THE PROINFLAMMATORY ROLE OF SERUM IL-6 IN ACUTE OTITIS MEDIA IN CHILDREN

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THE PROINFLAMMATORY ROLE OF SERUM IL-6 IN ACUTE OTITIS MEDIA IN CHILDREN (Abstract): Acute otitis media (AOM) represents the leading cause of health care visits and antibiotic prescriptions in children. In order to reduce the unnecessary antibiotic prescriptions and to avoid the burden of potential complications, new biomarkers may be useful for the diagnosis and follow-up of children with AOM. **Material and methods**: We performed a prospective study in pediatric patients with AOM hospitalized in a tertiary hospital in Iasi, Romania, during a 1-year period (between November 2021 and November 2022). We assessed the utility of serum interleukin-6, S100A12 protein, ESR (erythrocyte sedimentation rate) and CRP (C-reactive protein) in the diagnosis of children with clinical signs of AOM. **Results**: A total of 88 children, aged between 2 months old to 17 years old were included (46 with AOM and 42 healthy controls). The differences between the control group and AOM group were statistically significant ($p<0.001$) for ESR, CRP and IL-6 (median values being much higher in the case of patients with AOM). The differences between the control group and the one with AOM were only random ($p=0.326 > 0.05$) in case of S100A12 protein. ESR > 27 mm/hour was associated with a 2 times higher risk (RR=2.022) of having concomitant adenoiditis. **Conclusions**: IL-6 could offer a powerful tool for the objective evaluation of AOM. Further research is needed to fully elucidate the role of IL-6 in AOM and to determine whether it may be a useful biomarker for predicting treatment outcomes. **Keywords**: ACUTE OTITIS MEDIA, CHILDREN, IL-6, S100A12 PROTEIN.

Acute otitis media (AOM) is defined by the American Academy of Pediatrics as the rapid onset of signs and symptoms of inflammation in the middle ear, with or without otorrhea (1). In children, AOM represents an important healthcare issue due to high rates of antibiotherapy (2). Despite overall reduction in the incidence of AOM after the introduction of pneumococcal conjugate vaccines, Hu *et al.* reported at least one episode of AOM in 28.6% to 31.5% of the children younger than 2 years old in 2022 (3). In the United States, about 60% of children experience an episode of AOM before the age of 3 (4). In this context, AOM still represents the leading cause of health care visits and antibiotic prescriptions (5), with additional burden arising

from long-term complications, as hearing loss that impacts language acquisition and educational outcomes (6).

Currently, the diagnosis of AOM is based on signs of inflammation on otoscopy combined with clinical history and presenting signs and symptoms (7). As these methods may have a certain grade of subjectivity derived from the healthcare professional, but also from the perception of the parents on the pain and discomfort of their child, a more objective approach may be welcome. Microbiological culture and antibiotic sensitivity testing is useful in order to justify the antibiotic prescription, but the available guidelines do not routinely recommend performing a tympanocentesis (8). Also, blood tests (complete blood count, acute phase reactants such as C-reactive protein, erythrocyte sedimentation rate and fibrinogen, cultures) are performed only under specific circumstances (recurrent AOM, complications or treatment failure, follow-up) (9).

Recent discoveries emphasized the potential role of cytokines, a group of glycoproteins, in the modulation of inflammatory and immune reactions in AOM. Interleukin-6 (IL-6), an inflammatory mediator with pleiotropic effects, is highly produced during the initial stage of inflammation (10). Higher levels of serum IL-6 were found at the moment of diagnosis in children with AOM due to *Streptococcus pneumoniae*, but these results are difficult to extrapolate to other populations as the study sample comprised only 12 children (11). A less-studied molecule in the context of inflammation associated to AOM is S100A12 protein, also known as calgranulin C. Markedly overexpressed levels of S100A12 protein have been reported in inflammatory compartments, but the potential usefulness in clinical practice in the context of AOM has not yet fully established (12).

In order to reduce the unnecessary antibiotic prescriptions and to avoid the burden of potential serious complications, new biomarkers may be useful for the diagnosis and follow-up of children with AOM. Our aim was to assess the utility of serum interleukin-6 and S100A12 protein in the diagnosis of children with clinical signs of AOM.

MATERIAL AND METHODS

We performed a prospective study in pediatric patients with AOM hospitalized in "Sf. Maria" Emergency Hospital for Children, a tertiary hospital in Iasi, Romania, during a 1-year period (between November 2021 and November 2022). The study protocol was reviewed and approved by the Ethical Committees of "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania (No. 12292-22.07.2020) and of "Sf. Maria" Clinical Emergency Hospital for Children, Iasi (No. 34306/ November 5, 2020). Written informed consent was obtained from the parents of all study participants.

