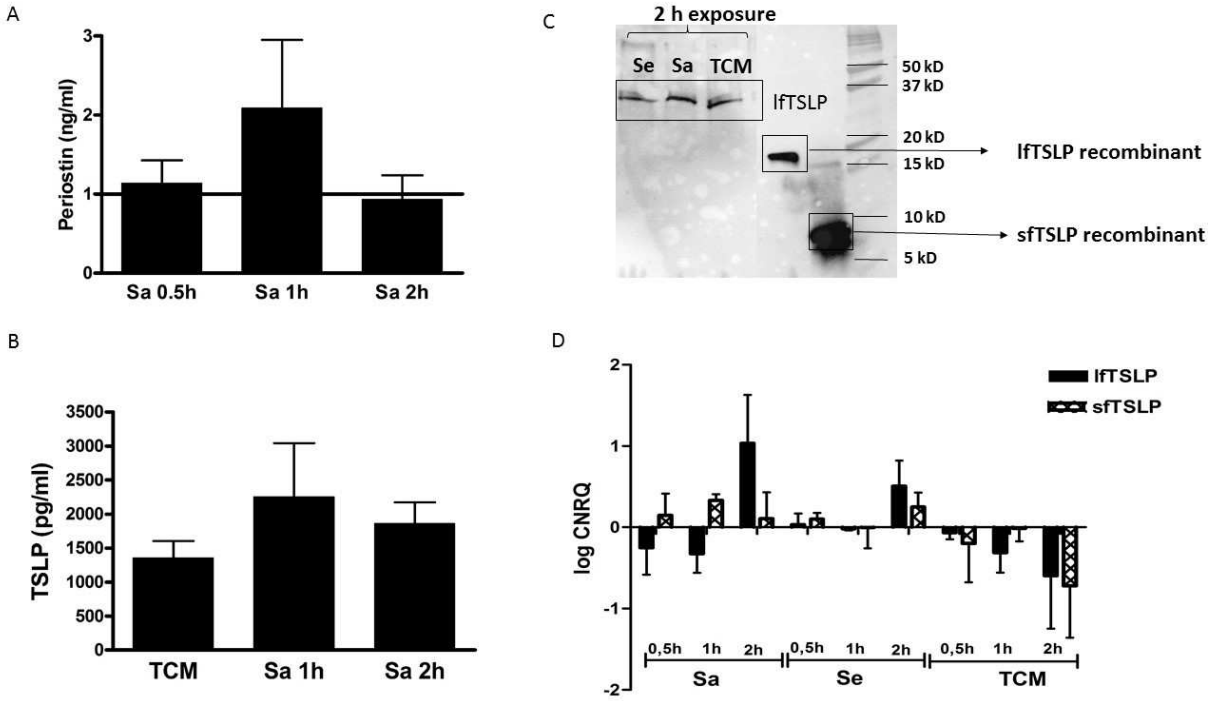

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Fig. S1 Characterization of the study population. Tissue levels of IgE (A), IL-5 (B), and ECP (C) were measured in all patients (CRSwNP, n=15) and controls (CTR, n=5) by means of ELISA and were found to be elevated in the CRSwNP patients. Eosinophils and neutrophils (D) were counted in tissue of controls and CRSwNP patients. Tissue slides stained for major basic protein in controls (E) and CRSwNP (F) showing the general distribution of eosinophils throughout the tissue (Scale bar = 100 µm). Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001

Fig. S2 Subepithelial eosinophils and damaged epithelium. *Immunohistochemistry was performed on subsequent tissue slides and the same region was selected for pictures A-B. The protein of interest is stained in red/purple, nuclei are stained with hematoxylin (blue).* (A) An immunohistochemical stain for MBP (red) shows the localization of eosinophils in polyp tissue. (Scale bar = 200 µm); (B) An immunohistochemical stain for caspase-3 (red) demonstrates very few apoptotic cells in the tissue. Inset image (B') shows no evidence for cell lysis. (B: Scale bar = 200 µm, B': scale bar = 10 µm)

Fig. S3 (A) Relation between EET formation and number of eosinophils in the tissues. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001
(B) Periostin levels in CRSwNP patients and controls. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001 (C) Scatterplot of periostin and IL-5 levels in CRSwNP patients with a significant correlation (p=0.013, R^2=0.665), as determined by Spearman Correlation. (D) Scatterplot of TSLP and % of eosinophils forming EETs with no correlation as determined by Spearman Correlation.

Fig. S4 (A) Relation between IL-5 and percentage of degranulated eosinophils. (B) Relation between periostin and percentage of degranulated eosinophils. (C) Relation between number of eosinophils and percentage of degranulated eosinophils. (D) Relation between *S. aureus* colonization and percentage of degranulated eosinophils.

