CLINICAL STUDY

EVALUATION OF THE PRESENCE OF CALPROTECTIN IN MIDDLE EAR EFFUSIONS FROM CHILDREN WITH OTITIS MEDIA WITH EFFUSION AND ITS RELATIONSHIP WITH TUMOR NECROSIS FACTOR-ALPHA AND INTERLEUKIN-8

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SUMMARY

Objective: The aim of this study is to assess the presence of calprotectin in middle ear effusions (MEEs) in patients with otitis media with effusion (OME) and to determine its relation to proinflammatory cytokines interleukin-8 (IL-8) and tumor necrosis factor-alpha TNF-α in effusions, as well as clinical and epidemiological data.

Materials and Methods: Thirty-three children (aged 2.5 to 14 years) diagnosed with chronic OME and scheduled for ventilation tube insertion were included in the study. A total of 41 MEEs and 33 plasma samples were collected during surgery and later, calprotectin, IL-8, and TNF-α levels in the samples were assessed by using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits. Total protein levels in MEEs were measured by bicinchoninic acid (BCA) protein Assay.

Results: Calprotectin, IL-8, and TNF-α were detected in 100%, 95.1%, and 43.9% of effusions, respectively. Mean levels of calprotectin, IL-8, and TNF-α determined in effusions were 581.4 ± 740 ng/mg total protein, 3379.2 ± 4329.6 pg/mg total protein, and 53.6 ± 69.7 pg/mg total protein, respectively. Mean calprotectin concentration in the plasma samples was 123.11 ± 59.40 ng/ml. A positive correlation was detected between measured values of calprotectin and TNF-α ($p < 0.05$). There was no correlation between the measured values of calprotectin, IL-8 or TNF-α and patient age or duration of effusion.

Conclusion: Calprotectin seems to participate in the inflammatory process during OME. However, further studies are needed to reveal the full extent of calprotectin's role in OME which may help to develop novel treatment strategies.

Keywords: Calprotectin; Cytokine; Interleukin-8; Otitis Media With Effusion; Tumor Necrosis Factor-α

EFÜZYONLU OTİTİS MEDİALI ÇOCUKLARDA ORTA KULAK EFÜZYONLARINDA KALPROTEKTİN VARLIĞININ DEĞERLENDİRİLMESİ VE TÜMÖR NEKROZ FAKTÖR-ALFA VE İNTERLÖKİN-8 İLE İLİŞKİSİ

ÖZET

Amaç: Bu çalışmanın amacı efüzyonlu otitis media (EOM) olan hastalarda, orta kulak efüzyonlarında kalprotektin varlığını değerlendirmek ve kalprotektinin efüzyonlardaki proenflamatuar sitokinler olan interlökin-8 (IL-8) ve tümör nekroz faktör-α (TNF-α), klinik ve epidemiyolojik verilerle ilişkisini belirlemektir.

Yöntem ve Gereçler: Kronik EOM tanısı alan ve ventilasyon tüpü yerleştirilmesi planlanan 33 çocuk hasta (2.5-14 yaşlar arası) çalışmaya dahil edildi. Ameliyat sırasında toplam 41 orta kulak efüzyonu ve 33 plazma örneği toplandı ve daha sonra numunelerdeki kalprotektin, IL-8 ve TNF-α seviyeleri, piyasada bulunan sandviç enzime bağlı immünosorban test (ELISA) kitleri kullanılarak değerlendirildi. Efüzyon örneklerindeki toplam protein seviyeleri bisinkoninik asit (BCA) protein testi ile ölçüldü.

Bulgular: Kalprotektin, IL-8 ve TNF-α, efüzyonların sırasıyla %100, %95.1 ve %43.9'unda saptandı. Efüzyonlarda saptanan ortalama kalprotektin, IL-8 ve TNF-α seviyeleri sırasıyla 581.4 ± 740 ng / mg total protein, 3379.2 ± 4329.6 pg / mg total protein ve 53.6 ± 69.7 pg / mg total protein idi. Plazma örneklerindeki ortalama kalprotektin konsantrasyonu 123.11 ± 59.40 ng/ml idi. Ölçülen kalprotektin ve TNF-α değerleri arasında pozitif korelasyon saptandı ($p <0.05$). Ölçülen kalprotektin, IL-8 veya TNF-α değerleri ile hasta yaşı veya efüzyon süresi arasında korelasyon yoktu.

