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RESEARCH

Revising the value of Antistreptolysin O titre in childhood and its interpretation in the diagnostic approach of rheumatic diseases

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Abstract

The burden of group A streptococcus (GAS) infection and its rheumatic sequelae remains dramatically high, especially in low-income countries. Recently, an increased number of Acute Rheumatic Fever (ARF) cases was documented in many regions of Italy. The diagnosis of rheumatic sequelae relies on clinical signs and on the evaluation of the Antistreptolysin O titre (ASO), whose variations are globally reported. To re-examine the standard reference value of ASO titre, by measuring either its upper limit of normal (ULN) in a population of healthy children (HC) or comparing these values with streptococcal antibodies registered in a cohort of patients affected by the rheumatic sequelae of GAS infection. We performed a multicenter retrospective study. We enrolled 125 HC, aged 2–17 years, and a total of 181 patients affected by ARF, acute streptococcal pharyngitis, post-streptococcal arthritis, Henoch-Schönlein purpura and erythema nodosum, divided into four groups. The levels of ASO and anti-deoxyribonuclease B (anti-DNase B) titres were analyzed and compared among the various groups. Moreover, the 80th percentile value was calculated and established as the ULN for ASO titre in HC group. The ULN for ASO titre in overall HC group was 515 IU/mL, resulting in higher than used in the routine investigation. The ASO titre was significantly higher in patients with rheumatic sequelae compared with HC group, with a peak in the age between 5 and 15 years.

Conclusion: Our study established a new ULN normal value of streptococcal serology in a childhood and adolescent population of Italy, suggesting the need to extend this revaluation to the critical areas, in order to avoid underestimating ARF diagnosis. The correct interpretation of ASO and anti-DNase B values in the context of rheumatic diseases has been discussed.

What is Known:

- *The global burden of disease caused by group A streptococcus is not known and remains an important cause of morbidity and mortality. Acute rheumatic fever continues to be a serious worldwide public health problem and a recent recurrence of group A streptococcus infection cases is observed.*
- *The streptococcal sequelae requires evidence of preceding streptococcal infection, commonly elevated streptococcal antibody titre, but the upper limit for these titres varies considerably based on age group, region, and origin.*

What is New:

- *This study provides population-specific values for streptococcal antibody titres in Italy.*
- *Interpret the results of group A streptococcal antibody tests within the clinical context.*

Keywords Acute rheumatic fever · Anti-deoxyribonuclease B · Antistreptolysin O titre · Acute pharyngitis · Rheumatic diseases · Upper limit of normal

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Abbreviations

ARF	Acute rheumatic fever
ASO	Antistreptolysin O titre
Anti-DNase B	Anti-deoxyribonuclease B
GAS	Group A streptococcus
HC	Healthy children
RHD	Rheumatic heart disease
ULN	Upper limit of normal

Introduction

Acute rheumatic fever (ARF), rheumatic heart disease (RHD), poststreptococcal glomerulonephritis, and post-streptococcal arthritis are non-suppurative sequelae of group A beta-hemolytic streptococcal (GAS) infection, due to an unregulated immunological response despite the clearance of the bacterium [1, 2]. Recently, we reported a significative increase of ARF incidence in Tuscany [3], as previously reported in other Italian regions [4, 5], suggesting that Italy may become a high-risk country, according to the new ARF diagnostic criteria [6]. To establish ARF diagnosis, the GAS identification is demonstrated by the rapid throat swab or of the GAS culture or by the evidence of elevated or rising anti-streptolysin O (ASO) or anti-deoxyribonuclease B (anti-DNase B) titres [1]. The isolation of GAS is uncommon, due to previous antibiotic therapy and to natural disappearance of beta-haemolytic streptococcus from the pharynx; indeed, a positive pharyngeal swab occurs only in 25% of patients with ARF [1, 7]. Therefore, the measurement of antibody count by the commercially available antibody assays ASO and anti-DNase B constitutes the most reliable method to confirm the presence of GAS infection [1].