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Primer	sequence
SDHA-FW	TGGGAACAAGAGGGCATCTG
SDHA-Rev	CCACCACTGCATCAAATTCATG
EF1-FW	CTGAACCATCCAGGCCAAAT
EF1-Rev	GCCGTGTGGCAATCCAAT
TSLP short form FW	CGTAAACTTTGCCGCCTATGA
TSLP short term Rev	TTCTTCATTGCCTGAGTAGCATTTAT
long form TSLP - FW	GGGCTGGTGTTAACTTACGACTTCA
long form TSLP - Rev	ACTCGGTACTTTTGGTCCCACTCA

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Investigated feature		CRSwNP (n/N)	Controls (n/N)
Degranulated eosinophils	< 1% eosinophils/not present	5/15	5/5
	< 10 % eosinophils	6/15	0/5
	< 20 % eosinophils	4/15	0/5
***S. aureus* presence**	Not present	7/15	4/5
	Planktonic < 2 spots	4/15	1/5
	Planktonic > 2 spots no biofilm	1/15	0/5
	Biofilm	3/15	0/5

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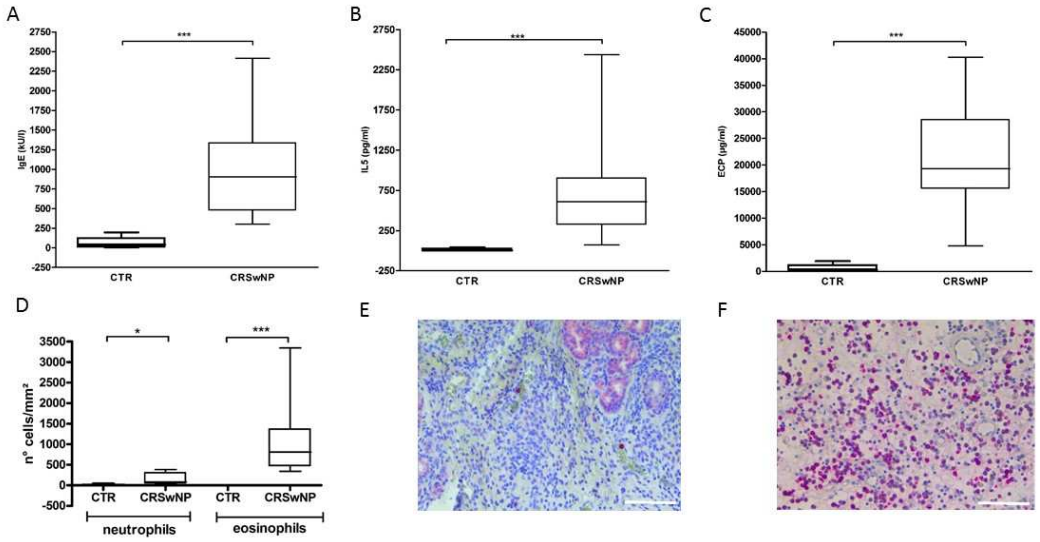

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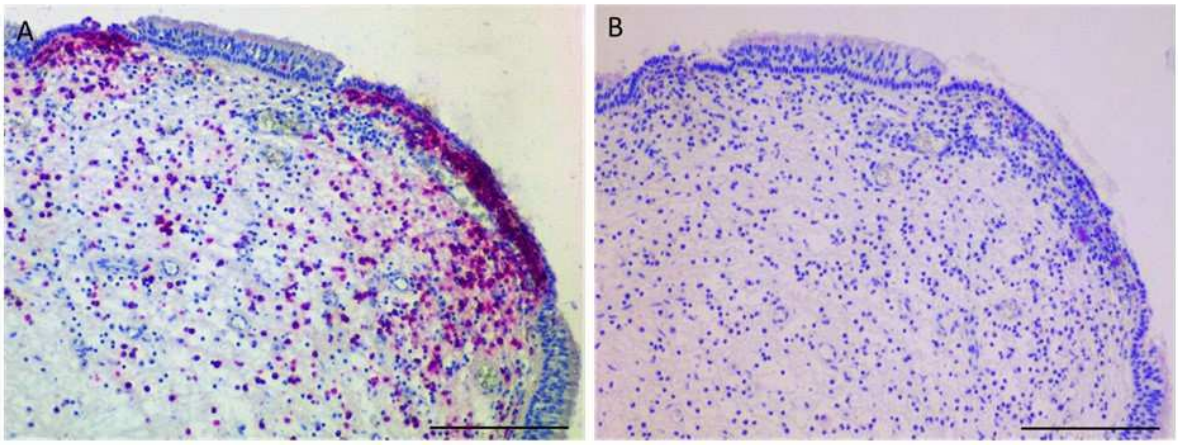