Sonuç: Kalprotektinin, EOM sırasındaki inflamatuar sürece katkıda bulunabileceği görülmektedir. Bununla birlikte, kalprotektinin yeni tedavi stratejilerinin geliştirilmesine yardımcı olabilecek EOM'daki rolünün tam olarak ortaya çıkarılması için, daha fazla çalışmaya ihtiyaç vardır.

Anahtar Sözcükler: Kalprotektin; Sitokin; Efüzyonlu Otitis Media; İnterlökin-8; Tümör Nekroz Faktör-α

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INTRODUCTION

Otitis media with effusion (OME) is a common disease that usually affects children and is characterized by the collection of fluid in the middle ear cavity in the absence of acute signs or symptoms of inflammation. OME is one of the foremost causes of hearing loss in children. Persistent symptoms in children may affect hearing and, subsequently, language, behavior,

and education, thus surgical intervention with ventilation tube insertion is necessary for patients with persistent symptoms [1].

Several etiological factors, especially eustachian tube dysfunction and infection that leads to chronic inflammation of the middle ear, are proposed for the pathogenesis of the disease[2]. Previous studies suggest that OME is primarily an inflammatory condition, and possible inflammatory stimuli presumed are bacteria, viruses, and allergy [3]. Infiltration of the middle ear mucosa by inflammatory cells is the first step in the inflammatory response with subsequent release of inflammatory mediators and pro-inflammatory cytokines. Inflammatory mediators such as prostaglandins (PGs), leukotrienes (LTs), platelet activating factor (PAF), and histamine, along with proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-8 (IL-8) have been shown to participate in this process [4,5]. Inflammation is supposed to be the cause of cell metaplasia and mucin secretion in the middle ear[6]. Thus, inflammation leads to structural alterations on tympanic membrane, ossicles, or middle ear epithelia, such as mucosal hypertrophy, adhesions, osteolysis, tympanic membrane deterioration, atelectasis, and consequently, hearing loss [7]. The precise etiology and molecular and cellular mechanisms that lay beneath the immunological and inflammatory response is still unclear, and this prevents the development of novel and more effective and noninvasive treatment options for the management of the disease.

Calprotectin (also known as S100A8/A9 complex) is a heterodimeric protein consisting of three non-covalently linked chains, two heavy and one light, that bind calcium and zinc [8,9]. It belongs to the calcium binding S100 protein family which is involved in different intracellular processes including cell cycle regulation, cell growth, cell differentiation, motility and secretion [10]. S100A8 and S100A9 are also termed myeloid related protein 8 (MRP8) and 14 (MRP14), as they are expressed primarily in myeloid lineage cells [8,9,11].Under inflammatory conditions, expression of these proteins are also induced in keratinocytes and epithelial cells [10]. Calprotectin is found in the cytosolic fluid of neutrophils, monocytes and macrophages [8,11,12]. It is either secreted actively from myeloid cells or released passively as a result of cell death [11,12]. Extracellular calprotectin has regulatory functions in the inflammation process [8,11] as well as broad spectrum antimicrobial [13] and antiproliferative properties [8,14]. During certain inflammatory conditions or infections, elevated levels of calprotectin were detected in various body fluids, including plasma, saliva, stool, synovial fluid, and urine [15]. Thus, calprotectin promises to be a useful clinical biomarker that correlates very well with disease activity [11]. Furthermore, there are a growing number of studies focusing on targeting S100A8/A9 complex for the modulation of inflammatory process, which may offer innovative options superior to conventional ones for the treatment of pathological inflammatory conditions [11,16].

The aim of this pilot study is to assess the presence of calprotectin in middle ear effusions and to determine its relationship with pro-inflammatory cytokines IL-8 and TNF-α in effusions, as well as with clinical features.

