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Impact of Glutathione (GSH) and some biochemical markers in β-thalassemia major patients with negative and positive Entamoebae histolytica.

Running title: Entamoebae histolytica and risk of biochemical markers in β-thalassemia major patients.

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Abstract:
Thalassemia syndromes are a broad category of hemoglobin diseases caused by the absence or reduced synthesis of normal globin chains. Glutathione S-transferase (GSH) activity has been found to be altered in individuals with Beta-thalassemia is a hereditary blood condition marked by a diminished or nonexistent synthesis of beta globin chains in hemoglobin. Glutathione (GSH) activity has been studied in individuals infected with Entamoeba histolytica, Studies have shown that GSH activity is present in E. histolytica, and plays a role in the parasite's detoxification and protection against oxidative stress. The goal of this study is to identify several biochemical markers and Glutathione S-transferase (GSH) values that predict outcomes in Iraqi β-thalassemia major who tested positive or negative for Entamoeba histolytica.
Patients and methods: *A total of 200 (150 with β-thalassemia patients and 50 healthy subjects) were involved in this study during their attendance at AL-Karama hospital on Baghdad. The research was carried out from January onwards 2024 to May 2024. The age range of all patients were from (15–35) years and the necessary information's were taken from all patients. Stool specimens were collected from patients with blood and /or mucus diarrhea and five ml disposable syringe was used to collect venous blood by vein puncture. blood was transferred to 10 ml sterile serum separator tubes (gel tube used for detection of: GSH by ELISA ALT, AST, and LDH by using biochemical specific auto-analyzer instrument.*
Results: *The results of biochemical markers showed an elevated levels in liver the enzymes (ALT, AST) and (LHD) was significantly higher than that observed in the control ($P<0.05$). According to serum concentration of GSH showed a lower for β-thalassemia patients' group ($P<0.0001$) (80.83 ± 77.7) as compared with control (320.9 ± 88.1). In the current investigation, there was no statistically significant difference ($P < 0.9042$) detected in the levels of GSH between patients with β-thalassemia and those infected with Entamoeba histolytica. Also, we found no significant difference ($P < 0.2472$) in LDH levels, but a significant increase ($P < 0.0001$) in ALT and AST was observed when comparing to patients with β-thalassemia those infected with Entamoeba histolytica.*

Conclusion: *Patients with β-thalassemia who are infected with Entamoeba histolytica exhibited a notable*

reduction in glutathione levels, along with a significant increase in certain biochemical markers.
***Keywords**: β-thalassemia, Entamoeba histolytica; GSH, ALT, AST, and LDH.*