However, several factors may influence the streptococcal antibody levels such as the age of population, the site and the time of onset infection [8, 9], the seasonal variation and particularly the streptococcal strain variability as well as the individual immune response [10]. Therefore, in order to review the normal upper limit (ULN) for ASO titre, the aim of the study was (a) to analyse the ASO antibody level in a large cohort of healthy children (HC) living in Tuscany; (b) to evaluate the ASO and anti-DNase levels in a cohort of children affected by rheumatic conditions and compared to those observed in HC; and (c) to emphasize that the ULN should be periodically revised and defined according to local epidemiology.

Methods

Study setting and samples collection

A total of 306 children and adolescents (61% males, aged 2–17 years with mean age 8.5 ± 3.8 SD) were included in a multicenter observational cross-sectional study (referred to the Pediatric Centers of Pisa and Florence) from January to June 2021. One hundred and twenty-five sex-matched HC.

The inclusion criteria were: (a) absence of a history of immunodeficiency or autoimmune diseases, (b) absence of evidence or history of sore throat or skin infections (erysipelas, cellulitis or impetigo), and (c) absence of intake of antibiotic therapy in the prior 4 weeks. The exclusion criteria were an elevated C-reactive protein (≥ 0.5 mg/dL) and the presence of clinical or subclinical carditis. Therefore, the whole HC group underwent a cardiologic assessment with two-dimensional color-flow Doppler echocardiography and was screened to exclude subclinical carditis. Subclinical carditis was defined as evidence of valvular involvement detected by echocardiography, in the absence of auscultatory findings of valvular dysfunction on clinical examination. Patients showing evidence or suspect of RHD, according to the 2012 World Heart Federation criteria [11] were excluded, while those with physiological mitral regurgitation or trivial aortic valve insufficiency, in the absence of other symptoms and with ASO titre or throat culture negative, were included into the HC group. Moreover, participants in the ARF group with poor adherence or compliance to penicillin prophylaxis were excluded. The study was carried out with blood samples obtained by routine examinations. The exams required and analyzed in the study are usually included in the routine clinical practice. Informed consent was obtained from all the participants' parents or legal guardians. The study was conducted according to the Declaration of Helsinki II. Overall, the data of the study were retrospectively collected and entered into a database anonymously.

Demographic and serological data of patients ($n = 181$) with post-streptococcal arthritis, Henoch-Schönlein purpura, erythema nodosum, bacterial pharyngitis, and ARF (based on the revised Jones criteria ACC/AHA 2015 [6]), were retrieved from the clinical records and entered into a dedicate database. In all patients, the previous streptococcal infection was demonstrated by the evidence of elevated or rising ASO titre or by the throat swab or culture positive for GAS. The acute GAS pharyngitis, due to current or recent GAS infection, was identified through history or clinical evidence of throat infection and confirmed by rapid throat swab or culture positive, according to Italian National Institute of Health guidelines, as previously described [12].

The overall cohort was classified into five groups. Group A included the HC ($n = 125$); group B and C were composed of 32 and 118 patients with acute streptococcal pharyngitis and ARF, respectively; group D included 8 children with post-streptococcal arthritis and group E 23 subjects with Henoch-Schönlein purpura or erythema nodosum (Fig. 1). The assessment of anti-DNase B titre was not evaluated in the HC population, due to the high executing cost.

Blood specimens were obtained using the conventional technique of venipuncture and were promptly placed in a temperature-controlled box on dry ice to maintain their integrity. Subsequently, these samples were carefully transported to the laboratory on the same day of collection, ensuring timely analysis. Upon reaching the laboratory, serum samples were maintained at − 20 °C until antibody testing; then, the samples underwent centrifugation to separate the serum, which was then employed for assessing the levels of antibodies through the testing process.

