Received: 22 August 2023 | Revised: 09 September 2023 | Accepted: 25 September 2023
DOI: 10.48309/ecc.2023.412850.1676
Eurasian Chemical Communications
SAMI

FULL PAPER

A comparative study of selected immunological markers on children with normal tonsils, simple hypertrophic tonsils, and recurrently inflamed tonsils

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The interest in the role of tonsils and tonsillar disorders in a variety of immunological adaptations have been increased, but their precise systemic immunological roles are not well-established. Samples obtained from 38 patients with palatine tonsillar hypertrophy (Group I, which was further classified into Group Ia including 16 patients with tonsillar hypertrophy without recurrent tonsillitis, and Group Ib including 22 patients with recurrent tonsillitis). Group II included 38 samples of apparently healthy controls were studied. The levels of ASOT, IL-10, and lactoferrin in venous samples were measured, in addition to histopathological examination and immunohistochemical assessment of the CD3 and CD20 expression on the excised samples. The serum level of ASOT among group Ib candidates were significantly higher than that in group Ia and group II. Furthermore, ASOT level showed significantly higher readings in group Ia than in group II. In addition, when comparing group Ia to group II, there was no statistically significant distinction in the levels of IL-10. In contrast, group Ib showed statistically higher levels of IL-10 than group Ia and group II. The group Ia demonstrated no significant statistical difference regarding the lactoferrin levels when compared with group II and statistically significant lower levels of lactoferrin in group Ib was seen when compared to group Ia and II. Recurrent tonsillitis of the hypertrophied tonsils has stronger effects on the systemic immune components than isolated hypertrophy. Further research and analysis are needed to determine the specific nature and individual variances of these influences.

KEYWORDS

Immune system; immunohistochemistry; interleukin 10; lactoferrin; recurrent tonsillitis; palatine tonsils.

Introduction

The palatine tonsils are ordinarily quite small at birth, gradually increase in size during the early years of childhood, and then tend to shrink in adolescence. Tonsils that are excessively hypertrophied exhibit a common and valued challenge in medical practice as they can cause several local and systemic unfavorable outcomes. Whereas recurring acute or chronic tonsillitis has been identified in many children as a frequent association, recurrent acute and/ or chronic inflammation has also been hypothesized as the etiology of tonsillar hypertrophy [1-4].

Currently, it is well-established that the lymphoid tissue of the Waldeyer's ring is functioning to influence the local pharyngeal humoral immune response. However, it should be highlighted that many researchers have different views on the nature of this humoral immune response in cases of tonsillar disorders [4-7].

The primary infectious agent that causes bacterial tonsillitis is, Group A *Streptococcus* species, expresses a variety of antigens, including streptolysin O, which drives the host to produce specific antibodies. Therefore, an antecedent Group-A *streptococcal* infection is indicated by an increasing antistreptolysin O titre (ASOT) [8]. It is well-recognized that the upper limit of normal ASOT readings varies with age, various geographic areas, season, and site of infection [9].

On the other side, the interleukin 10 (IL-10) is known for its potent immunosuppressive action. During infection, the fundamental role of IL-10 is to suppress the immune reaction against pathogenic microbes, thus abating tissue destruction and qualifying the immune response [10]. At the same time, many immune biofunctions, including tumor cells suppression, antibacterial effect, antiviral effect, and antiparasitic effect, are confined to the lactoferrin glycoprotein molecule [11].

Histologically, the palatine tonsil is composed of lymphoid follicles within a connective tissue stroma, with tonsillar crypts extending from the surface into the central follicle of the tonsil. The tonsils surfaces are lined with a kind of epithelial tissue known as stratified squamous epithelium, which is non-keratinized. This epithelium closely resembles the epithelium seen in the adjacent oropharynx. The safeguarding of this anatomical region is contingent upon immunological responses, encompassing both the innate and adaptive immune systems [12,13].

The palatine tonsils serve as the first sites for both humoral (B-lymphocytes) and cell mediated (T- lymphocytes) immune responses in the human body, owing to their lymphoid characteristics. Tonsils contain a substantial abundance of T cells, predominantly localized inside the extrafollicular regions. Despite its significant implications, a large number of tonsillectomy procedures are conducted to prevent complications arising from hypertrophy or recurrent inflammation [12]. However, it is not common practice for most head and neck surgical institutions to routinely perform histopathological examinations on these excised specimens [14].

