The Serum Level of Proinflammatory TNF-alpha Cytokine in Cyanotic and Acyanotic Congenital Heart Diseases in Mosul City

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ABSTRACT

Background: Tumor necrosis factor – alpha (TNF-α) has been proposed to play an important role in the etiopathology of congenital heart diseases (CHD) worldwide. However, no previous study about the role of TNF-α in the pathogenesis of CHDs in Mosul city / Iraq has been reported .

Objectives: 1) To evaluate the serum levels of TNF-α cytokine in cyanotic and a cyanotic congenital heart diseases (CHDs and to compare the results with control healthy children in Mosul city 2) To find any association between the level of this pro-inflammatory marker and other demographic parameters such as age and gender 3) To test the diagnostic validity of this cytokine for the diagnosis of CHD at different cut-off values.

Patients, materials and methods: A case-control study was conducted in the Department of Microbiology / College of Medicine / University of Mosul over two years and 3 months from April 2019 to July 2021. Twenty nine (29) child with a cyanotic congenital heart diseases and seventeen (17) child with cyanotic heart diseases were included. Another Thirty one (31) healthy child were also included as a controls. . All patients were collected from Al-Khansa teaching hospital in Mosul city. The serum TNF-α concentration was measured in all participants by using ELISA.

Results: Mean age of children with acyanotic heart diseases (2.7 ± 2.9 years) did not significantly differ from that of cyanotics (2.1 ± 1.9) or healthy controls (3.1 ± 1.7) , (P>0.05). The average TNF-α level in acyanotic heart diseases was 321.18 ± 325.71 ng/l compared to 120.63 ± 84.33 ng/l in cyanotics and 119.01 ± 139.71 in healthy controls. TNF- α was significantly elevated in acyanotic heart diseases in comparison to healthy children (P = 0.003). No significant difference was noted between acyaotics and cyanotic heart diseases in regards to TNF- α concentrations (P = 0.07). No age or gender effects were noted on TNF-α concentration in both acyanotic and cyanotic heart diseases (P>0.05). At the best cut-off value of 124 ng/l TNF-α had a specificity of 90.32% , sensitivity of 48.28% and accuracy rate of 39% as indicated by AUC-ROC curve .

Conclusion: The current study showed higher TNF- α in acyanotic (but not in cyanotic) heart diseases compared to healthy controls. TNF-α had poor diagnostic utility to discriminate between CHD and healthy individuals and therefore not recommended as valuable biological marker for the diagnosis of CHD.

Keywords: TNF- α , cyanotics and acyanotics, congenital heart diseases, Mosul city, Iraq.

المستوى المصلي للسايتوكاين الموالى للالتهاب - TNF الفا في امراض التشوهات القلبية الزراقية وبين امراض القلب الخلقية في مدينة الموصل

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الخلاصة

الخلفية العلمية للبحث: يلعب السايتوكاين الموالي للالتهاب TNF - الفا دورًا مهمًا في المسببات المرضية لأمراض القلب الخلقية في جميع أنحاء العالم . ومع ذلك ، لم يتم الإبلاغ عن أي دراسة سابقة حول دور TNF- الفا في التسبب في أمراض القلب الخلقية عند الأطفال في مدينة الموصل / العراق .

اهداف البحث : ١) تقييم مستوى السايتوكاين الموالي للالتهاب TNF - الفا في امصال الأطفال المصابين بأمراض التشوهات القلبية الزراقية والخلقية ومقارنتها بمستوياتها عند الاطفال الاصحاء في مدينة الموصل ٢) التحري عن وجود أي ارتباط بين مستوى هذا السايتوكاين الموالي للالتهاب ومعايير ديموغرافية أخرى مثل العمر والجنس ٣) اختبار الصلاحية التشخيصية لهذا السايتوكاين في تشخيص أمراض القلب الخلقية والزراقية .

