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Association between aryl hydrocarbon receptor and 4-hydroxynonenal in oxidative stress-mediated chronic rhinosinusitis with nasal polyps

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Abstract

Objective: Chronic rhinosinusitis with nasal polyps (CRSwNPs) is a distinct entity within the chronic rhinosinusitis group of diseases, which are chronic upper airway diseases with several pheno- and endotypes. Oxidative stress plays an important role in the pathogenesis of CRSwNPs. The aim was to assess the association between expression of the aryl hydrocarbon receptor (AhR) and 4-hydroxynonenal (4-HNE) in patients with CRSwNPs.

Methods: The study included 26 patients who underwent endoscopic sinus surgery: Fourteen patients with CRSwNPs, and 12 controls with healthy sinus mucosa. Expression of AhR and 4-HNE was assessed in tissue samples using immunohistochemistry. The level of 4-HNE in serum samples was measured using the ELISA assay. Total oxidative capacity (TOC) was assessed by measuring the peroxidase activity.

Results: Higher levels of 4-HNE expression were observed in tissues (3, range 1–3 vs. 0, range 0–0 $p<0.001$) and serum (27.7±11.5 vs 9.8±7.7 pmol/mg, $p < 0.001$) samples of CRSwNPs patients, as compared to healthy controls. Higher expression of AhR was found in inflammatory cells (plasma cells, lymphocytes, eosinopholes) of CRSwNPs patients, compared to controls (3, range 1–3 vs. 2, range 1–2, $p = 0.001$). There were no differences in TOC across groups (0.0285±0.0207 vs 0.02, 978±0.0197 μM H_2O_2 eq., $p = 0.848$). Patients with bronchial asthma (57%) had abundant eosinophil in tissue samples. Patients with recalcitrant CRSwNPs had higher 4-HNE serum levels, compared to non-recalcitrant cases (27.3 vs 24.2 pmol/mg, $p = 0.339$).

Conclusion: Patients suffering from CRSwNPs have oxidative stress–mediated overexpression of AhR, which is linked to a chronic inflammatory response in the paranasal sinus tissues.

Keywords

4-hydroxynonenal, aryl hydrocarbon receptor, chronic rhinosinusitis, oxidative stress, reactive oxygen species

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Introduction

Chronic rhinosinusitis (CRS) is a complex disease with several distinct phenotypes, each of them with a distinct underlying pathophysiology. It is generally agreed upon that there exist clinically relevant CRS phenotypes defined by an observable characteristic or feature, such as the presence or absence of nasal polyps.[1]

Reactive oxygen species (ROS) are highly protective molecules generated during the normal cellular metabolism, or from external sources.[2] When the level of ROS production exceeds the capacity of cellular antioxidants, an oxidative stress occurs. A study on the impact of second-hand smoking (SHS) on ROS generated in CRS patients has shown that SHS increases levels of ROS in paranasal sinus tissues and that these levels vary across CRS subtypes.[3] It has been shown that various effects of long-term uncontrolled oxidative stress are directly linked with the onset of numerous inflammatory cascades that reflect the up-regulation of inflammatory cytokines through different molecular pathways and with triggering of the intrinsic apoptotic pathway and caspase activation. Uncontrolled inflammatory response results in an extensive cell and tissue damage, giving rise to tissue destruction associated with chronic inflammation.[4,5]

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that has recently been identified as the critical physiological regulator of the immune response, capable of affecting both the innate and adaptive immunity.[6,7] Since the AhR signalling pathway represents an important link between environmental stimulators and immune-mediated inflammatory disorders, it has recently become an issue of great research interest.