We included the children aged 0-18 years old who were diagnosed with AOM in the ENT Department based on the following criteria: acute onset of the symptoms (fever, irritability, otalgia or otorrhea) and at least 2 signs of middle ear effusion confirmed by otoscopy (bulging or erythema of the tympanic membrane, air-fluid level behind the tympanic membrane, limited or absent mobility of tympanic membrane). Only children with a recent onset of AOM symptoms (the first 48 hours) who did not receive any antibiotic treatment were considered for our analysis. Patients with acute comorbidities which might have triggered a more powerful inflammatory

response - e.g., lower respiratory tract infections, gastroenteritis, urinary tract infections, meningitis or with chronic systemic conditions - e.g., diabetes mellitus, cancers or autoimmune disorders were excluded from our sample. We also set up a control group with children who presented in our hospital for a routine check-up, with no current infection or inflammatory disease.

At hospital admission, 3 mL blood samples were obtained from both AOM and control groups and collected in heparin-free tubes at room temperature. After centrifugation at 2000 x g for 15 minutes at 4 °C, we obtained serum samples which were immediately stored at – 80 °C. The Enzyme-Linked ImmunoSorbent Assay (ELISA) method was used to measure the IL-6 (code E-EL-H0102 ELISA Kit) and S100A12 protein (code E-EL-H1293 ELISA Kit) levels. Both kits were provided by Elabscience, United States of America. The micro-ELISA plate has been pre-coated with an antibody specific to Human IL-6 and Human S100A12, respectively. The samples were added to the micro-ELISA plate wells and combined with the specific antibody using the Sandwich-ELISA principle. Then, a biotinylated detection antibody specific for Human IL-6 and Human S100A12, respectively with an Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components were washed away, and the substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of stop solution which turned the color into yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. As the OD value was proportional to the concentration of IL-6 and S100A12 protein, respectively, we calculated these concentrations in the samples by comparing the OD of the samples to the standard curve.

Besides demographic data (age, gender), we also recorded data regarding significant commorbidies (adenoiditis), the hospitalization length, the potential complications and the readmissions to hospital in the following 5 months. We have also analyzed biologic markers - C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in both AOM and control group. The normal range provided by the laboratory of our hospital is less than 5 mg/L for CRP and less than 12 mm/hour for ESR.

Data obtained were analyzed using specific statistical functions provided by SPSS software, as the non-parametric Mann-Whitney U test, as well as Spearman's rank correlation coefficient. P values < 0.05 were considered statistically significant.

RESULTS

A total of 88 children aged between 2 months old to 17 years old were included in our analysis, among which 46 were diagnosed with AOM and 42 were healthy controls presented to our hospital for a routine check-up. The descriptive statistics revealed significantly higher values for ESR, CRP and IL-6 in children with AOM than in controls (tab. I). The distributions of the ESR, CRP, IL-6 and S100A12 protein in controls and cases are represented in first figure.

Our data were not normally distributed, and therefore, instead of using the t-test for comparing independent samples, we needed a non-parametric alternative test, which in this case was the Mann-Whitney U Test (tab. II), which does not require the assumptions of normality of variables and homogeneity of variances. The test showed that the differences between the control

group and AOM group are statistically significant ($p<0.001$) for ESR, CRP and IL-6 (median values being much higher in the case of patients with AOM, which is also illustrated in fig. 1). The values of the Rank-Biserial Correlation indicator showed an important effect of these differences between the control group and the otitis group for ESR, CRP and IL-6, their magnitude being above the threshold of 0.50. The differences between the control group and the one with AOM were only random ($p=0.326 > 0.05$) in case of S100A12 protein.

TABLE I.

Descriptive statistics of the main parameters analyzed in AOM cases and healthy controls

	ESR (mm/h)		CRP (mg/dL)		IL-6 (pg/mL)		S100A12 protein (pg/mL)	
	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
Median	5	27	2	13.920	3.885	118.815	9.495	9.795
MAD*	3	13	1	8.680	0.765	45.185	1.645	1.945
Shapiro-Wilk	0.912	0.886	0.942	0.762	0.749	0.83	0.946	0.935
P-value of Shapiro-Wilk	0.003	< .001	0.034	< .001	< .001	< .001	0.047	0.012
Minimum	2	10	0.100	1.38	2.88	44.1	1.360	0.4
Maximum	14	89	6	152.43	14.34	443.3	14.840	14.71