المواد وطرق العمل : نوع هذه الدراسة عبارة عن دراسة "الحالات والشواهد " وقد أجريت في قسم الأحياء المجهرية / كلية الطب / جامعة الموصل على مدى عامين و ثلاثة اشهر من نيسان / ٢٠١٩ إلى تموز / ٢٠٢١. شملت الدراسة تسعة وعشرون (٢٩) طفل مصاب بأمراض القلب الخلقية وسبعة عشر (١٧) طفلا يعانون من أمراض القلب الزراقية . كما تم تضمين واحد وثلاثين (٣١) طفلًا يتمتع بصحة جيدة كعناصر تحكم . تم جمع جميع المرضى من مستشفى الخنساء التعليمي في مدينة الموصل. تم قياس تركيز TNF - الفا في امصال جميع المشاركين باستخدام فحص الاليزا المناعي .

النتائج : لم يختلف متوسط عمر الأطفال المصابين بأمراض القلب الخلقية (٢.٧ ± ٢.٩ سنة) اختلافًا كبيرًا عن متوسط عمر الأطفال المصابين بأمراض القلب الزراقية (٢.١ ± ١.٩) أو الاطفال الاصحاء (٣.٤ ± ١.٧) ، (P> 0.05). كان متوسط مستوى مصل TNF – الفا في أمراض القلب الخلقية (٣٢١.١٨ ± ٣٢٥.٧١) نانوغرام / لتر مقارنة بـ (84.33 ± 120.63) نانوغرام / لتر في تشوهات القلب الزراقية و (١٣٩.٧١ ± ١١٩.٠١) في الاطفال الاصحاء. زاد مستوى TNF – الفا بشكل ملحوظ في تشوهات القلب الخلقية مقارنة بالأطفال الاصحاء (P = 0.003). لم يلاحظ أي فرق احصائي بين الاطفال المصابين بتشوهات القلب الخلقية والاطفال المصابين بتشوهات القلب الزراقية فيما يتعلق بمستويات TNF – الفا (P = 0.07). كما لم يلاحظ أي آثار للعمر أو الجنس على تركيز TNF – الفا في كلا من تشوهات القلب الخلقية والزراقية (P> 0.05) . عند أفضل قيمة قطع قدرها ١٢٤ نانوغرام / لتر كانت خصوصية TNF – الفا في تشخيص تشوهات القلب الخلقية ٩٠.٣٢٪ والحساسية ٤٨.٢٨٪ ومعدل الدقة ٣٩٪ وكما هو موضح بواسطة منحنى AUC-ROC .

الاستنتاجات : أظهرت الدراسة الحالية ارتفاع المستويات المصلية للسايتوكاين TNF – الفا في أمراض القلب الولادية الخلقية (ولكن ليس في أمراض القلب الولادية الزراقية) مقارنةً بالأطفال الاصحاء. ومع هذا كان لـ TNF – الفا فائدة تشخيصية ضعيفة للتمييز بين أمراض القلب الولادية الخلقية والاطفال الأصحاء ، وعليه لا يمكن استخدامه كعلامة بيولوجية فعالة في تشخيص أمراض القلب الولادية.

الكلمات المفتاحية : TNF – الفا ، امراض القلب الخلقية ، امراض القلب الزراقية ، مدينة الموصل ، العراق.

INTRODUCTION

Congenital heart diseases (CHDs) are among the commonest congenital abnormalities affecting children [1]. Their prevalence reaches up to 9 per 1000 live births and usually presented as defects in one or more structures of the heart that occur before birth. [2,3]. They are considered as one of the major causes of morbidity and mortality among children after birth [2, 3]. The CHDs are usually classified into cyanotic and acynotic congenital heart diseases [4].

Tumor necrosis factor alpha (TNF-α) is a cytokine with pro-inflammatory features that belongs to tumor necrosis factor super family [5] . It presents in two forms, free and transmembrane - bound forms [6]. The 17 kDa free TNF-α peptide that is composed of 157 amino acids is released after the cleavage of the bound transmembrane 26 kDa TNF-α peptide, which is composed of 233 amino acids via TNF-α cleavage enzyme [6, 7]. TNF-α level increases in the acute phase stage of inflammatory reaction and synthesized by different cells in the body as adipocytes, NK cells, granulocytes, mononuclear cells, neurons and muscles and many other cells [8]. It has a crucial importance in remodeling of bones, leucocyte trafficking, immune system response against various infections, autoimmune diseases, tumor immunology and insulin resistance [9,10]. TNF-α persuades its functions after binding to either of these two receptors, the TNF receptor (TNFR1) and TNF receptor 2(TNFR2) that are expressed on variable immune cells, endothelial lining of blood vessels and other body tissues [11,12] .