It has recently been shown that low expression of AhR in nasal polyp tissues can result in low expression of IL-22 in chronic rhinosinusitis with nasal polyps (CRSwNPs) patients, especially in recalcitrant cases, suggesting that the nasal respiratory epithelium is more severely affected in CRSwNPs as compared to other CRS subtypes, and emphasising the importance of the host immune system in these patients.[8,9] 4-hydroxy 2-nonenal (HNE) is one of the most abundant cytotoxic lipid-derived aldehyde and its biological activity is dependent on its intracellular concentration.[10] Lower intracellular levels (< 2 μM) elicit beneficial effects on cell survival and proliferation. Higher levels (10–60 μM) have genotoxic effects, since they lead to sister chromatid exchange, micronuclei formation and DNA fragmentation. At micromolar concentrations, HNE has consistently been shown to upregulate the expression of transcription factors such as Nuclear Factor Kappa B (NF-kB), which in turn regulates the expression of a variety of genes involved in cell proliferation and differentiation, such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). On the contrary, at higher concentrations, HNE has been shown to inhibit the formation of NF-kB, thereby exerting regulatory effects on inflammatory processes, mediated via intracellular concentration. Due to high binding affinity for cysteine, histidine and lysine, HNE forms relatively stable and hardly metabolized protein adducts.[11] Thus, peroxidation products are identifiable even after a long time. Findings of a recent study showed that HNE protein products are valuable bioactive markers of carcinogenesis in squamous cell carcinoma of the oropharynx.[12]

Despite its important role in the host innate and adaptive immunity, the contribution of oxidative stress to AhR expression in CRSwNP remains unclear. The aim was therefore to investigate the association between the expression of AhR and 4-HNE in CRSwNPs patients.

Patients and methods

Patients

The sinus mucosa samples of 26 patients who underwent endoscopic sinus surgery at the University hospital of Zagreb, Zagreb, Croatia were analysed at the Department of Molecular Medicine, Laboratory for Oxidative Stress, Ruder Bosković Institute. There were two subgroups of patients: cases were 14 patients suffering from CRSwNPs, and controls were 12 patients who underwent sinus surgery for reasons other than pathology of paranasal sinus. The study was granted approval by the Institutional Review Board and all patients signed an informed consent for participation in the study.

Blood was drawn from each patient (5 mL) and centrifuged at 3600 r/min for 15 min. The obtained serum was stored at −20°C. All patients who participated in the study had diffure CRS, as defined in the new EPOS 2020 CRS classification. All paranasal sinus mucosal samples had identical pathomorphological features typical of chronic inflammation. Tissue samples of the ethmoid and sphenoid sinus mucosa were harvested at the Department of ENT and Head and Neck Surgery, University hospital Zagreb, Zagreb, Croatia. A questionnaire was administered to all patients, to ask on allergies and smoking history, previous sinus surgery, sensitivity to NSAIDs and co-morbidities. Exclusion criteria were taking oral corticosteroids during past three months, cystic fibrosis, antrochoanal polyps and primary ciliary dyskinesia.

Assessment of expression of the oxidative stress-induced lipid peroxidation marker four-hydroxynonenal (4-HNE) in tissue samples

Fresh tissue samples were frozen at −80°C. Prior to immunohistochemistry all tissue samples were stained by haematoxylin and eosin. We are grateful to Prof. G. Waegu,

University of Graz, Austria, for the donation of the HNE-histidin antibody. Immunohistochemistry was performed to assess 4-HNE expression, using a monoclonal antibody in accordance with the manufacturer's instructions (Abcam, USA). The procedure uses the EnVision-HRP method. The Anti-HNE (monoclonal antibody) is diluted 1:10 in 1% bovine serum albumin (BSA) (in TBS solution) over night. The slides are rinsed 3 times for 5 minutes each in a TBST solution and then incubated for 30 min in ENVISION (Dako EnVision+System-HRP, Labelled Polymer Anti Mouse). The blocks are cut in 4 μm cuts and reviewed under the microscope with 200/400/1000 magnification. The inflammatory cells were delineated based on morphological features, as identified on the microscopical images.

Assessment of the lipid peroxidation marker 4-hydroxynonenal (4-HNE) in serum samples

Collected serum samples stored at −20°C were defrozen and then serum 4-HNE levels were measured using the ELISA assay. On the first day, the samples were rinsed five times each within 30 min in 200 μl wash buffer (0.1% Tween 20 in PBS and 200 μl peroxidase blocker). Then 50 μl H_2O_2 in 1 mL of solution was added (ex. 1 plate = 20 mL + 1 mL H_2O_2). Finally, a stop solution (50 μl 2M H_2SO_4) was added, then OD was measured at 450/620 nm. On the second day, protein concentration of standards was measured at 10 mg/mL (HNE-BSA standards range from 0 to 250 pmol of HNE-protein adducts/mg of proteins (0; 3,75; 7,5; 15; 30; 60; 120; 250 pmol/mg). Plasma/serum protein concentration was first measured using the Bradford method and adjusted to 10 mg/mL. All analyses were performed in triplicate or quadruplicate and the amounts of HNE-protein adducts measured by the ELISA were expressed as pmol HNE/mg of proteins. To eliminate sample background values, one well of each sample was incubated with 1% BSA in PBS (without primary antibody).

