# Online supplementary material

**(Differential release and deposition of S100A8/A9 proteins in inflamed upper airway tissue)**

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# METHODS

## Patients

Tissues, sera and nasal secretions were obtained at the Department of Otorhinolaryngology, Ghent University Hospital, Belgium during routine endonasal sinus surgery and approved by the local ethical committee. All patients gave their written informed consent before collecting material. All patients stopped oral and topical application of corticosteroids for at least 1 month before surgery. The diagnosis of chronic rhinosinusitis with nasal polyps was based on history, clinical examination, nasal endoscopy, and computed tomography (CT) of the paranasal cavities according to the current European[1, 2] and American Guidelines.[3] Nasal tissue (inferior turbinates) of subjects without chronic rhinosinusitis undergoing septoplasty because of anatomical variations or trauma served as controls. The patients' atopy status was assessed by means of a skin prick test (SPT) to common aeroallergens (histamine; positive control, saline; negative control, hazel, alder, birch, plane, cipres, grasses, olive, Artemesia, Ambrosia, Alternaria Alternata, Cladosporium, Aspergillus, . Parietaria, cat, dog, Derm. Pteronyssinus, Derm. Farinae, Blatella). CT scans were graded according to the Lund-MacKay method, yielding a final score that could range from 0 (complete lucency of all sinuses) to 24 (complete opacity of all sinuses).[4] Polyps were graded by size and extent in both the left and right nasal fossa on a scale of 0 to 3 (no symptoms, mild, moderate, severe), according to the Davos classification, and summed for both sides to give a bilateral score ranging from 0 to 6.[5] The patient groups' full clinical profile can be found in Table 1 of the main document. In Table E1 of the online supplementary material, the clinical data of the patients are provided in separate groups arranged per sample type (tissue, serum, nasal secretions).

## Nasal secretions

Nasal secretions were obtained by means of sinus packings (IVALON 4000 plus; 4.5 x 1.5 x 2.0 cm, Surgical Products M-Pact, Eudora, KS) that were placed bilaterally between the inferior turbinate and the septum for 5 minutes. The quantity of secretions collected was determined by comparing the weight of the sinus packings before and after insertion. Then, 3 mL of 0.9% NaCl solution was added to a tube containing the sinus packings and left at 4°C for 2 h, after which suspensions were centrifuged at 1,500 g for 10 minutes at 4°C. Aliquots were prepared and stored at -80°C for further analysis.

**Tissue homogenates**

Snap-frozen tissue specimens were weighed and suspended in a 10 times volume of 0.9% NaCl solution with protease inhibitor Complete Roche (Mannheim, Germany). Frozen tissue fragments were pulverized by means of a mechanical TissueLyser LT (Qiagen, Hilden, Germany) at 50 oscillations per second for 2 minutes in pre-chilled eppendorfs. The tissue homogenates were centrifuged at 15000 rpm for 5 minutes at 4°C and the supernatants were stored at –20°C until further analysis.

**S100A8, S100A9 and S100A8/A9 ELISA**

Commercially available ELISA kits (BMA Biomedicals, Augst, Switserland) were used to measure S100A8, S100A9 and S100A8/A9 protein levels. The S100A8 ELISA uses a combination of two discriminating monoclonal antibodies (clones 8-5C2 and S13.67), while the S100A9 ELISA uses clones S36.48 and S32.2, recognizing different epitopes on the same subunit, and designed in such a way that it yields high specificity for S100A8 and S100A9 proteins respectively, recognizing monomeric (an unstable form that does not occur *in vivo*) and homodimeric proteins but not the heterodimeric form. The heterodimeric S100A8/A9 ELISA uses a monoclonal antibody (clone 27E10) that recognizes an epitope that is only present on the heterocomplex.