Histopathological examination of the excised specimen in case of inflammation usually shows congested and hypertrophied tonsillar tissue with marked development of the germinal clear center and white-faint bogus membranes [13,15]. Detection of the lymphocyte type in lymphoid tissues requires ancillary investigations such as the immunohistochemistry where CD3 is a pan T-cell marker immunohistochemically expressed on most of the mature T-cells and CD20 is a pan B-cell marker expressed on the majority of the mature B-cells [16].

Several studies have reached the conclusion that individuals with positive serological tests for anti-streptolysin O (ASOT) antibodies exhibit an association with elevated levels of circulating T cells. This correlation may have implications for the

development of severe sequelae in cases of chronic tonsillitis. Nevertheless, a recommendation was made for numerous patients diagnosed with seronegative ASOT to have tonsillectomy [13]. Limited research has been undertaken to establish a correlation between the expression and distribution of CD3 and CD20 markers in both B-cell and T-cell populations within inflamed and simple hypertrophied resected tonsils.

The influence of tonsillar immune response in cases of hypertrophy alone (simple hypertrophy) with or without recurrent inflammation on the systemic immune profile in multiple levels on investigations provide opportunities for a more objective evaluation of the tonsillar immunological roles [1]. Therefore, our study aims to compare ASOT, IL-10, and lactoferrin values in normal children to those with a disease of tonsillar hypertrophy alone and patients of the same age group with tonsillar hypertrophy associated with recurrent tonsillitis in addition to the evaluation of CD20 and CD3 expression of both diseased groups.

Material and methods

A case-control study was conducted at Al-Azhar University Faculty of Medicine and the university hospitals in Cairo, Egypt, between January 2023 and June 2023 with direct and remote investigation of the participants and the related tissue material.

Ethical considerations

The patients or guardians of all the participants gave their informed consent for this study and prior to the study the Ethical Committee approval was provided. Candidates have the right to maintain their own confidential information, including research findings having the right to withdraw from the study at any time and three children were excluded from the study due to refusal or participation or refusal of some investigations.

Study population

The study included 38 children with age range from 3 to 18 years with grade 2 or more palatine tonsillar hypertrophy that presented to outpatient clinics during the period of the study (Group I). Also, 38 apparently healthy children were recruited from the outpatient clinics to serve as controls (Group II). The grading of tonsillar hypertrophy is evaluated according to one of the most famous and accepted grading scales proposed by Brodsky [17].

The patients within group I were subsequently divided into two subgroups: Group Ia, consisting of 16 children with tonsillar hypertrophy, but no prior instances of recurrent tonsillitis, and Group Ib, consisting of 22 children with tonsillar hypertrophy and a history of recurrent tonsillitis. The term "recurrent tonsillitis" was previously defined in the following manner: To meet the criteria for this condition, it is necessary to have a minimum of seven thoroughly documented, clinically significant, and appropriately managed instances of throat infection during the year prior. Alternatively, one must have experienced at least five such episodes in each of the two years preceding the current year, or three or more such occurrences in each of the three years preceding the current year. The mentioned definitions were integrated into the AAO-HNS (American Academy of the Otolaryngology Head & Neck Surgery) guidelines established by [18]. We excluded those with apparent malnutrition, active infections (e.g., urinary tract infections, otitis media, etc.), allergic disorders (e.g., allergic rhinitis, bronchial asthma, etc.), autoimmune disorders, or any condition that could affect the immune markers.

All participants were experienced the following: (a) comprehensive history taking, (b) full local and general clinical examination, (c) collection of blood samples and the

following markers were measured; ASOT, IL-10, and lactoferrin.

Laboratory assay and immunological markers

Venous sampling was performed to obtain the requested blood samples and all tests were carried out according to the user manual instructions of the manufacturer.

ASOT assay

The basis for automated quantitative ASOT measurement is the agglutination of human anti-streptolysin-O antibody with latex particles coated by streptolysin-O antigens. The Precipitate was measured turbidimetrically by Cobas e501/502 (Roche).