Assessment of AhR protein expression in tissue samples

Fresh tissue samples were frozen at −80°C, defrozen and then used to assess AhR protein expression by immunohistochemistry using rabbit polyclonal AhR antibodies in accordance with the manufacturer's instructions (Antibodies, USA).

The Anti-ACR-Lys 20 μg/ml mouse monoclonal antibody was diluted 1:10, then rinsed in PBS three times for 5 min each, then washed in 3% hydrogen peroxide (H_2O_2) solution for 20 min in dark and three times in TBS for 5 min each. Ab anti-mouse (Dako EnVision™ System-HRP Labelled Polymer anti-mouse) was used and the plates rinsed in TBST three times each for 5 min. The DAB (DakoLiquid DAB+Substrate Chromogen System) chromogen kit was applied until the solution turned brown.

Tissue samples from each study group were analysed under the microscope Olympus BX 51 magnified in 200/400 and 1000x using immersion oil. Positive cells number and intensity in tissue layer and cells, were divided into three categories: 0-negative positivity and intensity, 1 (0–30%) – weak positivity and intensity, 2 (30–50%) – intermediate positivity and intensity and 3 (> 50%) high positivity and intensity.[13]

4-HNE and AhR expression assessment

The assessment was performed according to criteria of intensity and number of positive cells in 1 mm^3 of tissue and graded as: 0-negative positivity and intensity; I – (0–30%) – weak positivity and intensity; II-(30–50%) – intermediate positivity and intensity; and III – (> 50%) high positivity and intensity.

Total oxidative capacity assay

Total oxidative capacity (TOC) was assessed using the peroxide reaction, as described in the literature.[14] A colorimetric/photometric fast test for the quantitative determination of peroxides in biological fluids was used. The measurement is based on a reaction between peroxides and peroxidase, followed by a colour reaction of the chromogenic substrate tetramethylbenzidine (TMB). The blue solution turns yellow after addition of the stop solution, after which a photometric measurement at 450 nm is performed. Quantification is achieved by serial dilutions of a standard peroxide solution. Per well, 10 μL of standards/controls/samples is pipetted and 200 μL Reaction mixture is added within 1 min. Optical density (OD) is measured at 450 nm (this is done because the buffer can precipitate plasma/serum proteins). The plate is incubated for 20 min, then 50 μL of the stop solution is added. The plate is left in place for 2–3 min before measurement and gently shaken to evenly distribute colour within the well. Second OD measurement at 450 nm is performed. The outcome of the assay is assessed as μM hydrogen peroxide equivalent.

Statistical analysis

Categorical variables were summarized as median (range), continuous variables as mean ± SD. Medians were compared using the Mann–Whitney *U*-test, means using the *t*-test. All tests were two sided. Power analysis was performed with serum 4-HNE levels set as outcome and analysis parameters set at: control mean = 9.8 ± 7.7; experimental group increase 100%; $\alpha = 0.05$; $\beta = 0.2$; power = 0.8. The minimum number of subjects for adequate study power was determined to be 10 per group. Alternatively,

Table 1. Patient characteristics.

	CRSwNP (n = 14)	Controls (n = 12)	p-value
Age (years)	41.9 ± 12.01	32.5 ± 14.0	0.073
Sex (female/male)	8/6	5/8	0.450
Skin prick test positive (y/n)	3/11	2/11	>0.999
Chronic obstructive lung disease (y/n)	1/13	0/12	N/A
Asthma	3/11	0/12	N/A
NSAID/aspirin sensitivity	2/12	1/11	>0.999
Previous sinus surgery (y/n)	8/6	4/9	0.252
Cigarette smoking (y/n)	4/10	3/9	>0.999
Oral corticosteroid use (y/n)	1/13	1/11	>0.999

Numbers are mean ± SD or absolute frequency; CRSwNP – chronic rhinosinusitis with nasal polyposis; y – yes; n – no; N/A – not available; NSAID – non-steroidal anti-inflammatory drugs.