MATERIAL and METHODS

1) Patient Population

This study included thirty-three children (aged 2.5 to 14 years) with chronic OME (of greater than 3 months), who were scheduled for ventilation tube insertion with adenoidectomy or adenotonsillectomy in a tertiary otorhinolaryngology clinic between 2012 and 2013. Diagnoses were made based on otoscopic findings, tympanograms, and audiograms. Children with known craniofacial anomalies, immune deficiencies, primary ciliary dyskinesia, hyperviscosity syndrome, and chronic systemic inflammatory disease were excluded. None of the patients had a history of upper respiratory tract infection or used antibiotics within three weeks prior to surgery. Clinical information from each patient, including age, gender, tympanic membrane retraction, effusion type, and duration of effusion were reviewed. Approval of this study was granted through the Local Institutional Ethical Committee (Approval Number 2012/49), and informed consent was taken from the parents of all participants.

2) Collection of Middle Ear Effusions

Forty-one middle ear effusions (MEEs) from 33 children were aspirated aseptically to polypropylene tubes under general anesthesia during ventilation tube insertion. All MEEs were collected before adenoidectomy or adenotonsillectomy. Samples were classified as mucoid or serous, depending on their general appearance, and stored immediately at -70°C until further assessment.

3) Collection of Plasma Samples

During the surgery, 5 ml venous blood sample from each patient (n=33) were collected into tubes with Ethylenediaminetetraacetic acid (EDTA) and centrifugated for 10 minutes at 3000 rpm and 4 °C to obtain plasma samples. Samples were stored in polypropylene tubes at -70°C until further assessment.

4) Effusion Preparation and Analysis

Prior to assessment, MEEs were thawed at room temperature and then each sample was diluted with 1.0 ml of phosphate buffered saline (PBS) and vortexed for 1 minute. Afterwards, samples were centrifugated for 15 minutes at 1500 rpm and 4 °C to obtain supernatants. The supernatants of MEEs were assayed by using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits for IL-8 (Quantikine Human IL-8 immunoassay; R&D Systems Inc, Minneapolis, USA), TNF-α (Quantikine TNF-α immunoassay R&D Systems Inc; Minneapolis, USA), and calprotectin (Human Calprotectin Assay; Hycult Biotech, Uden, Holland), incorporating monoclonal antibodies and an ELISA reader (ELx 800 UV; BioTek Instruments, Vermont, USA). Calprotectin and cytokine concentrations in the samples were determined by comparison with standard curves produced from standard concentrations provided by the manufacturers for each test. Cytokine and calprotectin concentrations in the samples were calculated from a logarithmic standard curve plotted as spectrophotometric absorbance (at 450 nm) versus the standard concentration. Values below the lowest standard were classified as not detectable. Detection limit for calprotectin, TNF-α and IL-8 were 1.6 ng/ml, 15.6 pg/ml, and 31.2 pg/ml respectively. We used the test off-label for calprotectin by utilizing the standard operating procedure that is provided by the company for measuring plasma calprotectin. Total protein levels in the samples were measured by bicinchoninic acid (BCA) protein assay (BCA Protein Assay Kit; Thermo Fisher Scientific, Illinos, USA). Actual protein concentrations were determined from a linear standard curve plotted as spectrophotometric absorbance (at 562 nm) versus the standard concentration and reported as mg/ml. Final calprotectin concentration was calculated by correcting for the dilution factor. The measured values of calprotectin were reported as the amount in nanograms per milliliter (ng/ml), whereas the values of IL-8 and TNF-α were reported as the amount of cytokine present in picograms per milliliter (pg/ml). Since the volume and dilution of each sample varied, measured values were normalized for volume effects by dividing the values (ng/ml or pg/ml) by the amount of protein measured in each effusion in milligrams per milliliter (mg/ml). Thus, the quantity of cytokines was expressed as picograms per milligram of total protein (pg/mg TP), whereas the quantity of calprotectin was expressed as nanogram per milligram total protein (ng/mg TP).