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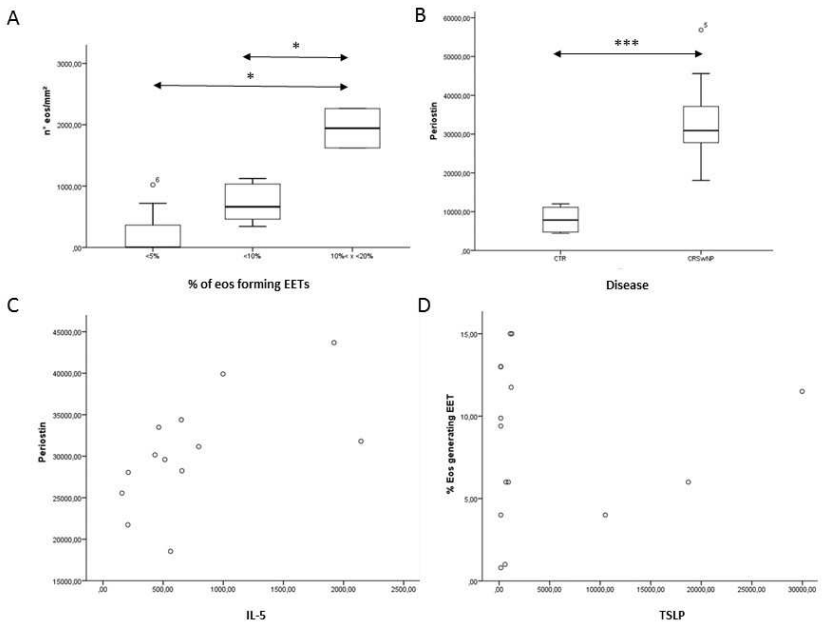

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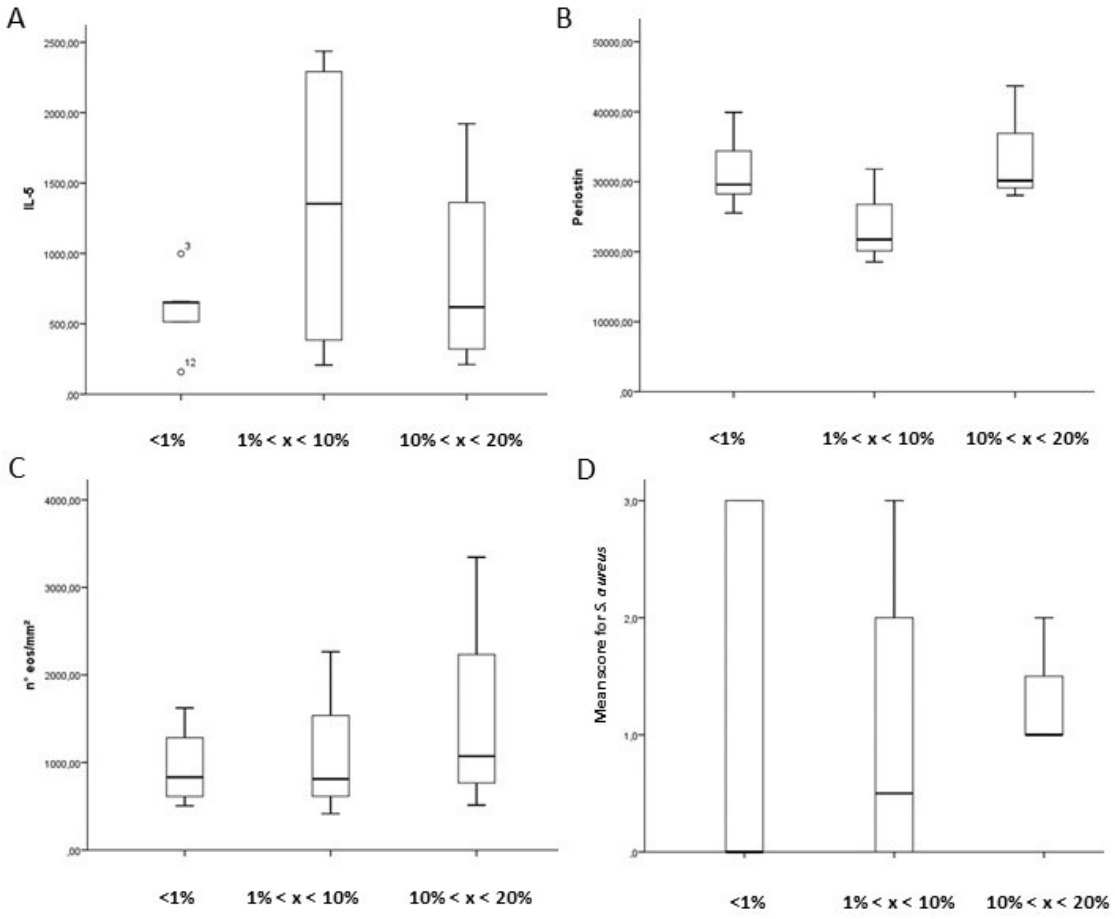
A
IL-5
2500,00
2000,00
1500,00
1000,00
500,00
,00
3
12
<1%
1% < x < 10%
10% < x < 20%
B
Periostin
50000,00
40000,00
30000,00
20000,00
10000,00
,00
<1%
1% < x < 10%
10% < x < 20%
C
n° eos/mm²
4000,00
3000,00
2000,00
1000,00
,00
<1%
1% < x < 10%
10% < x < 20%
D
Mean score for S. aureus
3,0
2,0
1,0
,0
<1%
1% < x < 10%
10% < x < 20%

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