4-HNE positivity was set as outcome and analysis parameters set at: incidence of high and medium positivity 86% (12/14); 60% decrease of incidence in the control group; $\alpha = 0.05$; $\beta = 0.2$; power = 0.8; the minimum number of patients per group was determined to be 13. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) Statistics software, version 25.0 (IBM Corporation, Armonk, NY, USA).

Results

Patient characteristics

Overall 14 patients with CRSwNPs and 12 controls were included in the study. There were no differences across groups with regard to age, sex, skin prick test positivity, NSAID/aspirin sensitivity, previous paranasal sinus surgey, cigarette smoking or oral corticosteroid use. In the CRSwNP group, there were cases of asthma and chronic obstructive lung disease, while there were no such cases in the control group. Patient characteristics are summarized in Table 1.

Lipid peroxidation marker 4-hydroxynonenal (4-HNE) over-expressed in tissue samples of CRSwNPs patients. Twelve tissue samples (86%) of the respiratory epithelium had grade III 4-HNE positivity; 10 samples (71%) had grade III positivity in the nuclei and grade I cytoplasmic positivity. Basal membrane and stromal positivity was grade I in 11 (78%) tissue samples. In the endothelium, grade I positive cytoplasms were seen in 10 tissues samples and four samples had nuclei grade 0–4-HNE (no staining). In the mucosal glands of eight CRwNPs samples, expression of 4-HNE was grade III in the cytoplasm and grade II in the nuclei. Plasma cells had grade III intensity in eight samples; eosinophil cells had grade III positivity in 10 samples. Nuclei of inflammatory cells were grade 0–4-HNE (no staining). One sample was identified with intraepithelial neutrophils grade III positivity.

In the control group, five samples (42%) of the respiratory epithelium were grade I positive cells were mostly in the lumen and the cilia. At the basal membrane, cytoplasm positivity was grade I in half of the samples and stromal cells were grade I positive to 4-HNE. Endothelial cell cytoplasms were grade II positivity in six tissue samples; nuclei of these cells were grade 0 4-HNE expression. No inflammatory cells were identified in control tissue samples. Exemplary 4-HNE tissue expression findings are shown in Figure 1 (haematoxylin was used as a control, negative stain).

Serum level of 4-HNE is significantly higher in CRSwNP patients

Among the CRSwNPs cases, the average concentration of 4-HNE was markedly higher, as compared to controls (27.7±11.5 pmol/mg vs. 9.8±7.7 pmol/mg, $p < 0.001$). Serum levels of 4-HNE in both groups is shown in Figure 2.

AhR protein expression is higher in tissue samples of CRSwNP patients

Nine (64%) CRSwNPs respiratory epithelium samples had grade III AhR cytoplasm positivity and grade 0 AhR (no staining) in the nuclei; respiratory cilia were grade III in five tissue samples and the remaining five samples (36%) were grade II positivity. The basal membrane was grade 0 AhR (no staining) in all tissue samples. Stromal cells were grade II AhR positivity in eight samples, and grade I in seven samples. In the endothelium, six samples had grade I cytoplasm and grade II nuclear AhR positivity. In the mucosal gland tissue, six samples had grade III cytoplasmic AhR positivity, and grade 0 nuclear AhR expression. In 9 (64%) CRSwNPs tissue samples, plasma cells had grade III AhR positivity and grade 0 nuclear expression; grade II of AhR expression was seen in cytoplasms of eosinophils and