5) Plasma Preparation and Analysis

Prior to assessment, EDTA-plasma samples were thawed at room temperature and vortexed for 1 minute. Plasma samples were assayed by using commercially available ELISA kit (Human Calprotectin Assay; Hycult Biotech, Uden, Holland) and ELISA reader (ELx 800 UV; BioTek Instruments, Vermont, USA). Calprotectin concentrations in the samples were calculated from a logarithmic standard curve plotted as spectrophotometric absorbance (at 450 nm) versus the standard concentration in a similar fashion for the calculation of calprotectin in MEEs. Final concentration was calculated by correcting for the dilution factor. Concentration of plasma calprotectin was reported as the amount in nanograms per milliliter (ng/ml). Detection limit for plasma calprotectin was 1.6 ng/ml.

6) Statistical Analysis

Statistical analyses were performed using SPSS for Windows (version 21.0; SPSS Inc., Chicago, IL, USA). For descriptive statistics, continuous variables were defined with median and means ± standard deviation (SD), whereas frequencies and percentages were used for discrete data. As the sample size was small, nonparametric tests were used for statistical analysis. Distribution of the data was assessed by the Kolmogorov-Smirnov test. The Chi-square/Fisher's Exact Test was used to identify difference between frequencies of the discrete data. The continuous data of two groups were compared with the Mann Whitney U test. Correlation of the continuous data was assessed with Spearman Test. A p value < 0.05 was considered statistically significant.

RESULTS

A total of 41 MEEs from 33 children (19 male, 14 female) were obtained and evaluated for the study. MEEs from both ears were obtained from eight children. The mean patient age was 80 months. All patients underwent ventilation tube insertion and adenoidectomy, whereas additional tonsillectomy was carried out on eight patients. None of the patients had a previous history of ventilation tube insertion. Average duration for effusion was 4.9 months. Of the 41 effusions, 85.4% (n=35) were mucoid and 14.6% (n=6) were serous. During the surgery, seven (17.1%) tympanic membranes were retracted at different levels, whereas 34 (82.9%) were normal (Table 1).

Calprotectin, IL-8, and TNF-α were detected in 100%, 95.1% and 43.9% of MEEs, respectively. The mean levels of calprotectin, IL-8, and TNF-α determined in MEEs were 581.4 ± 740 ng/mg.TP, 3379.2 ± 4329.6 pg/mg.TP, and 53.6 ± 69.7 pg/mg.TP, respectively. Mean concentration of protein in MEEs was 0.62 ± 0.189 mg/ml. Mean calprotectin concentration in the plasma samples was 123.11 ± 59.40 ng/ml (Table 1).

In mucoid effusions, mean levels of calprotectin, IL-8, and TNF-α were 597.8 ± 793.6 ng/mg.TP, 3457.6 ± 4632.1 pg/mg.TP and 49.7 ± 59.1 pg/mg.TP respectively. In serous effusions, mean levels of calprotectin, IL-8, and TNF-α were 485.8 ± 291.8 ng/mg.TP, 2921.7 ± 1939.9 pg/mg.TP and 76.1 ± 119.9 pg/mg.TP respectively. Mean levels of calprotectin, IL-8, and TNF-α in effusions from retracted ears were 942.4 ± 1319.9 ng/mg.TP, 2285.5 ± 1935.2 pg/mg.TP and 42.99 ± 53.4 pg/mg.TP respectively. Mean levels of calprotectin, IL-8, and TNF-α in effusions from non-retracted ears were 507.1 ± 560.1 ng/mg.TP, 3604.4 ± 4662.02 pg/mg.TP and 55.8 ± 73.0 pg/mg.TP respectively.

There was no correlation between the calculated values of effusion calprotectin, IL-8 or TNF-α and age of patients or duration of effusion. A positive correlation was detected between calculated values of calprotectin and TNF-α ($p < 0.05$). By contrast, there was no correlation either between calprotectin and IL-8 levels or between TNF-α and IL-8 levels ($p > 0.05$) (Table 2).