1. Introduction

Thalassemia syndromes are a broad category of hemoglobin diseases caused by the absence or reduced synthesis of normal globin chains. An estimated 1-5% of the global population is believed to carry a hereditary mutation associated with thalassemia., they are the most prevalent recessive disorder (**Brancaleoni *et al.*, 2016**). Thalassemia is identified by a variety of molecular abnormalities, including premature chain termination, mRNA instability, gene substitution or deletion, and start-up chain synthesis issues (***Nang, 2016; Sharma et al., 2017***). The word "thalassemia" originates from the Greek terms "thalassa," meaning sea, and "haema," meaning blood. This condition arises from genetic mutations that lead to the abnormal production of hemoglobin (Hb) subunits, which are inherited as pathogenic alleles of one or more globin genes located on chromosomes 11 (β) and 16 (α) (**Rachmilewitz *et al.*, 2011**). Thalassemia occurs in two types: α-thalassemia and β-thalassemia (Minor, Intermediate, and Major) (Moafi *et al.,* 2010). α-thalassemia, like sickle cell anemia and β-thalassemia, is predominantly found in tropical and subtropical areas (**Goh *et al*., 2020**). Beta-thalassemia is a hereditary anemia characterized by an autosomal-recessive pattern, resulting in either a complete absence or a significant reduction in the synthesis of β globin chains. This imbalance causes an accumulation of unbound α globin chains within the bone marrow, which in turn leads to the premature death of erythroid precursors and ineffective erythropoiesis. The clinical manifestations of β-thalassemia vary in severity, ranging from the severe, transfusion-dependent form known as thalassemia major to the more moderate, intermediate variants (**Danjou *et al.,* 2011**). In an earlier investigation, Gupta et al. found that the infection rate of intestinal parasites among patients with beta-thalassemia major was 21.9%. This research indicates that individuals suffering from beta-thalassemia major often harbor intestinal parasites. The most prevalent intestinal protozoan identified was Entamoeba histolytica/dispar, followed by Blastocystis hominis. Additionally, infestations of Ascaris lumbricoides and Ancylostoma duodenale were identified as helminthic infections (***Gupta et al.,2016***). Parasite infections also trigger the release of catecholamines, which may lead to the mobilization of red blood cells from the spleen or result in the enlargement of red blood cells as fluid shifts into the intracellular compartment (***Thachil & Bates 2017***). Glutathione S-transferase (GSH) activity has been found to be altered in individuals with Beta-thalassemia is a hereditary blood condition marked by a diminished or nonexistent synthesis of beta globin chains in hemoglobin (*Kotb et al.,2021*). Studies have shown that GSH activity is decreased in beta-thalassemia patients, particularly those with severe forms of the disease. The decreased GSH activity in beta-thalassemia patients has been attributed to oxidative stress, which is a major consequence of the disease. This oxidative stress leads to an imbalance between the production of free radicals and antioxidants, resulting in damage to cellular components and altered enzymatic activities *(**Tripathi et al.,2021**)*. Additionally, it has been suggested that GSH activity may play a role in the pathogenesis of beta-thalassemia, as abnormal levels of GSH activity have been observed in red blood cells and other tissues in these patients. However, the altered GSH activity in beta-thalassemia patients suggests that this enzyme may contribute to the development of the disease and could potentially be targeted for therapeutic interventions (***Bou-Fakhredin et al., 2022***). Infection with Entamoeba histolytica has been linked to increased levels of certain biochemical markers and changes in hepcidin and GSH levels in patients suffering from β-thalassemia major. The present study was undertaken to estimate the cutoff values for GSH and biochemical markers, specifically ALT, AST, and LDH, and to elucidate their correlation with beta-thalassemia who positive and negative for Entamoeba histolytica.

2. Materials and methodologies.

2.1. Populations studied

The present research encompassed 200 (150 with β-thalassemia patients and 50 healthy subjects) during their attendance at AL-Karama hospital and Ibn Al-Baladi hospital in Baghdad. The study was conducted between January 2024 to May 2024. The patients had suffered from β-thalassemia major from different period of time. The age range of all patients were from (15–35) years and the necessary information's were taken from all patients after taking their permission depending on the letter of approved by the Research Ethical Committee and Scientific Committee designated by Middle Technical University, the College of Health and Medical Techniques, and the Department of Medical Laboratory Techniques (No. 351/3) on January 21, 2024.

2.2. Laboratory tests

Stool specimens were collected from patients with blood and /or mucus diarrhea and five ml disposable syringe was used to collect venous blood by vein puncture. Blood was collected into 10 ml sterile serum separator tubes (gel tubes), subsequently centrifuged for 10 minutes at 6000 rpm, and the resulting serum was partitioned into multiple 0.5 ml aliquots, which were promptly frozen at -20°C until required for the measurement of GSH, ALT, AST, and LDH.

Serum concentrations of GSH were evaluated by using the technique enzyme-linked immunosorbent assay (ELISA). While, the alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). The evaluation was conducted utilizing an electro-chemiluminescence immunoassay with the Roche Cobas Integra 400 plus (Roche Diagnostics GmbH, Mannheim, Germany). Serum samples from all participants were processed through the instrument, which automatically determined the concentrations of ALT, AST, and LDH.

2.3. Data analysis (Statistical methods)

The statistical analysis was conducted utilizing GraphPad Prism version 9.2 (GraphPad Software Inc., LaJolla, CA). To evaluate the significance of group variance, Student's t-test and Two Way ANOVA (with Sidak's Multi-comparison Test) were employed. The Pearson correlation coefficient (r value) was used to assess the strength of correlation. Chi-square tests were applied to examine variances in counts. Receiver operating characteristic (ROC) curve analysis was carried out to ascertain the area under the curve (AUC) and to identify the optimal cut-off value for the markers that provided the best predictive capability. Quantitative parametric data underwent the Shapiro-Wilk test to verify normal distribution and were presented as mean ± SD, with statistical significance defined as * $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1 Age and Biochemical Parameters Value in β-Thalassemia Major Patients with or without Entamoeba histolytica and Control