As a proof of concept, some of our results could indeed confirm the selectivity of these ELISA kits; for example, non-selective detection of heteromeric S100A8/A9 proteins in human sinosnasal tissue homogenates with the S100A8 and S100A9 ELISA kits can be excluded because in the ex-vivo experiments on isolated human granulocytes -where we applied S aureus- we measured a mean concentration of approximately 110µg/ml S100A8/A9 with the S100A8/A9 kit (Fig E7; samples were 1000 times diluted to fit within the upper concentration range being to 200 ng/ml for this kit), while all these samples (undiluted) were below the detection limit of 0.3 ng/ml in both the S100A8 and S100A9 ELISA kit (results section in main document). A similar situation is true for the human upper airways tissue experiments as shown in Fig 6 A and B. The S100A8/A9 proteins that are present in the supernatants (panel A, left part) were not measurable with the S100A9 ELISA kit (panel B, left part) and S100A8 ELISA kit (reported in the results section of the main document and in the legend of Fig 6). The results on the tissue pellets from the same data set and from the data on serum and nasal secretions also show that the S100A8 ELISA kit did not detect S100A9 because no proteins at all could be measured with this kit in these samples (reported in the

results sections of the main document and the online supplementary material) while they clearly contain S100A9 proteins (Fig 6 B, right part and Fig E1, A).

**S100A8, S100A9 and S100A8/A9 immunohistochemistry**

Human sinonasal tissues were fixated in formalin (Fluka, Sigma-Aldrich, Bornem, Belgium) and embedded in paraffin. Paraffin sections (4-5 μm) were air-dried for 24 hours at 37°C. After deparaffinization in parasolve, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase in TRIS-buffered saline (TBS; pH 7.8) containing 0.001% NaN3 for 20 minutes at room temperature. For frozen section, sections were fixed in ice-cold acetone for 10 min and allowed to dry for 2 h. Both the frozen acetone-fixed and the deparaffinized formaline-fixed sections were washed with TBS for 10 minutes before being incubated overnight at 4°C with monoclonal Abs against S100A8 (clone S13.67; BMA Biomedicals, Augst, Switserland), S100A9 (clone IDCP1; Santa Cruz Biotechnology, Santa Cruz, CA) and S100A8/A9 (clone 27E10; BMA Biomedicals), or with polyclonal Abs against S100A8 and S100A9 (generated by Dr T. Vogl, Institute of Immunology, University of Muenster), or the respective isotype controls mouse $IgG_1$ (clone DAK-GO1; Dako, Glostrup, Denmark) or rabbit IgG (Dako). All monoclonal Ab were used at a final concentration of 5μg/ml TBS/5%BSA on paraffin section and at 1μg/ml on frozen sections, except for clone IDCP1 against S100A9, which was used at 0.025 μg/ml on frozen sections. The polyclonal Abs were used at 2μg/ml TBS/5% BSA on paraffin sections and at 1μg/ml on frozen sections. Next, the slides were washed for 10 minutes in TBS. Expression was detected using the LSAB+ technique conjugated with peroxidase according to the Manufacturer's instructions (labeled streptavidin-biotin; Dako). The peroxidase activity was detected using AEC Substrate chromogen (Dako), which results in a red stained precipitate. Finally, sections were counterstained with Hematoxylin for 2 minutes, washed in running tap water, and mounted in Aquatex (VWR International). In paraffin sections, double stainings were also performed with the monoclonal Ab against S100A9 after an antibody against MPO (rabbit IgG; 3μg/ml; Dako); for MPO, the peroxidase activity was detected using DAB+ Substrate chromogen (Dako) which results in a brown stained precipitate. For S100A9, New Fuchsin (Dako) was used, yielding red stainings.

Quantitative evaluation of the S100 expression level was performed by means of Image J software 1.48v (developed at the National Institutes of Health). Five equal squares on one cryo-slide without hematoxylin counterstaining were analyzed for S100 protein staining intensity and extent; the average was calculated per slide. Six slides per subject group were

assessed in such a way; the means of these data yielded the final results as presented. The data are expressed as arbitrary protein expression levels presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median.

**S100A8 and S100A9 gene expression analysis**

Total RNA was extracted using an Aurum Total RNA Mini Kit (Bio- Rad Laboratories). One µg of total RNA was reverse-transcribed to generate first-strand cDNA using an iScript cDNA Kit (Bio-Rad Laboratories). Two µl of a 5 times dilution of each cDNA reaction mix was used as a template for real-time PCR amplification performed on a LightCycler LC480 System (Roche) in the presence of 250 nM of forward and reverse primers, and 1X PCR Master mix (Roche) in a total reaction volume of 5 µl. The thermal cycling conditions were as follows: 95°C for 1 min, followed by 45 cycles of 3 s at 95°C, 30 sec at 60°C and 1 s at 72°C, and a dissociation curve analysis from 60°C to 95°C. β-actin (ACTB), hypoxanthine phosphoribosyltransferase 1 (HMBS-1) and elongation factor 1 (EF-1) were used as endogenous reference for normalization. Primer sequences for the ACTB and HMBS-1 were obtained from the public Real-Time PCR Primer and Probe Database of the Department of Medical Genetics, University of Ghent (http://medgen.ugent.be/rtprimerdb; RTPrimerDB ID1 for ACTB and ID4 for HMBS-1. The primers sequences (forward and reverse) for EF-1 are: CTGAACCATCCAGGCCAAAT and GCCGTGTGGCAATCCAAT; for S100A8: GCCAAGCCTAACCGCTATAA and CCCACCAGGTCTTCTGAAAG; for S100A9: GGCTTTGACAGAGTGCAAGA and AGGTGTTGATGATGGTCTCTATG. Relative changes in gene expression were determined based on the comparative $C_t$ method as described before.[6]