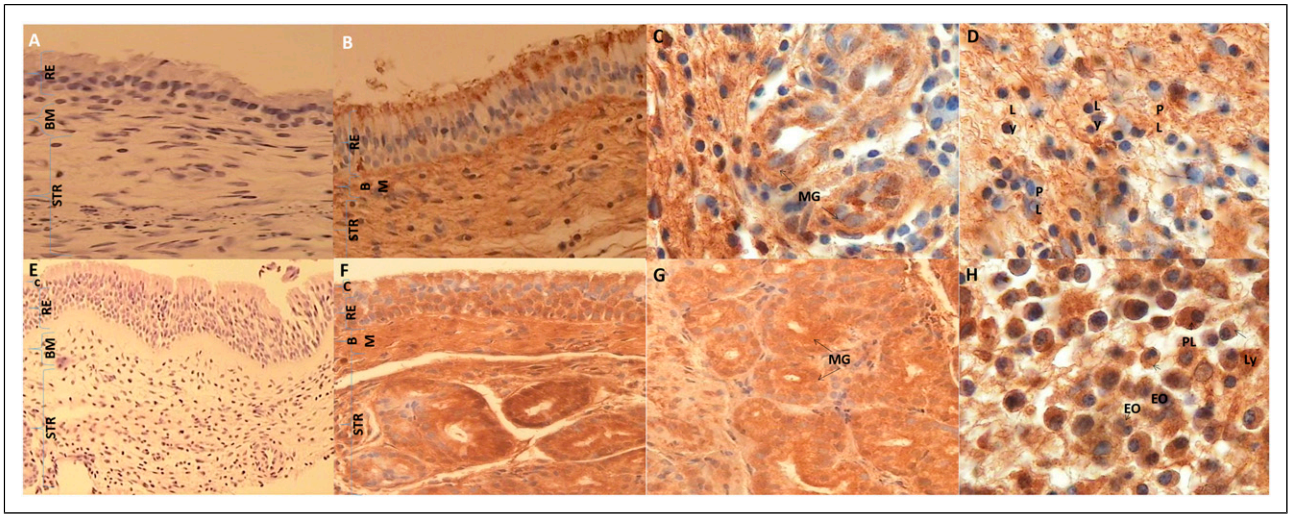

Figure 1. Healthy sinus tissue. **A** – negative control (stained by haematoxylin), C – cilia, RE – respiratory epithelium, BM – basal membrane, STR – stroma. **B** – 4HNE^{+} healthy sinus tissue layers. **C** – 4HNE^{+}, MG – mucosal glands. **D** – 4HNE^{+} cells. **E** – nasal polyp negative control (stained by haematoxylin). **F** – 4HNE $^{+}$nasal polyp tissue layers, Ly – lymphocyte, PL – plasma cell, EO – eosinophil. **G** – 4HNE^{+}, MG – mucosal glands. **H** – 4HNE^{+} cells. grade 0 – negative staining, grade I – weak positivity (0–30%) and intensity, grade II – intermediate positivity (30–50%) and intensity, grade III – high positivity (>50%) and intensity.

lymphocytes in 5 (36%) samples. Nuclei of all inflammatory cells were grade 0 AhR (no expression).

In the control group, 10 (83%) tissue samples of the respiratory epithelium had grade III AhR positive cytoplasms and grade I nuclei. All respiratory cilia had grade III AhR positivity. Basal membranes were grade 0 AhR expression and stromal cells were grade I positivity. Endothelial cells were grade III positivity in six samples and had grade II positive cells in six samples. Mucosal glands (7 samples) had grade III cytoplasmatic positivity and grade I nuclear positivity. Grade II cytoplasmic and nuclear positivity was observed in the majority of plasma cells in 8 (66%) samples. In four tissue samples, grade I AhR expression was observed in lymphocytes. Exemplary AhR tissue expression findings are shown in Figure 3.

Comparison between 4-HNE and AhR tissue expression into plasma cells of CRSwNP patients showed grade III positivity and intensity of both markers identified in nasal polyps (4-HNE+ in 57% patients and AhR+ plasma cells in 64% patients) compared to healthy controls (4-HNE^{+} 0% in plasma cells and AhR^{+}cells in 66% patients). All samples had grade II AhR positivity and intensivity in healthy controls.

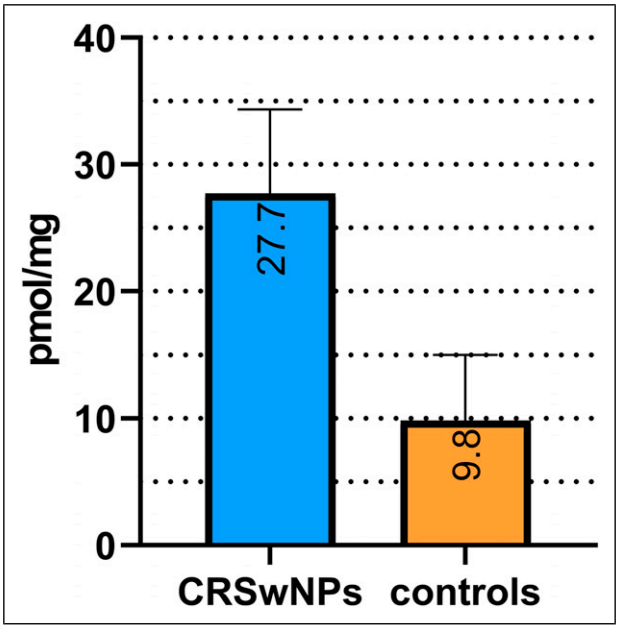

Figure 2. Serum levels of lipid peroxidation marker 4-HNE in CRSwNP patients and controls. Values in bars are means, whiskers are upper 95% CI (p<0.001, t-test).