IL-10 assay

The enzyme-linked immunosorbent assay (ELISA) approach was employed to measure the quantitative serum levels of IL-10 for all participants. The Quantikine ELISA kit from R&D Systems was utilized for this purpose. The methodology employed in this study utilizes the quantitative sandwich enzyme immunoassay approach. A microplate has been pre-coated with an anti-human IL10 monoclonal antibody. The process involves pipetting standards and samples into the designated wells, where the immobilized antibody selectively binds to any interleukin-10 (IL-10) molecules that may be present. Following the removal of any unattached chemicals through washing, a monoclonal antibody that is specific to human IL-10 is delivered into the wells using an enzyme-linked approach. Following a washing step to eliminate any unbound antibody-enzyme reagent, a substrate solution is introduced into the wells. The subsequent development of color is directly proportional to the IL-10 quantity that has bonded during the initial phase. The cessation of color proliferation is observed, and the magnitude of color intensity is monitored.

Lactoferrin assay

This assay makes use of the Human LTF/LF (Lactoferrin) ELISA Kit from Elabscience, which is an ELISA-based technique. This ELISA kit uses the Sandwich-ELISA technique. This kit's micro-ELISA plate has a human LTF/LF-specific antibody pre-coated on it. The relevant antibody and samples (or standards) are placed in the micro-ELISA plate's wells following the instructions. The Human LTF/LF concentration is negatively associated with the optical density value. We can determine the concentration of Human LTF/LF in the samples by comparing the optical densities of the samples to the standard curve.

Histopathology

The biological material of the tonsil was surgically obtained at Operation room under general anesthesia put in a 10% buffered formalin containing container and sent to the histopathology laboratory to be analyzed. According to previous related studies [19], the examined cases were evaluated for hypertrophy and inflammation taking in consideration the following histological criteria: number of lymphoid follicles/10 mm^2, type of lymphoid follicles, presence of chronic inflammatory infiltration, and individual leukocytes infiltrating the surface epithelium and subepithelial connective tissue, Ugras's abscess, erosions in its superficial layerand the fibrosis presence.

Immunohistochemical evaluation

The tissue block sections were prepared for immunohistochemical assessment using uniform thickness (3-4 μm) and were affixed to electrostatically treated glass slides. Deparaffinization was carried out by immersing the slides in a xylene solution at a temperature of 58 °C for 1-2 hours, followed by two additional immersions of 10-15 minutes each at room temperature. Subsequently, the sections were fixed in cold

acetone (4 °C) for 5 minutes and allowed to air-dry for 30 minutes. The slides were prepared with the Dako LSAB 2 System HRP kits. Subsequently, the chromogen-substrate, 3,3'-diaminobenzidine (DAB), was introduced in a light-restricted environment to produce a brown coloration. Following this step, the slides were stained with hematoxylin for duration of 3-4 minutes. In the present analysis, concentrated antibodies from Thermo Fisher Scientific were utilized. Specifically, the antibodies employed were CD20 mouse IgG monoclonal antibody (HI47) and CD3 mouse IgG monoclonal antibody (S4.1) (catalog number Q10484). Negative controls were performed by running prepared sections without the addition of primary antibodies as part of the routine procedure. The detection of the primary antibody binding was performed using avidin biotin peroxidase detection kits, following the instructions provided by the manufacturer.

Following the completion of immunostaining for both CD20 and CD3, the histopathologists and/or other investigators evaluated the cell count per 20 X field by analyzing a total of 20 fields for each slide. The number of positive cells was then tallied and documented. The grading system not absent, weak intensity, moderate intensity, and strong immunostaining was not considered as the major purpose was counting number of cells to analyze the distribution.

Statistical analysis

Statistical data was presented as mean standard deviation, range, median, or frequencies (number of cases and percentages), as applicable. When comparing two groups, numerical variables were compared using the Student t test for independent samples, whereas when comparing three groups, the One-way analysis of variance (ANOVA) test was used. To compare categorical data, a Chi-square (2) test was performed. Statistical significance was determined when the two-sided p-value was less than 0.05. IBM SPSS (Statistical Package for the Social Science) release 22 was used for all data analysis. This software was developed by IBM Corporation and is compatible with the Microsoft Windows operating system.

Results

Classification of the participants

After exclusion of 21 patients, the 76 participants who were included in this work were classified as follows: group I, which consists of the 38 children with tonsillar hypertrophy; and group II, which consists of the 38 apparently healthy children as a control group.

The candidates in group I were then divided into 2 groups: group Ia, which contained 16 participants with tonsillar hypertrophy but without a history of recurrent tonsillitis; and group Ib, which had 22 participants with tonsillar hypertrophy and recurrent inflammation.