**Human sinonasal *ex-vivo* tissue-cube fragment stimulation assay**

Fresh tissue fragments of human nasal polyps (+/- 0.9 $mm^3$) of CRSwNP patients were suspended as 0.04 g tissue-cubes/ml tissue culture medium (TCM) into 48-well plates in a total volume of 0.5 ml/well. Individual wells received 1 or 5 µg/ml human S100A8, S100A9 or S100A8/A9 proteins (provided by Dr T. Vogl, Institute of Immunology, University of Münster; see this article's online supplementary material for more detailed information) in the absence or presence of either a RAGE antibody that blocks the receptor-ligand interactions (10µg/ml; clone 176902; R&D systems, Minneapolis, Minn), a TLR4 functional blocking antibody (2µg/ml; clone HTA125; Abcam, Cambridge, UK), or the respective isotype

controls mouse IgG2b (clone 20116; R&D systems) and mouse IgG2a (clone MG2a-53; Abcam). The culture medium supernatants from the tissue-cube fragment stimulation assays were snap-frozen in liquid nitrogen and stored at -20°C until cytokine measurement.

In a separate set of experiments, where supernatants were sampled for S100A8/A9 measurements, the tissue-cubes were cultured in the absence or presence of either 2 µg/ml anti-human CXCL8/IL-8 antibody (R&D systems) in order to neutralize IL-8 bioactivity or a normal goat IgG control (R&D systems), $10^5$ colony forming units (cfu) of a clinical isolate of living or heat-killed frequent colonizers of the upper respiratory tract *Staphylococcus aureus* (*S. aureus*), or the bacterial products *S. aureus* enterotoxin B (SEB; 0.5µg/ml; Sigma-Aldrich, Bornem, Belgium), Lipoteichoic acid (LTA; 5 µg/ml) from *S. aureus* (Sigma-Aldrich) for 24 h. The experiments with living *S. aureus* were also performed in the absence and presence of the *de novo* proteins synthesis inhibitor cycloheximide (10 µg/ml), the endoplsmatic reticulum to Golgi complex transport inhibitor brefeldin A (100 ng/ml), the tubulin polymerization inhibitor demecolcine (1µM), and the actin polymerization/reversible phagocytosis inhibitor cytochalasin B (5µg/ml).

**Human S100A8, S100A9 and S100A8/A9 proteins**

S100A8/A9 proteins were isolated from human granulocytes, while the homodimers are recombinant proteins that were purified from *E. coli*.[7] The proteins were dialyzed against HBS-buffer in the presence of 0.01 mM DTT to avoid formation of artificial disulfide bridges. Possible endotoxin contaminations were excluded by a Pyrogent Plus Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD) and blocking experiments using 10µg/ml polymyxin B (PMB) sulfate (Sigma-Aldrich). Human nasal polyp tissue fragments were furthermore challenged with 1 ng/ml lipopolysaccharide (LPS) in the absence and presence of 10µg/ml PMB in order to assess the efficacy of PMB.

Tissue cube supernatants of above experiments were assayed for IL1β and TNFα levels by means of Luminex xMAP technology using the Fluorokine MAP Multiplex Human Cytokine Panel A kit (R&D Systems) on a Bio-Plex™ 200 Array Reader (Bio-Rad, Hercules, CA, USA).

**PBMC and granulocyte isolation**

Whole blood was routinely centrifuged over a Biocoll separating solution (Biochrom AG, Berlin) according to the manufacturer's guidelines. PBMCs were collected from the interphase. The bottom layer, containing granulocytes, was further treated by sequential hypotonic saline washes until all of the erythrocytes present were lysed.