Total oxidative capacity across groups

There were no differences in TOC across groups. In the CRSwNP group, TOC was 0.0285±0.0207 μM H_2O_2 eq., and in the controls TOC was 0.02, 978±0.0197 μM H_2O_2 eq. (p = 0.848). The comparison is shown in Figure 4.

Recalcitrant CRSwNP cases

Among the CRSwNPs cases, there were five patients (52%) with recalcitrant nasal polyposis and hypersensitivity to NSAIDs, who had undergone multiple FESS procedures and suffered from bronchial asthma.

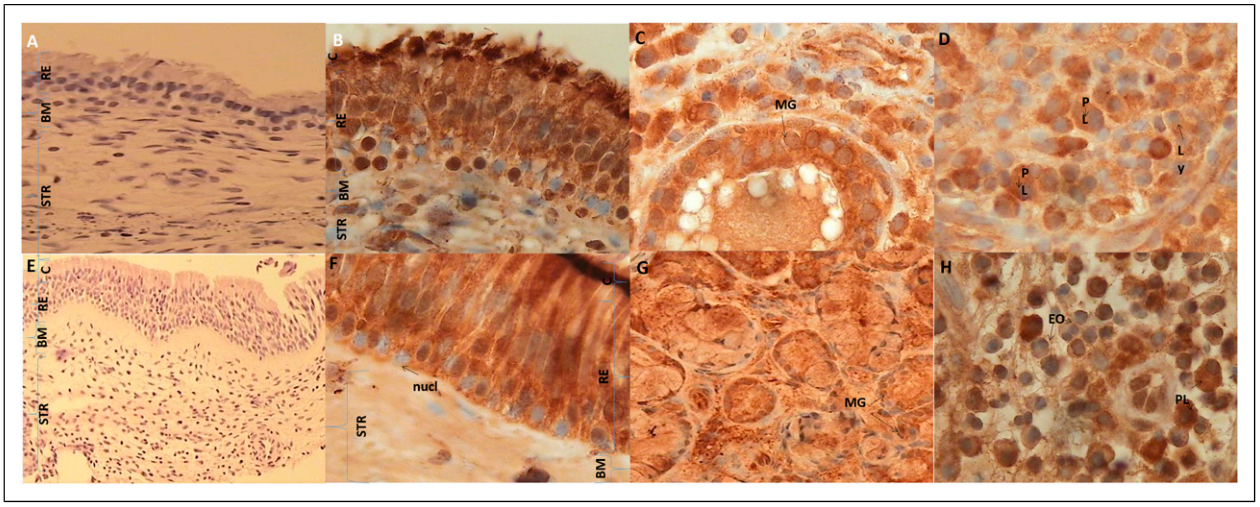

Figure 3. Healthy sinus tissue. **A** – negative control (stained by haematoxylin), C – cilia, RE - respiratory epithelium, BM – basal membrane, STR – stroma. **B** – AhR^{+} healthy sinus tissue layers. **C** – AhR^{+}, MG – mucosal glands. **D** – AhR^{+} cells. **E** – nasal polyp negative control (stained by haematoxylin). **F** – AhR^{+}nasal polyp tissue layers, Ly – lymphocyte, PL – plasma cell, EO – eosinophil. **G** – 4HNE^{+}, MG – mucosal glands. **H** – 4HNE^{+} cells. grade 0 – negative staining, grade I – weak positivity (0–30%) and intensity, grade II – intermediate positivity (30–50%) and intensity, grade III – high positivity (>50%) and intensity.