The findings of this study indicated that there was no statistically significant difference in age between patients with β-thalassemia major, who had an average age of 22.91 ± 5.6 years, and the control group, with an average age of 24.54 ± 5.5 years, as illustrated in Table 3-1 and Figure 3-1. From the same table and the accompanying figure indicate a highly significant increase in serum ALT, AST, and LDH levels in the patient group when compared to the control group. The mean values for ALT, AST, and LDH in patients with β-thalassemia were found to be significantly elevated ($P<0.0001$), with values of 94.28 ± 63.7, 77.33 ± 55.9, and 291.7 ± 107.2, respectively, in contrast to the control group, which exhibited mean values of 28.58 ± 8.8, 30.34 ± 9.3, and 171.0 ± 32.4,

respectively.

Table 3-1 Age and Biochemical Parameters Value in β-Thalassemia Major Patients with or without Entamoeba histolytica and Control

Parameter	Mean ± SD		Sig.	*p* Value
	Control	Patients		
Age	24.54 ± 5.5	22.91 ± 5.6	NS	0.0737
ALT	28.58 ± 8.8	94.28 ± 63.7	**	<0.0001
AST	30.34 ± 9.3	77.33 ± 55.9	**	<0.0001
LDH	171.0 ± 32.4	291.7 ± 107.2	**	<0.0001

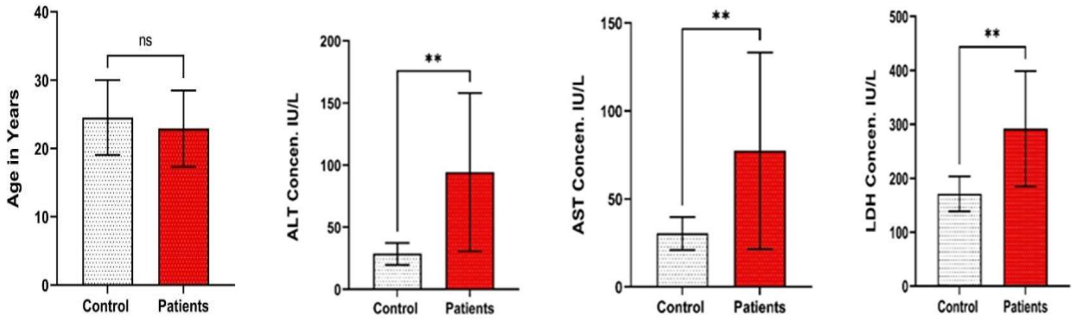

Figure (3-1). The age and certain biochemical indicators of β-thalassemia major in both patients and control groups.

In the present study found no significant difference (P < 0.2472) in LDH levels, but a significant increase (P < 0.0001) in ALT and AST was observed when comparing patients with β-thalassemia to those infected with Entamoeba histolytica, as illustrated in Table 3-2 and Figure 3-2.

Table (3-2). Concentration of ALT, AST and LDH in β-thalassemia major patients with negative and positive Entamoebae histolytica

Parameter	Mean ± SD		Sig.	*p* Value
	+ve	-ve		
ALT	118.3 ± 51.9	74.74 ± 64.43	**	<0.0001
AST	107.0 ± 52.77	49.17 ± 42.83	**	<0.0001
LDH	302.1 ± 115.6	281.8 ± 98.31	NS	0.2472

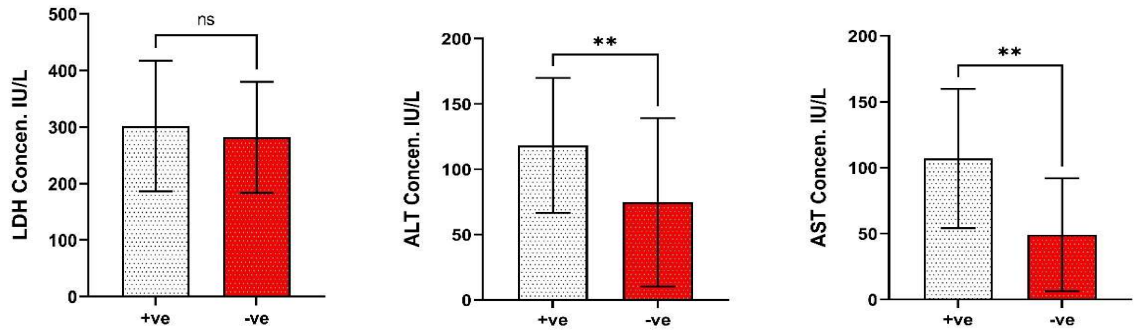

Figure 3-2 - ALT, AST and LDH in β-thalassemia major patients with negative and positive