Table 1: Clinical features of patient population and calprotectin, protein and cytokine levels in samples

		n (%)	Mean±sd	Median	Min-max
Age		33	79.97 ± 33.11*	75*	30 - 167*
Gender	Female	14 (%42.4)			
	Male	19 (%57.6)			
Effusion Type	Mucoid	35 (%85.4)			
	Serous	6 (%14.6)			
Duration of Effusion		41	4.93±1.96*	4*	3 - 9*
Concurrent Operation	Adenoidectomy	33 (%80.5)			
	Adenotonsillectomy	8 (%19.5)			
Timpanic Membrane Retraction	Present	7 (%17.1)			
	None	34 (%82.9)			
Effusion Protein *(mg/ml)*		41	0.62 ± 0.19	0.66	0.09 – 1.07
Effusion Calprotectin *(ng/mg TP)*		41 (100%)	581.4 ± 740.01	372.4	218.4 - 3917.7
Effusion IL-8 *(pg/mg TP)*		39 (95.1%)	3379.2 ± 4329.6	2649.6	22.8 - 28260.6
Effusion TNF-α *(pg/mg TP)*		18 (43.9%)	53.6 ± 69.7	25.6	18.0 -320.3
Plasma Calprotectin *(ng/ml)*		33	123.11 ± 59.40	121.44	55.41 - 296.52

**Months*

Table 2: Correlations between the evaluated variables

	Calprotectin *(ng/mg TP)*	**IL-8** *(pg/mg TP)*	**TNF-α** *(pg/mg TP)*
Calprotectin *(ng/mg TP)*	-	Rho: -0.025 p: 0.879	Rho: 0.445 **p: 0.004**
IL-8 *(pg/mg TP)*	Rho: -0.025 p: 0.879	-	Rho: 0.101 p: 0.530
TNF-α *(pg/mg TP)*	Rho: 0.445 **p: 0.004**	Rho: 0.101 p: 0.530	-
Age *(months)*	Rho: -0.107 p: 0.507	Rho: -0.037 p: 0.821	Rho: -0.257 p: 0.105
Duration of Effusion *(months)*	Rho: -0.269 p: 0.089	Rho: -0.017 p: 0.916	Rho: -0.049 p: 0.763

Spearman Correlation *A p value < 0.05 is accepted statistically significant*

DISCUSSION

Due to the developments in molecular biology and immunology, immunological and inflammatory factors and mechanisms which regulate OME process have become the field's focus for better understanding and treatment of the disease. Within the last three decades, several publications have appeared that focus on the subject of inflammatory mediators in the pathogenesis of OME. However only a few

studies evaluated the presence of S100 proteins in middle ear in OME.

As far as we know, this is the first study that evaluated the presence of calprotectin in MEEs and its relationship with TNF-α and IL-8 in OME. Calprotectin (S100A8/A9 heterodimer) was detected in 100% of effusions and a positive correlation was found between levels of calprotectin and TNF-α in effusions which supported our hypothesis that calprotectin may participate in the inflammatory process in OME.

It is shown from previous studies that extracellur calprotectin and its subunits has antiproliferative, antitumoral, antimicrobial, immunomodulator, and antinociceptive activities[8,11-14,17]. These functions may help to comprehend calprotectin's possible role in middle ear effusion during OME.

Calprotectin has been shown to regulate production of pro-inflammatory mediators such as cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide (NO) [16]. In a recent study, it was reported that secretions of pro-inflammatory cytokines, including TNF-α and interleukin-6 (IL-6) in cultured murine BV-2 line microglial cells were increased significantly by calprotectin [18]. Similarly, calprotectin was shown to induce expression of the pro-inflammatory cytokines IL-6, IL-8, TNF-α, and cyclooxygenase-2 (COX-2) on human periodontal ligament cells [19]. Moreover, Ahmad et al. reported that calprotectin stimulates IL-8 production in airway epithelial cells in vitro and thus enhances the neutrophilic inflammation in bronchial disease [20].