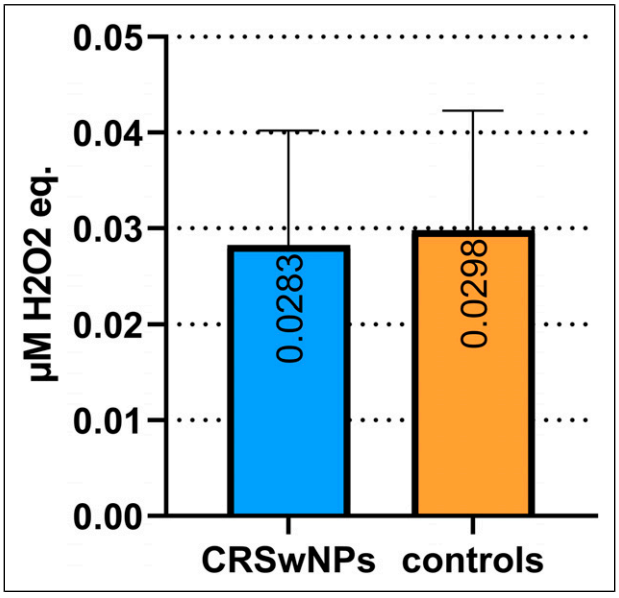

Figure 4. Total oxidative capacity in CRSwNP patients and controls. Values in bars are means, whiskers are upper 95% CI (p=0.848, t-test).

All of these patients had a damaged and desquamated respiratory epithelium, grade III 4-HNE positive eosinophils, grade III AhR positive cytoplasms and nuclei of plasma cells; by comparison, non-recalcitrant CRSwNPs patients had grade II 4-HNE positive eosinophils, and grade III cytoplasms and nuclei of plasma cells.

Discussion

4-hydroxynonenal is considered a major bioactive marker of lipid peroxidation across a wide spectrum of diseases. For example, it was shown that the levels of 4-HNE expression and NADPH oxidase isoform p67^{phox} are significantly increased in nasal polyp tissue when compared to healthy mucosa.[15] It is known that NADPH oxidase isoforms, such as NOX1 and NOX4, and the cellular source of superoxides are mainly localized in the epithelial layer, submucosal glands, vascular endothelium and inflammatory cells, both when it comes to the healthy and allergic nasal mucosa, as well as nasal polyps. Results of a study aimed to evaluate the influence of oxidative stress on the development of allergic rhinitis and nasal polyposis, have shown that NOX1 and NOX4 play an important role in reactive oxygen species production, hence contributing to the oxidative stress seen in allergic rhinitis and nasal polyposis.[6]

We did not find a difference in total oxidative stress between CRSwNPs cases and healthy controls; however, we did find a higher tissue and serum 4-HNE expression in CRSwNPs cases. The finding shows the important role of 4-HNE as specific oxidative stress marker of local tissue and general oxidative stress. Also, AhR expression is higher in inflammatory cells and tissue samples of CRSwNPs patients than in healthy controls. This result further shows the association between AhR and oxidative stress intensity.

Within the CRSwNP group, all patients with bronchial asthma had abundant eosinophil cells in tissue samples – we believe this findings delineates the endotype 2 CRS, as defined by the EPOS 2020 CRS classification criteria.

Moreover, all patients with this endotype suffered from a recalcitrant nasal polyposis. These patients had higher serum 4-HNE levels, as compared to non-recalcitrant patients. Finally, expression of 4-HNE in eosinophils of recalcitrant nasal polyposis patients was higher than in non-recalcitrant cases. This finding is suggestive of a central role of eosinophils in mediating the inflammatory response in the environment of oxidative stress in tissues of patients with CRSwnNPs. Expression of AhR in recalcitrant CRSwNPs cases was higher than in non-recalcitrant cases. This finding might be interpreted as an overexpression of AhR in this course of disease, as compared to a non-recalcitrant course.

It is known that certain compounds, such as flavonoids, inhibit AhR overexpression in inflammatory cells and thereby counteract the oxidative stress. Their role in the homeostasis of pro- and antiinflammatory mechanisms might be crucial in both pathogenetic as well as the therapeutic sense.

There are several limitations of our study. Firstly, the assessment of immunohistochemistry is semiquantitative and inherently susceptible to bias. Secondly, only two targets (4-HNE and AhR) were selected for the study. Assessment of more markers would yield more reliable conclusions; however, due to practical reasons we were only able to assess the two.

Conclusion

Patients suffering from CRSwNPs have oxidative stress–induced overexpression of aryl-hydrocarbon receptor which mediates a constant chronic inflammatory response in the paranasal sinus tissue.

Declaration of conflicting interests

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