TNF-α has been proposed to be implicate in the etiopathology of several kinds of cardiac diseases worldwide [13, 14,15,16,17] . However, previous reports about the role of TNF-α in the pathogenesis of CHDs in Iraq is somewhat lacking. Therefore, the current research was aimed :1) To assess the level of TNF-α in a sample of Iraqi children with CHDs in Mosul city. 2) To investigate its diagnostic validity and 3) To find any association of this cytokine with some other parameters such as gender and age.

MATERIALS & METHODS

2.1 Ethical Approval

Approval for this research was obtained from "Medical Research Ethics Committee (MREC)" of the College of Medicine / University of Mosul [(Ref. no: MREC/2019 (12)] on 26/3/2019. Parents of all children enrolled in this study gave written consent for participation on behalf of their children.

2.2 Subjects and Study Design

A Case – Control study conducted in the Department of Microbiology / College of Medicine / University of Mosul over two years and 3 month from April 2019 to July 2021. Seventy – seven (77) children were recruited from Al-Khansa teaching hospital in Mosul city to participate in this study. Some interruptions in samples collection were occur due to COVID-19 pandemic. The patients included (29) children with acyanotic heart diseases and (17) children with cyanotic heart diseases in addition to 31 healthy children as controls. The inclusion criteria included children with confirmed diagnosis of CHD which was made by specialist pediatric echo-cardiologists and verified by ECHO cardiograph. Exclusion criteria included 1) children with acquired heart diseases such as rheumatic heart diseases 2) other co-diseases that might affect TNF-α level such as co-existent acute infections, sepsis, juvenile rheumatoid arthritis and the presence of autoimmune diseases as determined by specialist pediatrics. Demographic data for patients such as age, sex, residence, presenting symptoms, medical history, family history and drug history were all documented. In addition past obstetric history such as time of birth and mode of delivery were also detailed and reported.

2.3 Methods

2.3.1 Blood Sampling

Human venous blood were collected from all patients and controls in plain tubes. After collection, 1 ml of the blood were centrifuged at 4000 rpm within 1-2 hours for serum dissociation. The serum was frozen at -20 ºC for estimation of TNF-α by ELISA.

2.3.2 Determination of Serum TNF-A Protein by ELISA

The TNF-α levels were determined in the sera of children with CHDs and healthy controls by quantitative sandwich TNF-α 96-well ELISA kit (Bioassay Technology, China, Cat. No E0082Hu) according to manufacturer instructions. This kit has a minimum sensitivity of 1.52ng/L.

2.4 Statistical Analysis

Mann-Whitney test was implemented to compare the means of TNF-α concentrations between patients with CHDs and healthy individuals. Chi square test was used to compare frequencies of nominal data. "Area under the Receiver Operating Characteristics (AUC-ROC) curve" was employed to test the validity of TNF-α in the diagnosis of CHD at different cut-off values. Pearson Correlations co-efficient was utilized to evaluate correlations. MedCalc® Statistical Software version 20 (Belgium) was adopted to perform statistical analysis and construct figures and graphs throughout this study.

RESULTS

3.1 Sample's Demographics

Table 1 summarizes the demographic characteristics of the samples in this study. There were 29 children with acyanotic CHDs, 17 with cyanotic CHDs and 31 healthy controls. Among the studied populations, acyanotic heart diseases were seen more predominantly in females (n = 17, 58.6%) than in males (n = 12, 41.4%), whereas cyanotic heart diseases were more prevalent in males (n =12, 70.6%) than in females (n = 5, 29.4 %) respectively. However, no real difference was reported between females and males in both acyanotic and cyanotic heart diseases ($\chi 2$ = P = 0 .0556). In addition, the mean age of children with acyanotic heart diseases (2.7 ± 2.9 years) did not significantly differ from that of cyanotics (2.1 ± 1.9) or healthy controls (3.1 ± 1.7) (P>0.05). Interestingly, more than 82 % of children with CHD were with history of normal vaginal delivery compared to less than 18% with history of cesarean section. Ventricular septal defect (VSD) was the most frequent acyanotic heart diseases while tetralogy of fallot (TOF) was the most common cyanotic heart disease among our patients. Most patients with acyanotic heart diseases (86.2%) and cyanotic heart diseases (76.5%) had no other associated congenital abnormality.