Demographic data of the candidates

There were no statistically significant differences regarding the age and sex distributions between the three groups (Ia, Ib, and II), where the p-values were 0.752 and 0.878, respectively (Table 1).

TABLE 1 Demographic criteria of the candidates

Number		Group Ia 16	Group Ib 22	Group II 38	Level of significance (P)
Age (years)	Mean	7.48	8.19	8.33	F = 0.286
	SD	2.90	4.08	3.98	(P =0.752)
Gender	Female	6 (37.50%)	9 (40.91%)	17 (44.74%)	$\chi 2$ = 0.260
	Male	10 (62.50%)	13 (59.09%)	21 (55.26%)	(P = 0.878)

Comparison of ASOT readings for different groups

There were significantly higher serum levels of ASOT in the tonsillar hypertrophy-associated recurrent tonsillitis group (group Ib) than in group Ia with isolated tonsillar hypertrophy and control group (group II) [495.2 ± 157.6 IU/ml vs. 265.8 ± 38.4 IU/ml and 151.1 ± 80.6 IU/ml; P < 0.001 and p < 0.001, respectively]. In addition, there were significantly higher readings of ASOT in the isolated tonsillar hypertrophy group (group Ia) than in control group (group II) [265.8 ± 38.4 vs. 151.1 ± 80.6; P < 0.001]. Also, there were significant statistical differences regarding ASOT levels when comparing the three groups' results (P < 0.001). Moreover, most of the isolated tonsillar hypertrophy group (group Ia) had their ASOT rank between 200 and 400 IU/ml (13 out of 16), while most of the tonsillar hypertrophy-associated recurrent tonsillitis group (group Ib) had their ASOT rank between 400 and 800 IU/ml (12 out of 22). At the same time, most of the control group (group II) had their ASOT levels below 200 IU/ml (31 out of 38). There were significant differences between the 3 studied groups regarding the ASOT ranking [P < 0.001], as indicated in Table 2.

TABLE 2 ASOT levels within the studied groups

Number		Group Ia 16	Group Ib 22	Group II 38	Level of significance
ASOT (IU/ml)	Mean	265.8	495.2	151.1	F = 76.88
	SD	38.4	157.6	80.6	**(P < 0.001)**
ASOT ranking (IU/ml)	<200	1	1	31	χ^2 = 66.79 **(P < 0.001)**
	200-400	13	6	5	
	400-800	1	12	1	
	800-1200	1	3	1	

Comparison of inflammatory markers for different groups

When comparing children with isolated tonsillar hypertrophy (group Ia) to the control group (group II), there was no statistically significant distinction in the levels of IL-10 [p = 0.102]. In contrast, group Ib (tonsillar hypertrophy-associated recurrent tonsillitis) had statistically higher levels of IL-10 than group Ia (isolated tonsillar hypertrophy) and group II (control) [P = 0.025 and p < 0.001, respectively]. The results of the three groups also revealed statistically significant variations in serum IL-10 levels [P < 0.001], as presented in Table 3.

TABLE 3 Interleukin 10 levels of the studied groups

Number		Group Ia 16	Group Ib 22	Group II 38
IL-10 (pg/ml)	**Mean**	10.48	14.93	7.41
	SD	4.29	6.65	6.81
P value	**Ia vs. Ib**		**0.025**	
	Ia vs. II		0.102	
	Ib vs. II		**< 0.001**	
	Three groups		**< 0.001**	

Our results indicated that the children with isolated tonsillar hypertrophy (group Ia) demonstrated no significant statistical difference in regards to the levels of lactoferrin when compared with the control group (group II) [p = 0.724]. On the other hand, there were statistically significant lower levels of lactoferrin in the tonsillar hypertrophy-associated recurrent tonsillitis group (group Ib) than in group Ia with isolated tonsillar hypertrophy and the control group (group II) [P = 0.024 and 0.027, respectively]. Importantly, no significant statistical differences were noted regarding serum lactoferrin levels when comparing the three groups' results [P = 0.059], as provided in Table 4.