The ASO titres were measured using automated nephelometric assay (N Latex ASL, BN II System, Siemens Healthcare Diagnostics, Germany), according to the manufacturer's instructions and were expressed in IU/mL, as previously described [13]. The anti-DNase B titres were measured using automated nephelometric immunoassays (N latex ADNase B, BN II System, Siemens Healthcare Diagnostics, Germany), based on manufacturer's recommendations and were expressed in IU/mL [13]. Standardized controls provided by the manufacturer were used for calibration of both tests. The evidence of streptococcal infection was expressed as ASO titre > 250 Todd Units and Anti-DNase B titre > 640 Todds Units, applying standardized normal values.

Determining ULN values for ASO: cut-off selection

The ULN cut-off for ASO titre of HC was achieved by separating the upper 20% from the lower 80% of the group distribution through a dichotomous approach. We determine the 80th percentile as the ULN for streptococcal antibody above in which levels are considered positive for streptococcal infection. Furthermore, the ULN values for ASO titre were stratified on the base of various age-specific ranges.

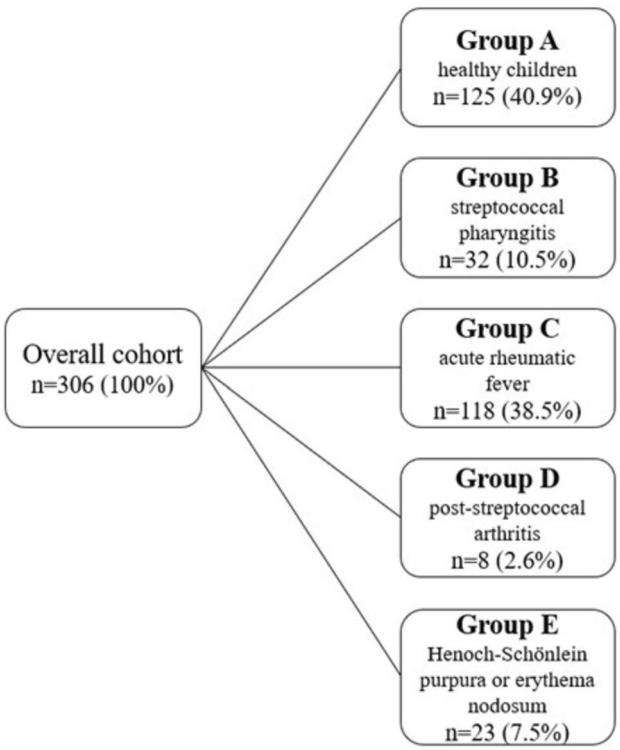

Fig. 1 Flow chart of patients enrolled for study

Statistical analysis

Normally distributed variables were presented as means ± standard deviations (SD); non-parametric variables were expressed as median and interquartile range (IQR). The raw data for both ASO and anti-DNase B titres were transformed into normally distributed through a logarithmic function and there were no extreme outliers. A linear regression was applied to compare the values of ASO titre of various groups versus healthy subject group. Parametric test (Mood's test) was used to compare median values of various groups where appropriate. The most appropriate cut-off value for ASO was determined by the Receiver Operator Characteristic (ROC) curve analysis. A p-value < 0.05 with 95% confidence interval (CI) was considered statistically significant. Data analysis was conducted using Stata V.14.0 Software.

Results

Determining ULN values for ASO and comparison of ASO antibodies values in various study groups

The ULN for ASO value computed for the overall cohort of HC was 515 IU/mL. The ULN values distributed in the four age groups are shown in detail in Table 1. A peak titre was observed in the groups including children and adolescents aged between 5 and 15 years, with a gradual decrease in the following aged group. Table 2 shows the ASO median value of the five groups. The comparison between the ASO median value of each patient's group and the HC cohort revealed that the levels of the ARF group were significantly raised ($p < 0.001$); similarly, the ASO median value of the subjects with acute tonsillitis and post-streptococcal arthritis, when compared to HC group, resulted significantly raised ($p < 0.001$ and $p = 0.011$, respectively). Moreover, no significant difference of ASO values was observed between HC population and patients affected by rheumatic conditions related to a previous documented streptococcal infection ($p = 0.171$) (Table 3).