* MAD, median absolute deviation

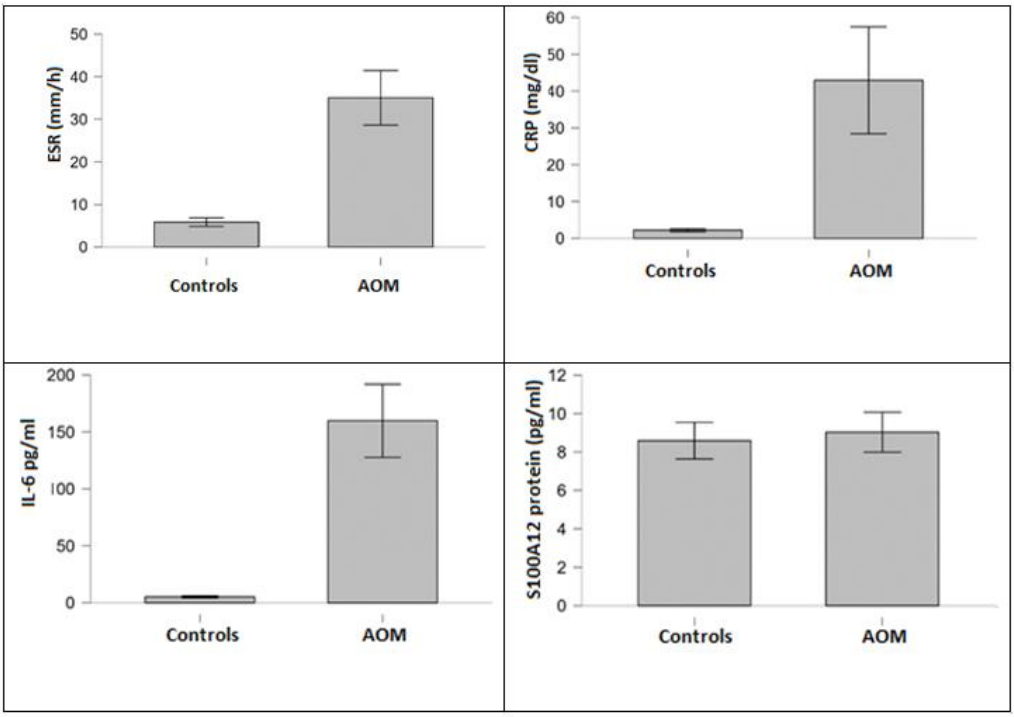

Fig. 2. Distribution of the ESR, CRP, IL-6 and S100A12 protein in controls and cases

As the assumption of normality of the sample was previously violated (the Shapiro-Wilk test registers statistically significant values in all situations, respectively $p < 0.05$), we used Spearman's correlation coefficient instead of Pearson's coefficient to highlight the existing correlations between ESR, CRP, IL- 6 and S100A12 (tab. III). A matrix of correlation coefficients between the 4 variables, which also contains the level of statistical significance associated with each variable was generated (tab. III). A strong direct (positive) relation between CRP and ESR ($rs = 0.630$, $p < 0.001$) and a direct link of medium intensity between IL-6 and ESR ($rs = 0.3090$, $p < 0.05$) were observed. No statistically significant correlation was revealed between the other pairs of variables.

The association between the presence of adenoiditis and the value of ESR > 27 mm/hr was statistically significant ($\chi^2=5.841$, $p<0.05$). Moreover, an ESR > 27 mm/hr was associated with a 2 times higher risk (RR=2.022) of having concomitant adenoiditis (fig. 2).

Table II.

Mann-Whitney U test

	W df	p	Hodges-Lehmann Estimate	Rank-Biserial Correlation	SE Rank-Biserial Correlation
ESR	9.000	**< .001**	-22.000	-0.991	0.123
CRP	37.000	< .001	-11.840	-0.962	0.123
IL-6	0.000	< .001	-114.324	-1.000	0.123
S100A12	848.000	0.326	-0.650	-0.122	0.123
Note. For the Mann-Whitney test, effect size is given by the rank biserial correlation.					

TABLE III.

Correlation Matrix for AOM group

		ESR	CRP	IL-6	S100A12 protein
ESR	Spearman's rho	—			
	p-value	—			
CRP	Spearman's rho	0.630	—		
	p-value	< .001	—		
IL-6	Spearman's rho	0.309	0.261	—	
	p-value	0.037	0.080	—	
S100A12 protein	Spearman's rho	0.195	0.185	0.229	—
	p-value	0.193	0.219	0.125	—

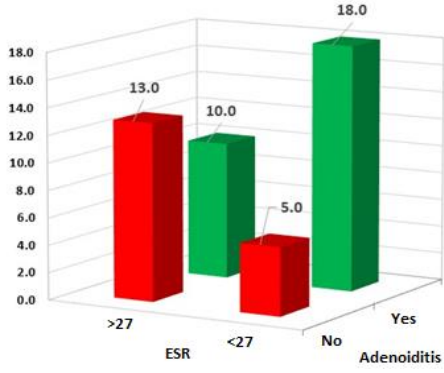

Fig. 2. Correlations between adenoiditis and ESR>27 mm/h

No statistical significance was found between adenoiditis and CRP > 50 mg/L (χ^2=2.741, p=0.098) or between bilateral AOM and CRP > 50 mg/L (χ^2=0.063, p=0.801).