# RESULTS

## S100A8, S100A9 and S100A8/A9 protein levels in serum and nasal secretions of CRSwNP and tissue of CRSsNP

S100A8/A9 was found to be significantly more present in serum (Fig E1, A) and nasal secretions (Fig E1 B) of CRSwNP patients than in controls. In serum and nasal secretions, the median levels of S100A9 were not significantly different from those in controls (8.52 ng/ml; range 4.99-19.89 ng/ml vs. 7.17 ng/ml; range 3.95-10.87 ng/ml and 333.03 ng/ml; range 120.68-819.81 ng/ml vs. 368 ng/ml; range 121.90-851.97 ng/ml, respectively) while the levels of S100A8 were too low to yield trustable data for analysis (data not shown).

S100A8 and S100A9 levels in ethmoidal tissue from CRSsNP patients were not significantly different from those in controls (median 70.2 ng/ml; range 0.0-668.3 ng/ml vs. 55.0 ng/ml; range 0.0-499.5 ng/ml and 1247 ng/ml; range 236.7-4340.2 ng/ml vs. 1089 ng/ml; range 143.2-4171.6 ng/ml, respectively) while S100A8/A9 levels were significantly increased compared with control inferior turbinate (median 59.8 µg/ml; range 19.9-137.6 µg/ml vs. 37.0 µg/ml; range 4.5-133.9 µg/ml, $p<0.05$) but remaining significantly lower than observed in CRSwNP tissue (Fig 1 C, $P<0.01$).

## S100A8, S100A9 and S100A8/A9 immunohistochemistry

In contrast with the very pronounced S100A9 immunoreactivity of the ECM structures in human nasal polyps tissue from CRSwNP patients (Fig 3, C in this article's main document), acetone-fixed cryo-sections showed no or very locally limited S100A9 immunoreactivity in ethmoidal tissue of CRSsNP patients (Fig E2, A). Similar to CRSwNP (Fig 3, $E_1$ and $F_2$ in this article's main document), in formalin-fixed paraffin sections from CRSsNP tissue, the same antibody against S100A9 clearly stained neutrophils (Fig E2, B).

S100A8/A9 stainings with a monoclonal Ab (clone 27E10) only worked in acetone-fixed sections, showing almost no immunoreactivity in control tissue (Fig E2, C), while staining both ECM structures and inflammatory infiltrates in CRSwNP tissues (Fig E2, $D_1$) but only inflammatory cells in CRSsNP tissues (Fig E2, $D_2$).

Mouse $IgG_1$ isotype and rabbit IgG controls (data not shown) yielded no immunosignals in both cryo (Fig E2, $E_1$) and paraffin (Fig E2, $E_2$) sections of human polyp tissue.

**Human S100A8, S100A9 and S100A8/A9 proteins**

The endotoxin levels in the recombinant S100 proteins were confirmed to be extremely low and were at least less than 5 pg LPS/1 µg S100 protein. Any functional effect potentially mediated by traces of LPS in our assay was furthermore excluded as the proinflammatory responses by 5µg/ml S100A8 and S100A9 were not different in the absence or presence of 10 µg/ml PMB (Fig E5). On the other hand, 10 µg/ml PMB abolished the effects of 1 ng/ml LPS almost completely (Fig E6) on IL1β and TNFα release. Because 1 ng/ml LPS induced a response that was comparable in magnitude to the response by 5µg/ml S100A8 and 5µg/ml S100A9 on IL1β and TNFα levels, while it exceeds at least 20 times the maximal possible LPS contamination that can be present in 5µg/ml S100 proteins, furthermore indicating that a functional effect by traces of LPS in our assay can be excluded.

## FIGURE CAPTIONS

FIG E1. Quantification of S100A8/A9 protein levels in serum **(A)**, and nasal secretions **(B)** by means of ELISA in controls and CRSwNP. Values are expressed as µg/g tissue or µg/ml serum/nasal secretion and presented as scatter plots showing the median as a line. *P < 0.05; ***P < 0.001. The corresponding patients' clinical data can be found in Table 1 of the main document and table E1 of this online supplementary material.