Seasonal distribution and analysis of ARF group

The ASO levels of healthy subjects were slightly higher in winter than in the other seasons. No significant seasonal variations of ASO and anti-DNase titres were registered in ARF cases. In the ARF patients' group, the mean time between GAS infection and onset of clinical manifestations was 25.5 days; it was considerably higher in patients with rheumatic chorea ($n = 24$) than those affected by rheumatic carditis ($n = 16$): 42.4 vs 21.5 days. Nevertheless, the ASO titre reached a higher value in patients with rheumatic carditis (mean 1450.7 ± 742.5 IU/

Table 1 Upper limit of normal (80th centile) reference values for serum streptococcal antibody titre in healthy children by age group

Age (years)	Subjects (%)	ASO titre (IU/mL) 80% ULN
2–4	28 (22.4)	373
5–10	61 (48.8)	549.5
11–15	25 (20)	503.5
> 15	11 (8.8)	359
Total	125 (100)	515

ASO Antistreptolysin O, *ULN* upper limit of normal

mL) than that one observed in patients with rheumatic chorea (mean 1252.4 ± 618.8 IU/mL).

Anti-DNase titre was analysed in a limited number of patients ($n = 77$, 65.2%) belonging to ARF group, resulting in median value of 1084.3 IU/mL (IQR 519.7–1818.5). Similar values were exhibited in the comparison between patients with rheumatic carditis and those ones with rheumatic chorea (mean 1247.4 ± 966 IU/mL vs 1260.6 ± 1050.7 IU/mL).

We identified the best ASO titre cut-off at 696 IU/mL with a sensitivity of 85.6% (95% CI 0.78–0.91), a specificity of 75% (95% CI 0.68–0.81), an accuracy of 79%, a positive predictive value of 68%, and a negative predictive value of 89% (Fig. 2). Of note, the non-parametric area under the curve (AUC) was 0.86 (95% CI 0.82–0.90).

Discussion

The incidence of ARF persists high in low-income countries and among marginalized sections of high-income countries and in the South Pacific, becoming an important cause of morbidity and mortality [14–16]. In Italy, the incidence of ARF has been scarcely investigated; moreover, we recently demonstrated that Tuscany, according to AHA revised ARF diagnostic criteria, is a high-risk area, according to previous studies performed in other regions of North and Central Italy. This confirms an inversion with respect to the past century, when a decline in the incidence of ARF in developed countries has been described. The measurement of ASO titre is a common and important parameter in the pediatric daily clinical practice to recognize GAS-associated pathologic conditions, being negative only in the 20% of patients who received ARF diagnosis [17]. Furthermore, the knowledge of the ULN for ASO and its interpretation are relevant for the diagnosis of post-streptococcal, non-suppurative sequelae, especially in patients with atypical clinical history or GAS undetectable. Of note, in children with chorea presentation, the evidence of the host immune response against GAS remains the only demonstration of a GAS infection. It is known that ULN for ASO titre is extremely variable in relation to geographic area, age of population (higher titres being observed between the ages of 5 and 12 years), seasonal variation (especially during winter and early spring months), site and time of onset of infection [8, 9, 18], and particularly the streptococcal strain variability and in the individual immune response [10]. Concerning the host immune response, it has been reported that an innate state of immune responsiveness to streptococcal antigens, genetic determinants of HLAs and the presence of certain markers such as B-cell alloantigens influence the GAS immune response [19]. Additionally, it is relevant to underline that immigrant from developing countries and/or marginalized children as well as certain populations, including aboriginal and Pacific islanders [16], are more susceptible to GAS infection, depending on crowded living conditions, inadequate access to the healthcare system, and socioeconomic disadvantage [20]. In particular, the immigrant children in Europe constitute a reservoir of *Streptococcus pyogenes*, causing new epidemic outbreaks either the circulation and the persistence of new strains or variants of the bacterium in certain regions. Thus, the knowledge of the prevalence of GAS strains is of crucial importance for planning appropriate four-level prevention strategies to reduce RHD incidence. The measures to the surveillance of GAS infection should include universal disease registration systems, the tracking