The average hospitalization length was 5 days and no serious complications or readmissions to hospital have occurred in the following 5 months in the AOM group.

DISCUSSION

The role of IL-6 in the diagnosis of AOM

IL-6 is a pleiotropic cytokine, commonly referred as a primary mediator of the acute phase response, which plays an important role in the inflammatory reaction to bacterial infections (13). Up to the present, its important role in inflammation, innate and adaptative immune responses has been recognized in various studies (14, 15). During the resolution phase of the infections, IL-6 promotes the reduction of the neutrophil infiltrate and initiates an acquired immune response (16). In contrast to other cytokines, the production of IL-6 may continue even after the initial stimulus has been removed, being eliminated in a lower rate than other cytokines (17). This may explain why higher levels of IL-6 were found in the sera from patients with AOM in the early phases of the disease in comparison with healthy controls.

Several studies have investigated the role of IL-6 in AOM in children, but the exact role of IL-6 in the pathogenesis of AOM has not been clearly established yet. However, it was almost 35 years ago when Heikkinen *et al.* already reported that low levels of IL-6 cannot solely exclude the bacterial etiology of acute otitis media (18). Our results regarding IL-6 serum levels are in accordance with those of Scharer *et al.*, who found higher mean concentrations of IL-6 in a sample of 10 children with AOM compared to healthy controls (p = 0.05) (11). Also, our findings were consistent with another study performed in a sample of 14 children with AOM (19). However, these results are difficult to interpret and extrapolate as in pediatrics, there are often difficulties in developing studies on larger samples.

The optimal cut-off value for serum IL-6 to discriminate AOM from healthy controls has yet to be clearly established. However, the role of IL-6 has been better described in chronic otitis media. For instance, in a study comparing the role of interleukin 1α (IL-1α), IL-6 and interleukin 8 (IL-8) in the chronic evolution of otitis media, Serban *et al.* reported that IL-6 serum values recorded the highest mean level in chronic suppurative otitis media ranging from 57.23 to 193.33 pg/mL (20). In our sample, the serum IL-6 levels ranged from 44 to 443.3 pg/mL, with a medium level of 118.815 pg/mL, which might suggest that higher levels of IL-6 can be correlated to acute rather than chronic otitis media. It has been shown that IL-6 levels also decrease after antibiotic treatment, suggesting that IL-6 is involved in the response to bacterial infection (11).

In vitro studies have shown that IL-6 is produced by middle ear epithelial cells and fibroblasts in response to bacterial components, such as lipopolysaccharides (LPS), and that IL-6 production is enhanced by other cytokines, such as TNF-α and IL-1 β. IL-6 can also stimulate the production of other cytokines, such as IL-8, which promote neutrophil recruitment and activation in the middle ear (21). The role of IL-6 in AOM has been also investigated in animal models, such as mice and chinchillas. In these models, IL-6 levels were found to be elevated in the middle ear after bacterial

inoculation, and IL-6 blockade with antibodies or genetic deletion resulted in reduced neutrophil recruitment and less severe middle ear inflammation (22). IL-6 has also been investigated as a potential therapeutic target in AOM. Blockade of IL-6 signaling has been shown to reduce middle ear inflammation and improve clinical symptoms in animal models (23).

S100A12 protein in the diagnosis of AOM

Firstly, described in 1995 by Guinard *et al.* (24), S100 calcium-binding protein A12 (S100A12 protein), also known as calgranulin C, MRP6, or EN-RAGE (extracellular newly identified receptor for Advanced glycation end products binding protein), belongs to the S100 family of low molecular weight proteins (25). S100A12 protein is predominantly localized in the cytoplasm of myeloid cells such as neutrophils (26), but recent data suggest that, under inflammatory conditions, it can also be present in epithelial and endothelial cells and in pro-inflammatory macrophages (27). Besides its role in calcium homeostasis, S100A12 protein express chemo-attractive properties to immune cells, leading to a pro-inflammation state via TLR-4 and RAGE pathways (28). Recent data reveals the role of S100A12 protein in the evaluation and assessment of several inflammatory disorders, including Behçet's disease (29), familial Mediterranean fever (30), Kawasaki's disease (31), inflammatory bowel disease (32) and AOM (33).