Entamoebae histolytica

3-2 Concentration of GSH in β- thalassemia major patients with or without Entamoeba histolytica and Control

The levels of GSH exhibited a markedly significant reduction (P<0.0001) in individuals diagnosed with β-thalassemia (80.83 ± 77.7) when contrasted with the control group (320.9 ± 88.1), as illustrated in figure (3-3). Furthermore, this study found no statistically significant difference (P < 0.9042) in GSH levels between β-thalassemia patients and those infected with Entamoeba histolytica.

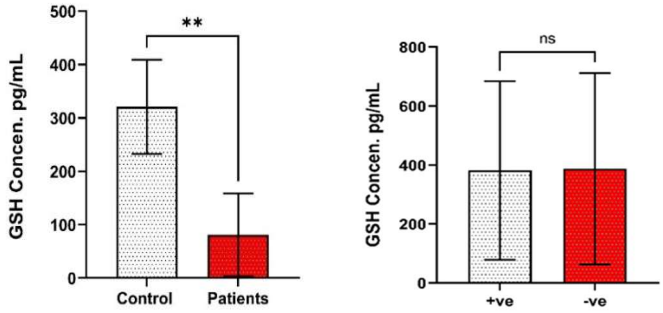

Figure (3-3). GSH levels in β-thalassemia patients and β-thalassemia major patients with negative and positive

Entamoebae histolytica were compared to controls.

Table 3-3 Concentration of GSH in β-thalassemia major patients and with negative and positive Entamoebae histolytica

Parameter	Mean ± SD		Sig.	*p* Value
	Control	Patients		
GSH	320.9 ± 88.1	80.83 ± 77.7	**	<0.0001

Concentration of GSH in β-thalassemia major patients with negative and positive Entamoebae histolytica.

To evaluate the effectiveness of various biochemical markers in predicting disease presence in individuals both positive and negative for Entamoeba histolytica, Receiver Operating Characteristics (ROC) Curve analysis was utilized. This method involves plotting the true positive rate (sensitivity) against the false positive rate (1 - specificity). The area under the ROC curve (AUC) was computed, serving as an indicator of a test's predictive validity. An AUC of less than 0.600 signifies that a test is ineffective as a predictor, while an AUC ranging from 0.600 to 0.700 is considered a sufficient predictor. An AUC between 0.700 and 0.800 is classified as good, from 0.800 to 0.900 as very good, and an AUC greater than 0.900 indicates an excellent predictive test. Based on these AUC thresholds, S. ALT and S. AST emerged as good predictors of the disease, with AUC values of 0.7946 and 0.7595, respectively. LDH was identified as a very good predictor, with an AUC of 0.8867, while GSH was recognized as an excellent predictor of the disease, achieving an AUC of 0.9696 (Figure 3-4).

Parameter	Mean ± SD		Sig.	*p* Value
	+ve	-ve		
GSH	381.3 ± 302.6	387.4 ± 324.5	NS	0.9042