Table 2 Antistreptolysin O titre levels in the studied groups

Group	Subjects (%)	Median ASO (IU/mL)	IQR
Healthy children	125 (40.8)	289	97–493.5
Acute follicular tonsillitis	32 (10.4)	793	520.5–635
Acute rheumatic fever	118 (38.5)	1360	917–1904
Post-streptococcal arthritis	8 (2.6)	1150	879–2645
Other rheumatic conditions[a]	23 (7.5)	432	176–1070

ASO Antistreptolysin O, *IQR* interquartile range

[a]Henoch-Schönlein purpura or erythema nodosum

Table 3 Comparison the Antistreptolysin O titre between the different pathological groups and healthy children group

	Acute follicular tonsillitis (*P*-value)	ARF (*P-value*)	Post streptococcal arthritis (*P-value*)	Henoch-Schönlein purpura / erythema nodosum (*P-value*)
Healthy Children group	< 0.001	< 0.001	0.011	0.171

ARF acute rheumatic fever

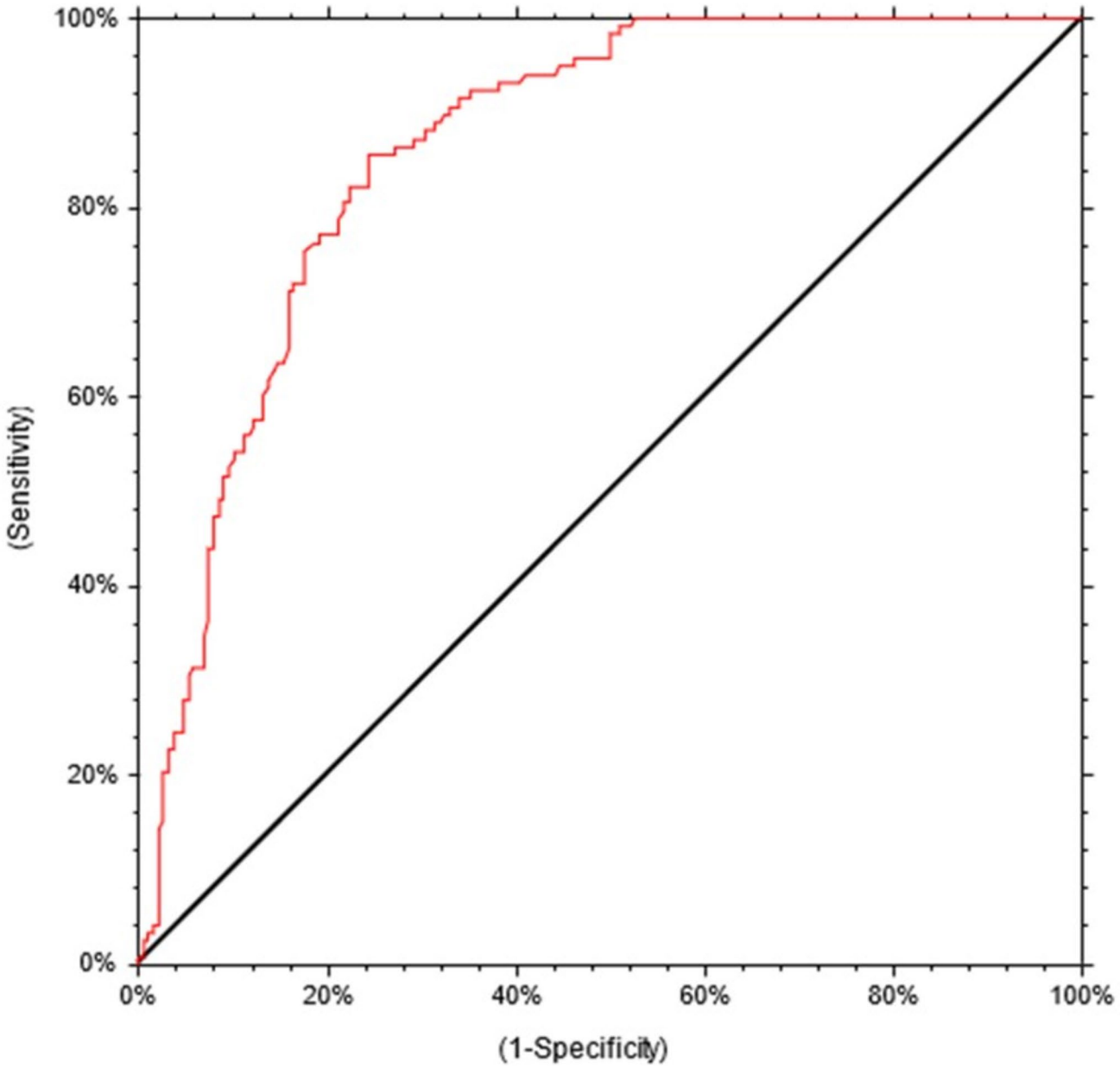

Fig. 2 The receiver operating curve (ROC) of Antistreptolysin O titre, showing an area under the curve (AUC, red line) equal to 0.86

of the disease transmission, and the identification of high-risk populations [21].

To date, age-stratified ULN values were established in the healthy pediatric populations of America, Australia, India, Africa, and Pacific region [10, 13, 18, 22–24]. No investigations in HC living at our latitudes were performed, and the normal values routinely used in Italy are conventionally carried out. Therefore, in this study, we analyzed a large cohort of HC in order to determinate the ULN for ASO titre in a targeted area that we recently identified at high risk of ARF [3]. Interestingly, we found that the ULN was higher than that one conventionally used. It has been demonstrated that the frequency of streptococcal infections and reinfections substantially influence the streptococcal antibody level in healthy populations [25], and that non-rheumatic individuals living in an area with a high incidence of ARF had inappropriately elevated levels of streptococcal antibodies compared to nonrheumatic ones living in an ARF low incidence area [19]. This phenomenon could be related to the heterogeneity and resurgence of rheumatogenic strains and the different distribution of M types, due to the mobility of GAS strains on a global scale [26]. Additionally, it is of note to underline that our healthy pediatric population encompasses the carriers of GAS, whose rate is increasing with child's age,