Table 1 : Demographic characteristics of the samples

	Acyanotics	**Cyanotics**	**Controls**	
Gender	**n = 29**	**n = 17**	**n = 31**	
Female	17 (58.6%)	5 (29.4%)	12	
Male	12 (41.4%)	12 (70.6%)	19	* P = 0 .0556
Age (years)				
Mean ± SD	[a]2.7 ± 2.9	[b] 2.1 ± 1.9	[c]3.1 ± 1.7	**a vs. b, P = 0.451 **a vs. c, P = 0.514 **b vs. c, P = 0.068
Mode of delivery				
Normal	24 (82.6%)	14 (82.4%)	-	
Cesarean section	5 (17.4%)	3 (17.6%)	-	
Type of abnormality				
VSD	8	-	-	
ASD	6	-	-	
PDA	4	-	-	
BiCuspid aortic valve	1	-	-	
Aortic stenosis	1	-	-	
ASD + VSD	5	-	-	
VSD+PDA	2	-	-	
ASD + Pulmonary stenosis	1	-	-	
ASD+Arotic stenosis	1	-	-	
TOF	-	12	-	
Ebstein abnormality	-	2	-	
Endocardial cushing defect	-	1	-	
Total venous return	-	1	-	
AVF	-	1	-	
Other abnormalities				
- ve	25 (86.2%)	13 (76.5%)	-	
+ ve	4 (13.8%)	4 (23.5%)	-	
Family history of CHD				
ve	29	17	-	
+ ve	0	0	-	
Drug history during pregnancy				
ve	29	17		
+ ve	0	0	**-**	**-**
TNF-α (ng/l)				
Range	59.43 -1213.47	37.65 - 339.34	29.32 - 611.38	
Mean	321.18	120.63	119.01	
95% CI for mean	198.04-445.84	77.00- 163.71	67.76 - 170.26	
SD for mean	325.17	84.33	139.71	
Median	113.90	92.11	80.98	
Skewness	1.16	1.64	2.99	

*Chi square test , ** Mann Whitney test, VSD = Ventricular septal defects, ASD=Atrial septal defects, PDA = Patent ductus arteriosus TOF = Tetralogy of fallot,

3.2 TNF- α concentration in acyanotic CHDs, cyanotic CHDs and healthy controls

For children with acyanotic heart diseases the mean TNF serum level ± SD was 321.18 ± 325.71 ng/l compared to 120.63 ± 84.33 ng/l in cyanotics and 119.01 ± 139.71 in healthy controls (**Figure 1**) . No significant difference was noted between acyaotics and cyanotic heart diseases in regards to TNF- α concentrations (P = 0.07). However, TNF-α was remarkably increased in acyanotic heart diseases compared to healthy controls (P = 0.003) , but not between cyanotics and healthy controls (P = 0.27) .

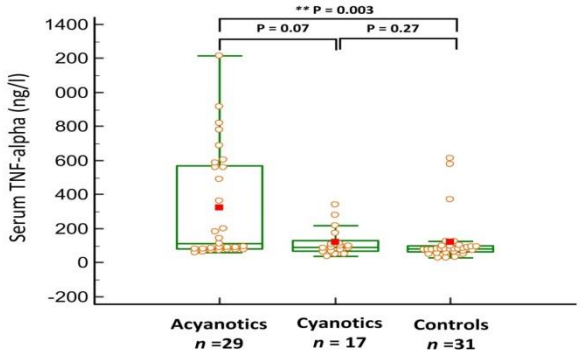

Figure 1 : TNF-alpha concentration in acyanotic CHD, cyanotic CHDs and healthy controls. TNF-alpha significantly increased in acyanotic heart diseases compared to healthy controls (*P* = 0.003). No statistical difference was found between acyanotic and cyanotic groups (*P* = 0.07). Red squares represent the means of each of the three groups respectively.

3.3 TNF - A Concentration According to Gender and Age

In acyanotic group, the mean TNF- α concentration among females (396.05 ± 372.17 ng/l) did not significantly differ from that of male population (216.94 ± 219.21) , P = 0.27 (Figure 2). Similarly TNF- α concentration was not significantly different among females (106.47 ± 64.75) and males (126.14 ± 93.25) in cyanotic groups (P = 0.75).