TABLE 4 Lactoferrin levels of the studied groups

Number		Group Ia 16	Group Ib 22	Group II 38
Lactoferrin (ng/ml)	**Mean**	388.41	345.54	398.13
	SD	61.65	50.20	101.65
P value	**Ia vs. Ib**		**0.024**	
	Ia vs. II		0.724	
	Ib vs. II		**0.027**	
	Three groups		0.059	

Immunohistochemical expression

The comparative study of immunohistochemical expression of CD20 (follicular) and CD3 (extra-follicular) on the excised specimens (Figure 1), after histopathological examination of 9 simple hypertrophic tonsillar specimens and 11 clinically and histologically inflamed tonsils revealed a significant difference of the positively stained CD3 cells between both groups, where the expression in the inflamed tonsils was significantly higher than the simple hypertrophied specimens. However, no significant difference was found in the expression of CD20 (Table 5).

TABLE 5 CD20 expression

Number		Hypertrophy alone 9	Recurrently inflamed 11	P-value
CD20	**Mean**	515.5	492.5	>0.05
	SD	92.2	65.3	
CD3	**Mean**	228.5	41	<0.05
	SD	159	25.5	

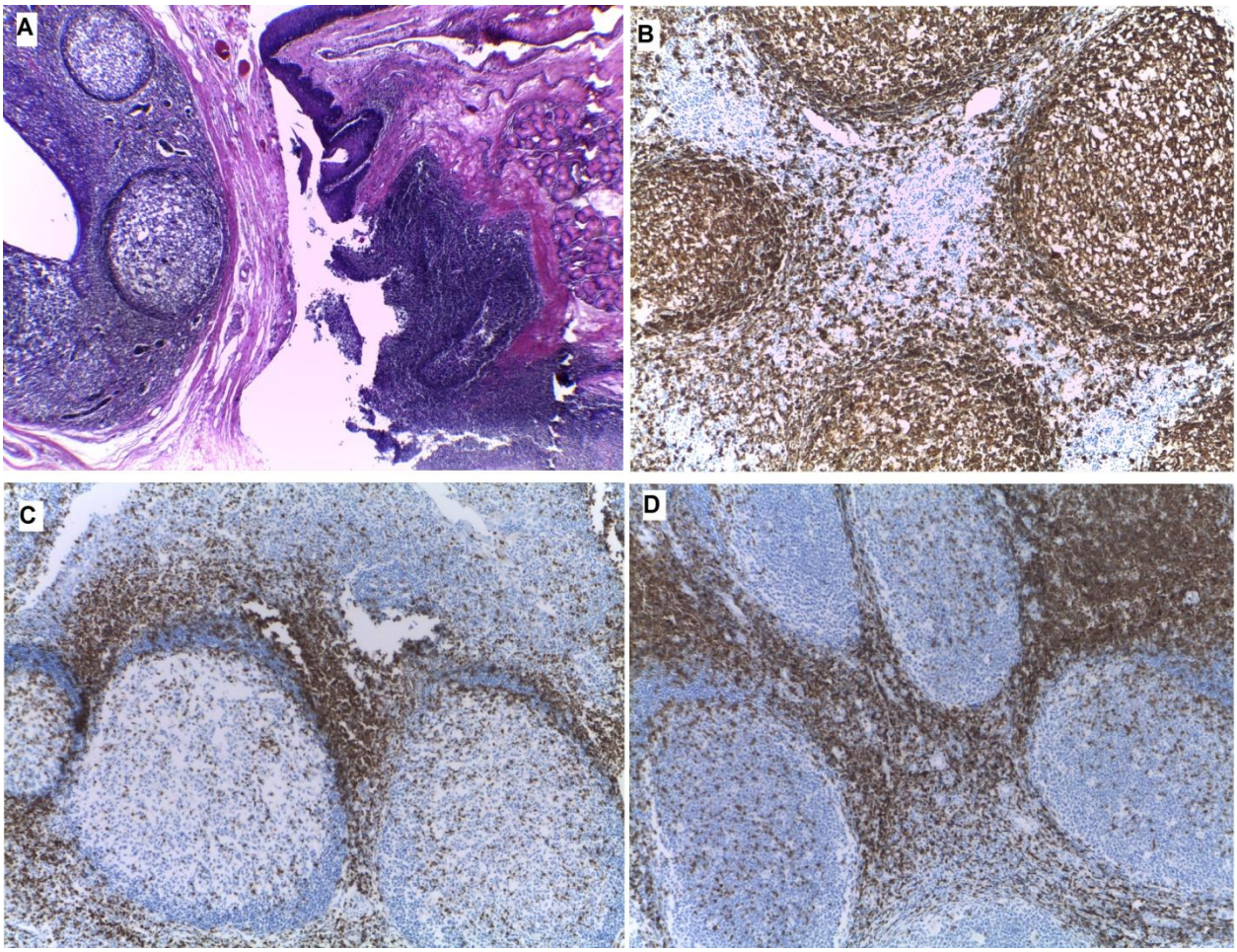

FIGURE 1 (A) Histological picture of a case of tonsillar hypertrophy (H&E, 100x). (B) Immunohistochemical expression of CD20 (100x). (C) Immunohistochemical expression of the CD3 in acase of simple hypertrophy (100x). (D) Immunohistochemical expression of CD 3 in a case of hypertrophy with recurrent inflammation (100x)

Discussion

In this study, we looked at how tonsillar lymphatic tissue affected the systemic immune profile in cases of hypertrophy with or without recurring inflammation. Therefore, the levels of ASOT, IL-10, and lactoferrin were investigated in the sera of 38 children presenting with tonsillar hypertrophy who had or did not have recurrent tonsillitis, in addition to another 38 apparently healthy children enrolled as a control group. The immunohistochemical expression and distribution of CD20 and CD3 on the excised specimens of two groups (hypertrophied only and the inflamed tonsils) also evaluated.

The cutoff point for a normal ASOT in various populations has been researched by numerous authors. It was 200 Todd's units in Sweden [20], 333 Todd's units in Minnesota [21], 240 IU/mL in another study done in several American states [22], 326 IU/mL in Korea [23], 305 IU/mL in Mumbai [24], 239 IU/mL in another part of India [25], and 200 IU/mL in Tanzania [26]. The majority of these readings were higher than the 200 IU/mL threshold that laboratories normally establish [27,28]. Furthermore, another Egyptian study noted that the cutoff point for normal ASOT was significantly higher, up to 400 IU/mL, due to frequent and improperly treated streptococcal tonsillitis in our population [9].