and represents the major reservoir of GAS in the community [10, 27]. Some individuals harbor the same strain of GAS in the upper respiratory tract for long periods of time ranging from a few weeks to many months without evidence of any symptoms. Their ASO or Anti-DNase titres are elevated, but remain steady over time or show a slight decrease, but usually they do not increase. Interestingly, an analysis of GAS persistence following initial acquisition revealed that 48% of subjects continued to harbor the homologous serotype for > 13 weeks, 32% for > 26 weeks, and 16% for > 52 weeks. Within the subjects harboring GAS for > 26 weeks, none had significant increase in ASO or Anti-DNase titres during the period of GAS persistence [7]. The interpretation of ASO titre for diagnostic purpose is intriguing in these cases, leading to a possibility of false-positive diagnosis of GAS infection. We point out that the interpretation of ASO titre must refer not only to the grade of risk of a specific population of a geographic area, but also to the patient's clinical context. The "carrier state" phenomenon remains poorly understood; prospective studies of the long-term kinetics of the immune response in patients with uncomplicated GAS infections as well as in GAS carriers are required. Moreover, as a previous meta-analysis documented that the pooled prevalence of GAS carriers in healthy subjects with variable socioeconomic levels was 12% [28], their inclusion in our study does not substantially influence the ULN for ASO titre found in our cohort. Due to presence of asymptomatic GAS colonization or carriage in the pharynx of several school-aged children, primary prevention strategies remain controversial and difficult to implement [21]. Additionally, a single cut-off value for age groups was determined, showing a minimal variability in the year-by-year values in the cohort aged 5 to 15 years.