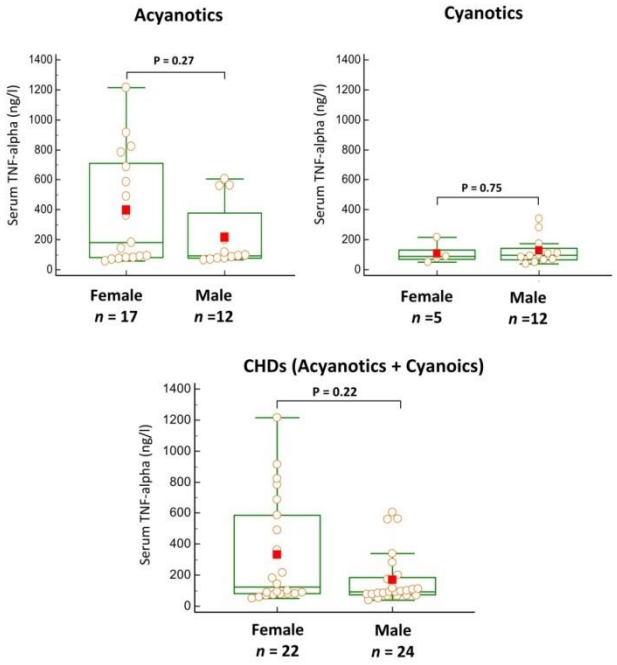

Figure 2 : Serum TNF-alpha concentration according to gender. A) Acyanotic group B) Cyanotic group. No gender effect was noted on TNF-alpha concentration in both groups (P > 0.05). Red squares represent the TNF- alpha means in different studies groups.

To assess the effect of age on TNF- α, we divided the children with CHDs into two age groups, those with ≤ 2 and those > 2 years since most of our patients fell into these two age categories. The average TNF- α concentration in acyanotic children with ≤ 2 years was (250.26 ± 268.42) compared to (423.48 ± 382.17) in older group (Figure 3A) . No significant difference was noted between the two age categories in regard to TNF- α concentration (P = 0.22) (Figure 3B). Similar results were reported in cyanotic children (88.91 ± 38.09 in ≤ 2 years compared to 165.28 ± 113.06 in > 2 years, P = 0.17). To further confirm the effect of age on the serum concentration of TNF- α, we performed a correlation study using Pearson correlation co-efficient to see if the TNF- α serum level was influenced by age in both cyanotic and acyanotic groups. Overall, in confirming to above results, there was no considerable correlation between TNF – alpha and age in both studied populations (r =0.2413, P=0.2073 in acyanotics and r = - 0.2582, P=0.3170 in cyanotics) respectively (Figure 3 C & D).

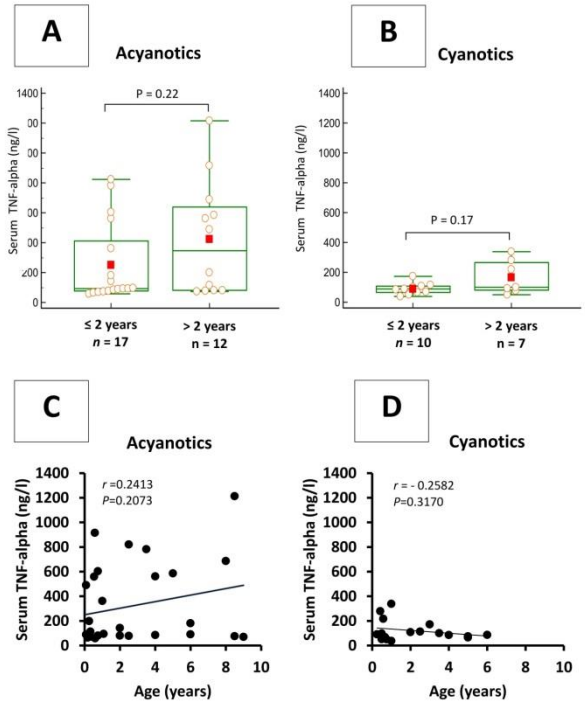

Figure 3 : Effect of age on the serum TNF-alpha. No significant difference was found in the TNF-alpha levels in children ≤ 2 and > 2 years in both acyanotic and cyanotic groups (A &B) . Overall , no correlation of TNF-alpha cytokine with age was noted in both groups (P > 0.05)