In contrast, the current research indicates that 81.6% (31 out of 38) of the healthy youngsters in the control group exhibited ASOT levels < 200 IU/ml. The presented data demonstrate regions of agreement between the reference ranges provided by Eid *et al.* [29] and the findings of our study. Specifically, a significant proportion (82%) of the control group in their study had ASOT levels below 200 IU/ml, which aligns with our own results. Our study revealed a notable increase in serum levels of anti-streptolysin O titer (ASOT) among individuals with recurrent tonsillitis associated with tonsillar hypertrophy, in comparison to the control group. Several research publications demonstrated similar findings [9, 30-31].

The findings of our study indicate that the immune response involving antibody formation against microbial pathogens, specifically Streptococcus *hemolyticus*, is more pronounced in individuals with tonsillar hypertrophy-associated recurrent tonsillitis compared to those with isolated hypertrophy. To the best of our knowledge, there were a limited number of articles in the existing body of literature that undertake a comparative analysis of this particular subject matter. In addition, the investigation conducted by Bredun and Kosakivska [1] has successfully discovered a significant disparity in blood ASOT levels between children diagnosed with chronic tonsillitis and those presenting with hypertrophy as the sole condition.

While it was observed that the candidates with isolated tonsillar hypertrophy had higher levels of IL-10 compared to the children in the control group, it is important to note that there was no significant difference between the two groups (P = 0.102). In comparison to the isolated tonsillar hypertrophy group and healthy children in the control group, children with tonsillar hypertrophy-associated recurrent tonsillitis exhibited notably elevated levels of IL-10 in their sera (p = 0.025 and p < 0.001, respectively).

This finding can be attributed to the significant participation of IL-10 in two major aspects of infectious disorders. On the one hand, IL-10 stops the spread of immunopathological reactions brought on by an exaggerated immune response to chronic or acute infections. On the other hand, by hindering protective innate and adaptive immune responses, IL-10 plays a crucial role in the persistence and recurrence of microbial agents [31]. Moreover, a review of the published research reveals that there is no research concerned with serum levels of IL-10 in cases of recurrent tonsillitis, except for one study that investigated serum IL-10 levels in children with chronic tonsillitis and those with tonsillar hypertrophy, where their results showed that IL-10 was found only in the serum of children with chronic tonsillitis [1].