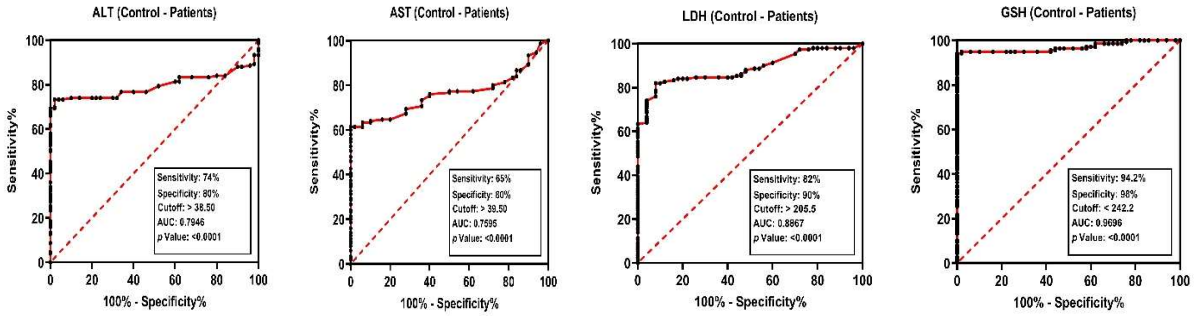

Figure 3-4: The Receiver Operating Characteristics (ROC) curve assesses the validity of S. ALT, S. AST, S. LDH, and S. GSH in predicting disease severity.

4. Discussion

The age of the patients did not exhibit a statistically significant difference when compared to the control group. This outcome may be attributed to the sample selection process. Various studies have indicated differing average ages, with **Chirico et al. (2013)** noting that the mean age of the patients was 34.4 years. **Ang et al. (2014)** indicated that the average age of patients with β-thalassemia was 36 years, with a range from 18 to 59 years. Furthermore, **Poggi et al. (2016)** reported a mean age of 39.9 years among β-thalassemia patients. The discrepancies in these age figures may stem from variations in selection criteria, patient demographics, and lifestyle factors. Additionally, it is conceivable that age may not significant role in the onset of β-thalassemia, as suggested by **Rai & Stuart, (2024).** Further investigation is warranted to better understand the connection between age and β-thalassemia.

In our results noticed the maximum LDH levels were significantly higher (P- value <0.0001**)** in β-thalassemia patients compared to control and no significant difference (P < 0.2472) in LDH levels between patients with β-thalassemia and those infected with Entamoeba histolytica. Lactate dehydrogenase (LDH) can be physiologically quantified in serum as a result of normal cellular turnover, with five isoenzymes identified. Among these, LDH-1 and LDH-2 are specifically found in red blood cells (RBCs). A decrease in the count of red blood cells (RBCs) is a defining indicator of anemia, one might expect a correlation between LDH levels and the presence of anemia (**Klein *et al*.,2020**). Heller and Venger have noted that elevated LDH activity, in conjunction with normal or mildly increased transaminase levels, is commonly associated with megaloblastic anemia (**Heller and Venger 1962**). Research conducted by Albahout et al. indicated that individuals diagnosed with B-thalassemia major exhibited markedly elevated titer of LDH in comparison to those suffering from sickle cell disease and beta thalassemia intermedia (**Albahout *et al*.,2023***)*. Hemolytic anemia in individuals with thalassemia major frequently results in elevated levels of LDH, serving as an indicator of intravascular hemolysis. (**Kato et al.,2006**). Several research studies indicate that the increased levels of LDH in individuals with thalassemia major are attributed to ineffective erythropoiesis (Toren *et al.,* 1996). In thalassemia major, hemolysis combined with IE leads to anaerobic glycolysis, which causes elevated LDH levels (**Musharraf et al.,2017**).

The results of the present study demonstrated a statistically significant increase in the levels of AST and ALT enzymes in thalassemia patients, regardless of whether they were infected with Entamoeba histolytica or not, when compared to the control group, as illustrated in Table 3.2 and Figure 3.2. Iron overload, characterized by elevated serum ferritin levels, is recognized as a contributor to liver dysfunction, as evidenced by increased levels of ALT and AST. Our findings regarding highly liver enzymes among thalassemia patients were agreement with previous reports from Iraq (Mohammad, 2012). Hashim et al. indicated in their research conducted in Maysan that the variation in Transferase concentrations, when compared to healthy individuals, is attributed to excessive hemolysis