3.4 Validity of TNF - α as a diagnostic marker in CHDs

ROC curve was used to evaluate the validity of TNF- α as a diagnostic marker in acyanotic heart diseases since its level was increased significantly over healthy individuals. The results were summarized in (table 2 , Figure 4) . In general TNF - α had poor diagnostic utility in this study . A clear cut from healthy controls was noted at a cut-off >124 ng/l (AUC = 0.717, P = 0.001). Although this cut-off value carried a good degree of specificity (90.32%), but it had low sensitivity of 48.28% .

Table 2: sensitivity and specificitty of TNF-alpha for the diagnosis of CHD at different cut-off values .

Criterion	Sensitivity	Specificity	Youden index (Accuracy rate %)
>29.6574	100.00	6.45	0.06 (06%)
>33.4461	100.00	9.68	0.10 (10%)
>48.4984	100.00	12.90	0.13 (13%)
>53.1578	100.00	19.35	0.19 (19%)
>64.8720537	93.10	25.81	0.19 (19%)
>74.8394481	82.76	32.26	0.15 (15%)
>83.8832	68.97	64.52	0.33 (33%)
>95.11530524	51.72	74.19	0.26 (26%)
>98.7964	51.72	80.65	0.32 (32%)
>102.0761	51.72	83.87	0.36 (36%)
>113.9016786	48.28	83.87	0.32 (32%)
*>124.698	48.28	90.32	0.39 (39%)
>143.5006949	44.83	90.32	0.35 (35%)
>181.9904672	41.38	90.32	0.32 (32%)
>199.3788787	37.93	90.32	0.28 (28%)
>361.9305772	34.48	90.32	0.25 (25%)
>490.3369107	31.03	93.55	0.25 (25%)
>560.2493174	27.59	93.55	0.21 (21%)
>611.3747	17.24	100.00	0.17 (17%)
>686.7834705	13.79	100.00	0.14 (14%)
>782.2271384	10.34	100.00	0.10 (10%)
>821.7746514	6.90	100.00	0.07 (07%)
>915.7876636	3.45	100.00	0.03 (03%)

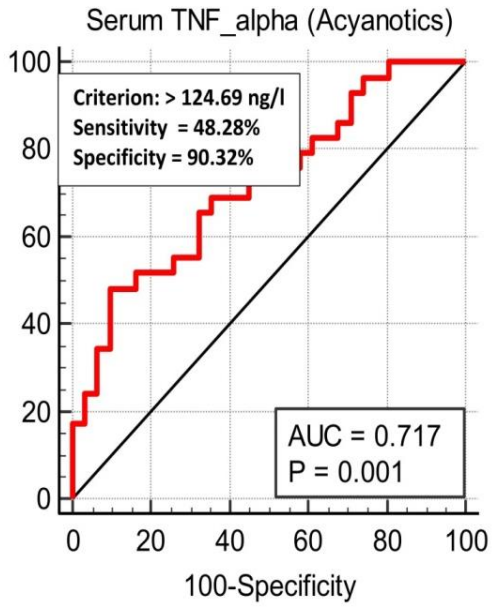

Figure 4: AUC-ROC curve of TNF-alpha in acyanotic heart diseases. A) Sensitivity and specificity of TNF-alpha for the diagnosis of acyanotic diseases at different cut-off values . * represent the optimal cut-off value . B) At 124. 69 ng/l cut-off TNF-alpha has a specificity of 90.32% and a sensitivity of 48.28% and accuracy rate of 39% (AUC = 0.717, P = 0.001).