Also, the lactoferrin level was investigated in the sera of the candidates in the three groups of the study, where we found that there were statistically significant lower levels of lactoferrin in the tonsillar hypertrophy-associated recurrent tonsillitis group than in candidates with isolated tonsillar hypertrophy and the healthy control group [P = 0.024 and 0.027, respectively]. Similarly, Bredun and Kosakivska [1] noted that the serum lactoferrin level in the chronic tonsillitis group is lower than in the group with tonsillar hypertrophy. These findings may be confined to the high level of the anti-inflammatory cytokine IL-10 in the sera of the chronic or recurrent tonsillitis group.

In this study, we evaluated the expression of CD20 (B cell marker) and CD3 (T cell marker) in both groups examined histologically post tonsillectomy, no significant difference in CD20 was seen; however, CD30 was expressed significantly higher in the tonsillectomy specimens associated with recurrent inflammation. A recently published study [13] evaluated the expression of tonsillar B lymphocytes (CD20) and T-cell (CD3) markers in relation to serological levels of ASOT in cases of chronic tonsillitis and found a

significant difference, however the study did not compare between inflamed and non-inflamed tonsils.

Conclusion

The present study concluded that recurrent tonsillitis accompanied by tonsillar hypertrophy had more intense influences on the systemic immune components than did isolated hypertrophy. On the other hand, the precise nature and individual variations of these influences need further study and investigation.

Acknowledgements

None.

Conflict of interest

The authors declare that there is no conflict of interest in this article.

Funding sources

None.

Ethical approval

The ethical approval was provided from research Ethical Committee at the faculty of medicine, Al-Azhar University, Cairo under ID number: His_395Med.Research00000113.

Informed consent

Provided.

Authors' Contributions

Study design, A M.A.H, A.E.H, H.M.F.E, M.R.Z, M.A.D, M.M.Al, K.R.E, N.M.A, S.A.N, E.M, A.A.A, and A.H; methodology, M.A.H, A.E.H, R.M.G, G.S.S,M.N, M.A.M.I,M.A.A, M.M.Ab, E.M, and A.H; software, A M.A.H, A.E.H, M.A.M.I, M.A.A, M.M.Al, M.M.Ab, H.M.F.E, M.R.Z, M.A.D, E.M, and A.H; investigation, M.A.H, A.E.H, R.M.G, G.S.S,M.N, M.A.M.I, M.A.A, N.M.A, S.A.N, E.M, A.A.A, and A.H; resources, M.A.H, M.A.A, M.M.Ab, M.M.A, H.M.F.E, M.R.Z, M.A.D, M.M.Al, K.R.E, and A.H; data curation, M.A.H, A.E.H, R.M.G, G.S.S,M.N, M.A.M.I, M.A.D, M.M.Al, K.R.E, N.M.A, A.A.A, S.A.N, E.M, and A.H; writing-original draft preparation, M.A.H, A.E.H, R.M.G, M.A.A, M.M.Ab, H.M.F.E, M.R.Z, M.A.D, M.M.Al, E.M, and A.H; writing-review and editing, M.A.H, A.E.H, R.M.G, G.S.S,M.N, M.A.M.I,M.A.A, H.M.F.E, M.R.Z, M.A.D, M.M.A, K.R.E, N.M.A, S.A.N, E.M, A.A.A, and A.H.; supervision, M.A.H and A.H.

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How to cite this article: Mohammed A. Hassan, Ahmed El-Sayed Hammour, Rasha M. Goud, Gehan S. Shalaby, Mohamed Nady, Mostafa Ali M. Ibrahim, Mohammed A. Alghamdi, Rajab A. Alzahrani, Essam Mandour, Hossam M. Farid El Zamek, Mohamed Ramadan Zohri, Mohamed A. Doma, Mohammad M. Alkherkhisy, Ali Abdullah. Alshehri, Noha M. Aly,Suhaib A. Naeem, Ayat Abu-elnasr Awwad, Mohamed Mahmoud Abdellah, Abdulkarim Hasan. A comparative study of selected immunological markers on children with normal tonsils, simple hypertrophic tonsils, and recurrently inflamed tonsils. *Eurasian Chemical Communications*, 2023 5(11), 987-999. **Link:** https://www.echemcom.com/article_180360.html