**FIG E2.** Immunolabeling on acetone-fixed cryo-sections **(A, $F_1$)** and paraffin sections **(B, C, D, E, $F_2$)** with antibodies directed against S100A9 in ethmoidal mucosa from a CRSsNP patient **(A and B)** and against the S100A8/A9 heteromer **(C and D)** in inferior turbinate tissue (IT) from a control subject **(C),** in a nasal polyp (NP) from a CRSwNP patient **($D_1$)**, and in ethmoidal mucosa from a CRSsNP patient **($D_2$).** Isotype $IgG_1$ on cryo **($E_1$)** and paraffin **($E_2$)** sections showed no staining. (magnification x 200).

**FIG E3.** S100A8 **(A)** and S100A9 **(B)** mRNA expression in sinonasal tissue of controls (inferior turbinates) and CRSwNP (nasal polyps) patients. Data are expressed as normalized relative copy number and presented as scatter plots showing means as a line with standard error of the mean.

**FIG E4.** Effect of exogenously added 5µg/ml S100A8, S100A9 and S100A8/A9 proteins on the release of IL-6 **(A)**, TGF-β **(B)** and ECP **(C)** from human nasal polyp tissue in an ex-vivo tissue assay. Data are presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, and the median (n=6).

**FIG E5.** Potential functional effects mediated by traces of LPS in our ex-vivo CRSwNP tissue responses were excluded as IL-1β **(A and B)** and TNF-α **(C and D)** release by 5µg/ml S100A8 **(A and C)** and S100A9 **(B and D)** were not different in the absence or presence of 10 µg/ml polymyxin B (PMB). Data are presented as Box-and-Whisker plots showing the minimum and the maximum value, the lower and the upper quartile, the median (as a line), and the mean (as a cross) (n=6).

**FIG E6.** 10 µg/ml polymyxin B (PMB) abolished the effects of 1 ng/ml LPS on IL-1β and TNF-α release. Data are presented as Box-and-Whisker plots showing the minimum and the

maximum value, the lower and the upper quartile, the median (as a line), and the mean (as a cross) (n=6). *P < 0.05; **P < 0.01.

**FIG E7.** Effect of exogenously added $10^5$ cfu *S. aureus* on the release of S100A8/A9 proteins (left axis) and on cell death assessed as the level of lactate dehydrogenase (LDH) in the supernatants (right axis) from isolated human granulocytes ($2.10^6$ cell/ml). HI means heat inactivated *S. aureus*. Data are means ± S.E.M. (n=5). **P < 0.01.

**FIG E8.** Evaluation of the effect of $10^5$ cfu *S. aureus* on cell death in our *ex-vivo* tissue assay, assessed as levels of lactate dehydrogenase (LDH) in the supernatants. HI means heat inactivated *S. aureus*. Data are means ± S.E.M. (n=6-7).

**FIG E9.** Effect of inhibition of *de novo* proteins synthesis (10 µg/ml cycloheximide), endoplasmic reticulum-Golgi complex protein transport (100 ng/ml brefeldin A), tubulin polymerization (1µM demecolcine), and actin polymerization (5µg/ml cytochalasin B) on the release of S100A8/A9 induced by $10^5$ cfu *S. aureus* in a human nasal polyp tissue *ex-vivo* tissue assay. Data are means ± S.E.M. (n=7). # P < 0.05 vs. baseline without *S. aureus*; *P < 0.05 vs. baseline with *S. aureus*.

# REFERENCES


1 Fokkens W, Lund V, Mullol J. European position paper on rhinosinusitis and nasal polyps 2007. *Rhinol Suppl* 2007; 1-136.

2 Thomas M, Yawn BP, Price D, Lund V, Mullol J, Fokkens W. EPOS Primary Care Guidelines: European Position Paper on the Primary Care Diagnosis and Management of Rhinosinusitis and Nasal Polyps 2. *Prim Care Respir J* 2008 Jun; 17: 79-89.

3 Meltzer EO, Hamilos DL, Hadley JA, *et al*. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol* 2004 Dec; 114: 155-212.

4 Lund VJ, Kennedy DW. Staging for rhinosinusitis. *Otolaryngol Head Neck Surg* 1997 Sep; 117: S35-S40.

5 Fokkens W, Lund V, Bachert C, *et al*. EAACI Position Paper on Rhinosinusitis and Nasal Polyps Executive Summary. *Allergy* 2005 May 12; 60: 583-601.

6 Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. *Methods* 2001 Dec; 25: 402-408.

7 Vogl T, Leukert N, Barczyk K, Strupat K, Roth J. Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. *Biochim Biophys Acta* 2006 Nov; 1763: 1298-1306.