DISCUSSION

The main positive finding observed in this study was significantly higher TNF- α levels in acyanotic heart diseases compared to healthy children. Higher TNF-α levels in acyanotic diseases might be attributed to the increase secretion of this cytokine by diseased heart since normal human heart does not express TNF-α cytokine whether at mRNA or protein levels [18,19]. Due to its catabolic effect, TNF-α (together with low calorie intake) has been implicated as a possible mechanism for cardiac cachexia syndrome in advanced cases of heart diseases [15]. Moreover, increased circulatory level of this cytokine has been suggested as one of different dysfunctional mechanisms that account for cardiac decompensations seen in complex heart diseases [18]. Evidence has been accumulated during last two decades indicated that TNF-α of cardiac source rather than systemic TNF-α is the main contributor (in an autocrine manner) to myocardial dysfunctions and myocytes apoptosis in variable heart diseases such as myocardial infarction, chronic heart failure and myocarditis [19, 20]. Moreover, TNF-α tends to suppress cardiac contractility in both nitric oxide dependent and independent manners, and thus further implicated to cardiac pathophysiology in heart diseases [21, 22].

The results of the current research also showed that TNF- α levels were elevated in acyanotic in comparison to cyanotic children although no significant clear-cut difference was found between the two populations. Moreover, a non- significant distinction was also found between cyanotic group and healthy individuals. These findings were somewhat weird since TNF-α was suggested to correlate positively with chronic hypoxemia[23] which is more prominent in cyanotics than in acyanotic heart diseases [24]. However, higher levels of TNF-α in acyanotics in regards to cyanotics patients was also reported by other researchers [16]. Although the explication for these findings might be complexed and multifactorial, however, two possible explanations were suggested 1) *Time of presentation of the illness* : Due to apparent presentations, cyanotic heart diseases tends to show up earlier than acyanotic heart diseases which tend to be diagnosed late. In support for this assumption, Shiva et al [16] found that cyanotic patients were significantly younger than acyanotic patients. 2) *Body mass index(BMI):* Although the association of TNF-α with BMI is still a matter of controversy [25], but there is a good evidence of correlation between this cytokine and weight loss and cachexia found in severe cases of heart diseases [26]. Interestingly, Noori et al [17] found that the lowest levels of the BMI was noted in acyanotic rather than cyanotic patients.

Age and gender might affect the impact of the diseases in children. Despite of good evidence of age and gender influences on diseases outcome in children, the information on age and gender influences on CHD are still scanty [27]. Meanwhile, investigations on TNF-α levels in CHD according to these variables are relatively sparse. In this study, no associations and/or correlations between TNF-α serum levels and sex or age in CHD were found. This indicated that the level of this cytokines did not differ significantly in cyanotic and acyanotic patients in terms of age or gender. Similar findings were reported by Shahramian and colleagues [25] who did not find any association either.

To investigate the usefulness of TNF-α as a biological marker for the diagnosis of CHD , AUC-ROC curve analysis was performed. Since TNF-α was only different in acyanotic group compared to healthy children in this research, therefore this analysis was done to examine the potency of this cytokine to efficiently discriminate between these two groups at different cut-off values. The current results showed substandard diagnostic validity of TNF-α for the diagnosis of acyanotic congenital heart diseases. At the best cut-off value of 124. 69 ng/l, it had a specificity of 90.32% but a low sensitivity of 48.28% with overall accuracy rate of 39%. These results were in disagreement with other scholars who concluded that TNF-α is a good marker in CHD [13, 14,15,16,17.] On other hand, Miyoshi et al [28] found that maternal TNF-α alone at a cut-off of 68 pg/ml (ng/l) had a sensitivity of 50.0% and specificity of 93.4% for the diagnosis of heart failure in fetuses with congenital cardiac defects or arrhythmias. Nevertheless, they concluded that if TNF-α was combined with other markers such as vascular endothelial growth factor-D and heparin-binding epidermal growth factor-like growth factor the sensitivity increased to 100% but on the expense of decreasing specificity to 80.3%. On the light of these observations we recommend further investigations to highlights the importance of combination of TNF-α with other biomarkers for early diagnosis of CHDs.

CONCLUSIONS

Up to the best of our knowledge, this study is the first report that highlights the role of tumor necrosis factor – alpha (TNF-α) in the diagnosis of cyanotic and acyanotic congenital heart diseases (CHD) among a sample of Mosul city children. The data of the current study did not support the use of TNF-α as a *biomarker* in the diagnosis of CHD among our children. Further research regarding the benefits of TNF-α in other clinical aspects such as follow up of patients with CHD, response to various medications or referral for cardia surgery is suggested.

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Conflict of Interest

The authors have no personal or financial conflict of interest to declare.

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