Calprotectin also impacts inflammatory processes via interaction with inflammatory cells. Eue et al. reported that calprotectin regulated transendothelial migration of monocytes by enhancing monocyte adhesion to endothelial cells [17]. Moreover, Ryckyman et al. demonstrated that an injection of calprotectin or its subunits in the murine air pouch induced an inflammatory reaction, and rapid and transient accumulation of neutrohils was observed, suggesting that this migration is associated with chemotactic and proadhesive properties of myeloid related proteins towards neutrophils in vitro [21].

On the other hand, calprotectin also shows anti-inflammatory effects under specific circumstances to avoid tissue damage as a consequence of extreme inflammation. It is suggested that calprotectin has a capacity to bind proinflammatory cytokines IL-1B, IL-6, and TNF-α, which contributes to its anti-inflammatory function [16,22]. Furthermore, it is shown that calprotectin inhibits oxidative metabolism of polymorphonuclear neutrophils (PMNs) in vitro[23].

Calprotectin (S100A8/A9 complex) exhibits antimicrobial activity against several microorganisms by binding and, thus, controlling the levels of essential trace metals, such as zinc and manganese, which are needed for proliferation [24,25]. Moreover calprotectin was shown to inhibit bacterial binding of Listeria monocytogenes and Salmonella serovar Typhimurium to epithelial cells thus reducing the invasion of these pathogens [13]. Although, antimicrobial activity of calprotectin against species including Escherichia coli, Staphylococcus aureus, Candida albicans, Staphylococcus epidermidis, Klebsiella pneumonia, Salmonella typhimurium, and Listeria monocytogenes has been shown in different studies [12,13,24-26], its antimicrobial activity against otitis media pathogens, such as Streptococcus pyogenes, Moraxella catarrhalis, Streptococcus pneumoniae, and non-typeable Haemophilus influenzae is unknown and should be investigated in further studies.

In a recent in vitro study carried out on human airway cells, it was shown that S100 protein S100A12 and calprotectin subunits S100A8 and S100A9 induce the production of mucin protein (MUC5AC) [27], which has also been identified along with other mucin proteins in MEEs during OME in previous studies [3]. Furthermore, calprotectin was associated with dermal fibroblast activation and fibrosis [28]. Regarding the pathological changes seen in OME, it can be speculated that calprotectin may also contribute to the pathophysiology of OME with these functions.

Apart from calprotectin's aforementioned suggested biological functions in inflammation, significantly elevated calprotectin levels in blood

and in certain body fluids at sites of inflammation were shown to correlate well with disease activity in various inflammatory conditions such as rhematoid artritis, systemic lupus erythematosus, Sjögren syndrome, cystic fibrosis, systemic sclerosis, infections and in inflammatory bowel diseases. Thus, this has encouraged the use of calprotectin as a biomarker for assessing disease progression and treatment response [8,11,12,14-16,29]. It has been reported that calprotectin concentration is less than 1 µg/ml (1000 ng/ml) in healthy human serum/plasma [14]. In the present study, calprotectin levels in all plasma samples were within normal range, with a mean concentration of 123.11 ± 59.40 ng/ml. This is consistent with the absence of systemic inflammatory response and localized characteristic of OME, which hampers the possible use of plasma calprotectin as a biomarker for OME.

In the present study, a positive correlation was found between calprotectin and TNF-α levels. TNF-α is produced by middle ear mucosal cells and accumulating inflammatory cells, primarily by macrophages during OME. It is associated with mucus secretion and severe histopathological changes in the middle ear and is considered as primary cytokine that contribute to chronic OME [30]. TNF-α is presumed to be a strong inducer of IL-8 [5]. On the other hand, no correlation was found between calprotectin and IL-8 levels and between calprotectin and duration of effusion or age. IL-8 is produced by macrophages, fibroblasts, epithelial cells and lymphocytes [5] and is presumed to be responsible for the accumulation of inflammatory cells, especially neutrophils in middle ear tissues and effusions, and was shown to correlate with total number of neutrophils in effusions [28]. These results could be interpreted as indirect evidence of possible similar cellular origin or a mutual relationship between calprotectin and TNF-α during OME. On the other hand, there may be other complex immunological mechanisms regulating IL-8 production in OME.