(**Hashim et al., 2020**). Excessive red blood cell breakage (hemolysis) or the need for peptide chain synthesis may cause a difference in transportable enzyme concentrations compared to healthy individuals. These enzymes are effective in transmitting amine groups in amino acids (**Al-Khashli and Al-Shawi,2013**). ALT and AST are common in many tissues of the human body. AST enzymes are more effective than ALT enzymes. It is more prevalent in the heart, liver, skeletal muscles, and kidneys. The liver contains a large amount of ALT, as do the kidney, heart, and skeletal muscles. Elevated iron levels in the serum of patients may enhance enzyme effectiveness, potentially resulting in the breakdown of fats within specific organ cells. This phenomenon occurs due to the formation of free radicals, which can damage essential molecules, including lipids, proteins, and cellular DNA. (**Hashim et al., 2020; Bushra *et al*., 2013, Ellis, 2010**). Sateriale and his colleagues (2011) discovered that approximately 50 million infections of the E. histolytica parasite affect the liver each year. Amoebic liver abscesses result from the consumption of food or water that has been contaminated with human feces. Nevertheless, the most prevalent symptomatic infection associated with this condition is amoebic dysentery. In rare cases, trophozoites can invade the intestinal mucosa and spread blood, resulting in extraintestinal disease. Aside from the intestine, the most common symptom is liver abscess. The trophozoites enter the liver via the portal venous circulation and cause significant damage (**Tharmaratnam et al., 2020**). The present results are consistent with earlier studies that demonstrate elevated serum liver enzyme levels in individuals diagnosed with amebiasis (**Pluta & Pluta, 2008**). Moreover, ALT and AST were good predictors of the disease with AUC 0.7946 and 7595 respectively.

Regarding serum Glutathione S-transferase (GSH), in our study found β-thalassemia major patients with and without Entamoeba histolytica had lower levels of serum GSH compared with control the differences were statistically significant (P. value <0.0001) and no statistically significant difference (P < 0.9042) was observed in the levels of GSH between patients with β-thalassemia and those infected with Entamoeba histolytica table 3-3 and **figure 3-3**.

Reduced glutathione (GSH) levels result in increased production of free radicals. Glutathione is essential for protecting proteins required for nucleic acid synthesis, as well as contributing to DNA repair. Furthermore, it contributes significantly to the immune system by supporting white blood cells and preserving the integrity of red blood cells (**Chaudhary et al.,2023; Lin et al.,2024**). Glutathione (GSH) serves as a significant intracellular reducing agent that exhibits high sensitivity to oxidative stress. It is essential for protecting bodily tissues and cells from harm inflicted by reactive oxygen species (ROS). Glutathione (GSH) aids in preserving the integrity of the plasma membrane by preventing lipid peroxidation, modulates gene expression, counteracts oxidative agents, and prevents their generation (**Diez et al., 2015**). Our findings are agreement with the findings by **Muanprasat *et al.*** and **Muaid and Zaidan** in their study reported that the level of GSH is reduced in cases of β-thalassemia major (**Muanprasat et al., 2013 ; Muaid and Zaidan ,2015**). Moreover, the analysis of the ROC curve demonstrated that serum GSH possesses outstanding prognostic accuracy for patients with β-thalassemia major, regardless of their Entamoeba histolytica status, with an AUC of 0.9696. Our study found a significant reduction in serum GSH levels in β-thalassemia major patients with E. histolytica infection. This suggests that the incidence of this parasite may be related to significant oxidative stress, resulting in a decrease of GSH due to an increase in the quantity of free radicals

(Halliwell and Gutheridge,2015). The levels of GSH recorded in this study align with findings from two earlier investigations concerning parasitic infections in humans, specifically those involving T. gondii (Karaman et al., 2008) and (Kaya et al., 2008). These studies indicated that individuals afflicted with fascioliasis exhibited lower glutathione peroxidase activity (an enzyme that facilitates the conversion of H2O2 into water and alcohols utilizing reduced glutathione) in both serum and erythrocytes compared to non-infected individuals. Entamoeba histolytica is a pathogenic organism in humans that is unable to produce glutathione independently; however, it can assimilate this compound from the surrounding growth media or potentially from its human host (Ondarza et al.,1990)

The reduced concentration of glutathione can be linked to the insufficient availability of the substrate required for its synthesis during oxidative stress. This includes factors such as NAD pH, which serves as a crucial stimulating agent for the activity of glutathione reductase. This enzyme plays a vital role in converting the inactive form of glutathione back into its active state (**Muller et al.,2003**). Additionally, the resistance of the parasite to phagocytosis may be attributed to an increase in free radicals, which subsequently results in a reduction of glutathione levels in the serum of patients (**Yazar et al.,2003**).

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