Several studies depicted its antimicrobial role, mainly via a nutritional immunity – based mechanism, as essential transition metals for the growth and resistance of the microorganisms are depleted during the infections. For instance, S100A12 protein exerts inhibitory activity against *Campylobacter jejuni* (34) and *Helicobacter pylori* through a zinc+-dependent sequestration (35). Moreover, S100A12 expresses antiparasitic properties, by enhancing the production of superoxide possibly through the binding of copper (36).

Contrary to our results, higher levels of S100A12 protein were reported in children with AOM than in healthy controls. According to Liu *et al.*, increased serum levels of S100A12 protein were found at the onset of acute otitis media due to *Streptoccocus pneumoniae* or nontippable *Haemophilus influenzae* (37). Upper respiratory viral infections which may coexist during the episodes of acute otitis media did not significantly influence the concentrations of S100A12 protein, but the authors noticed a decreasing level of S100A12 protein in children who recovered from acute otitis media (37).

Up to the present, no consensus regarding the reference values for serum levels of S100A12 protein has been reached. Using enzyme-linked immunosorbent assay (ELISA), several authors reported mean serum S100A12 protein levels in healthy adults ranging from 10.7 to 75.0 ng/mL (38-41). Liu *et al.* were the first to provide a reference value for serum S100A12 protein levels in children: the mean serum concentration was 12.1 ng/mL ± 19.8 ng/mL in normal healthy subjects and 36.7 ± 72.4 ng/mL in children with acute otitis media (37). More recently, Hinze *et al.* reported a mean value of 44 ng/mL for S100A12 protein levels in healthy individuals aged between 4 to 20 years old (42). Recently, Pichichero *et al.* proposed a biomarker score based on age at diagnosis and serum concentrations of S100A12 protein and IL-10, which enables the clinicians to distinguish between upper respiratory infections with and without middle ear involvement

(43). However, further studies are required to fully understand the mechanisms involved in the activation of S100A12 protein in AOM and its implications in the development of the disease.

Limitations of the study

There are a number of limitations in this study that ought to be acknowledged. A major limitation of this study is the small sample size, but in pediatrics it is often difficult to recruit a high number of participants for research purpose basically due to ethical issues (44). Moreover, we recruited less patients than expected probably due to the lower incidence of the AOM in the Covid-19 era. There are several studies reporting a reduction in the incidence rate of AOM during the pandemic era and probably the protective measures we adopted to combat Covid-19 (45, 46). Another limitation is the lack of serum re-evaluation after the antibiotic treatment. This was mainly due to the favorable evolution of AOM cases, which did not require another blood sample to confirm the efficacy of the treatment. Also, it would have been useful to have the microbiological spectrum of the middle ear effusions, but a tympanocentesis was not routinely performed as it is an invasive procedure which weather was not recommended by the ENT specialist, or the parents were often reluctant to accept additional or invasive investigations.

CONCLUSIONS

Our study reveals that the inflammatory response related to AOM may be rather specific, than global, as the serum levels of IL-6 were higher in AOM group than in healthy controls ($p<0.001$), but with no significant difference regarding serum S100A12 protein between the two groups. Therefore, IL-6 could offer a powerful tool for the objective evaluation of AOM. IL-6 plays an important role in the pathogenesis of AOM in children, through its pro-inflammatory and immune-modulatory effects. Further research is needed to fully elucidate the role of IL-6 in AOM and to determine whether it may be a useful biomarker for predicting treatment outcomes.

Although it is only theoretical at this point, this different response may be specific to the children prone to AOM, and further studies are needed in order to establish if the risk of developing AOM is related to specific cytokine responses. Understanding the intracellular intricacies in relation to inflammation may contribute to newer therapies for AOM and even to the reduction of the complications and economic burden. We are hopeful that advances in the understanding of the inflammatory background of AOM may provide opportunities to create more targeted and effective treatments in the future.

Abbreviations

AOM: acute otitis media, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, HRP: Avidin-Horseradish Peroxidase, IL-1α: Interleukin-1α, IL-1β: Interleukin-1β, TNF-α: Tumor necrosis factor, IL-6: Interleukin-6, IL-8: Interleukin-8, OD: optical density, LPS: lipopolysaccharides.

CONFLICT OF INTEREST AND FUNDING

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All the authors had equal contributions

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committees of "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania (No. 12292-22.07.2020) and of "Sf. Maria" Clinical Emergency Hospital for Children, Iasi (No. 34306/November 5, 2020).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical issues.

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