Due to the dynamics of streptococcal antibody response, the ASO titre starts to rise approximately from the first week after GAS infection, with a peak at about the third–sixth week, whereas anti-DNase B titre rises from the first–second week with a slightly late peak from the sixth–eight week [18]. As expected, the median titre of ASO in acute pharyngitis group, ARF and post streptococcal arthritis were significantly increased in comparison with the healthy group. Concerning the group of patients affected by Henoch-Schönlein purpura, the rise of ASO titre confirms that *Streptococcus pyogenes* is the most prevalent trigger of the disease. Indeed, its identification is relevant, as well as the clearance of the infection significantly reduces the relapse or recurrence of purpura [29].

ASO and anti-DNase B titres found in ARF patients were variably raised depending on the time elapsed from GAS infection to clinical presentation, with notable variations between acute carditis and chorea. According to the late presentation of chorea, ASO values were lower, even if not significantly, when compared with rheumatic carditis patients; anti-DNase B titres are in the normal range in a quarter of patients with chorea. Furthermore, the modality and the severity of ARF presentation are not related to the ASO levels tested, as previously described [30]. Kotby et al. reported that, in contrast to the cases of acute rheumatic carditis, levels of the streptococcal antibodies in patients with residual rheumatic heart valve disease were reduced (data not available in our cohort) [22]. It is possible to argue that the penicillin prophylaxis, by avoiding GAS re-infection, can modulate the immune response to restrain the magnitude of antibody response. Moreover, only 16% of patients monitored up to 1 year following an ARF episode continued to exhibit elevated ASO levels, without manifesting recurrent infections [1]. Even in the absence of GAS positive culture, the antibody decay rate following an immune response to GAS infection is often remarkably prolonged. Hence, the correct interpretation the ASO titre in the diagnostic approach of streptococcal sequelae is crucial, in order to avoid overdiagnosis or inappropriate/overuse of antibiotics. We emphasize that this condition characterizes a state of immunological memory response to a previous acute exposure to streptococcal bacterium, therefore not indicating active disease.

Although no cases of skin sequelae to GAS infection are included in our study, previous observations indicated that patients with impetigo commonly develop a brisk anti-DNase B and a feeble ASO response in comparison to patients with pharyngeal infections [10, 31], due to the binding of free cholesterol in the skin to the streptolysin O molecule, with consequent decrease of the antigenic burden [32].

Conclusion

The burden of GAS infection remains elevated in childhood, leading to the increase of ARF incidence and consequently to the progressive definition of "high risk" for many geographic areas (also in developed countries). Therefore, its diagnosis is extremely important to avoid the GAS severe sequelae and the evaluation of ASO titre cardinal in the differential diagnostic process, mainly in the patients in which the chorea is the unique clinical manifestation. The higher ULN titre for ASO, compared to the value conventionally used, as we reported in a cohort HC living in an area at ARF high risk, suggests the need to extend its revaluation to critical areas, in order to avoid underestimating ARF diagnosis. The knowledge of the different distribution of the ASO titres found in different rheumatic conditions, as above mentioned, helps in correct interpretation.

Finally, if the measurement of antibodies to specific streptococcal antigens is necessary to confirm the evidence of the antecedent GAS infection, we point out that it is not by itself diagnostic of GAS acute infection or sequelae, being in some cases the result of an immunological memory

response to a previous GAS contact. Therefore, we recommend that its interpretation should be performed with extensive critical reasoning, within the patient's clinical context.

Authors' contributions Prof.ssa Consolini conceptualized and designed the study. Dr. Alberio and Dr.ssa Biagini contributed to the data collection and to the review of the literature and drafted the initial manuscript. Dr. Di Gangi contributed to the statistical analysis of data and actively participated in manuscript drafting. Prof. Simonini and Prof. Peroni designed the data collection instruments and actively participated in manuscript drafting. Prof.ssa Consolini and Dr.ssa Pagnini reviewed and critically revised the final manuscript. All authors read and approved the final manuscript.

Availability of data and materials Data supporting the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval Data relevant to the study were analysed and reported anonymously. This is an observational and retrospective chart review study design; thus, the ethical research committee approval was waived. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Consent to participate Informed consent was obtained from all subjects included in the study.

Competing interests The authors declare no competing interests.

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