In previous studies, TNF-α and IL-8 were detected in various rates and correlations between these two cytokines and clinical features differ among authors and these discrepancies may be related to differences in sample size, patient selection, effusion type, preoperative antibiotic use, age of individuals, duration of OME, and features of the measuring methods [6,7,31-33]. In the present study, IL-8 and TNF-α were detected in 95.1% and 43.9% of the samples which is coherent with the literature. Maxwell et al. reported a positive correlation between TNF-α and IL-8 levels in MEEs. However they did not find a correlation between age and TNF-α level[31]. On the other hand, Yellon et al. reported a positive correlation between level of TNF-α and age [32]. In the present study, we found no correlation either between the levels of TNF-α and IL-8 or between cytokine levels and age or duration of effusion. Similarly Bozena et al. did not find a significant correlation between TNF-α and IL-8 concentrations in MEEs from OME patients or between cytokine levels and clinical features [7]. In a study by Matkovic et al., it was shown that, while there was a different interlink between cytokines within the samples from both ears obtained from the same individual, concentrations of TNF-α and IL-8 within samples from both ears were not correlated in patients with bilateral OME [34]. Furthermore, Jurkiewicz et al. found a statistically significant correlation between TNF-α and IL-8 concentrations in MEEs and between TNF-α concentrations and age in non-atopic patients with OME, while there was no correlation between the aforementioned parameters in atopic patients. They concluded that cytokines may play different roles in atopic and non-atopic patients with OME [35]. Since we did not evaluate our patients for atopy, lack of correlation between these cytokines in the present study may also be associated with each patient's allergic profile.

Our findings are supported by a recent study by Hong et al., which evaluated the gene expression of S100 proteins in human middle ear epithelial cells exposed to an otitis media pathogen in vitro, as well as in the middle ear epithelial cells of patients with recurrent otitis media and chronic OME [36]. Elevated gene expressions of S100A8, S100A9 and S100A12 were found in Streptococcus pneumoniae infected middle ear epithelialium culture cells whereas only S100A8 and S100A9 gene

expressions were elevated in the middle ear epithelial cells of otitis media patients. It was suggested that S100 proteins take a part in innate immunity in chronic otitis media [36]. Similarly, Kerschner et al. reported significantly high levels of S100A8 and S100A9 expression in nontypeable Haemophilus influenzae infected chinchilla middle ear mucosa [37]. Furthermore, Val et al. demonstrated the presence of neutrophil extracellular traps (NETs) in MEEs from children with OME. They performed a detailed proteomic analysis of MEEs and emhasized that proteins derived from NETs, including calprotectin subunits S100A8 and S100A9 were predominant mediators of innate immune response in chronic otitis media [38]. Thus, the high positivity of calprotectin detected in our study may be associated with local release by inflammatory cells as well as active secretion by middle ear epithelial cells.

This study had some limitations. First, our sample size was small, and this prevented the possibility of performing subgroup analysis according to viscosity of effusions and tympanic membrane condition. Second, we could not determine whether the origin of calprotectin was middle ear mucosa or inflammatory cells because of the study design. Third, MEEs were not analyzed for leukocytes. Since contaminating leukocytes could influence calprotectin concentration, MEEs should be analyzed for such cells in future studies. Fourth, due to the off-label use of the ELISA test for the detection of calprotectin in MEEs, there might have been unknown factors interfering with the accuracy of our measurements. Thus, conventional immunohistochemical methods should be applied in further studies. Finally, cross-sectional study design precluded inference of any causality relation from the findings.

CONCLUSION

Calprotectin was detected in all MEEs in OME patients and a positive correlation was found between calprotectin and TNF-α levels which suggests calprotectin may contribute to the disease process. However, the biological function of calprotectin in OME and the mechanism by which it contributes to the pathogenesis of the disease remains unclear. Further studies are needed to reveal calprotectin's role in OME, which may help to develop novel treatment strategies.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Reference Number 2012/49) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of Interest: No conflict of interest was declared by the authors.

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