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Association between selected immune parameters and thalassemia: An analytical study

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Abstract

Existing genetic blood disorders are referred to as hereditary anemia. Such disease is especially high in the Mediterranean area. In Iraq, the prevalence of carriers was estimated between 4.5% -5% of the general population. The disease can be clinically presented by mild anemia up to severe manifestations that need transfusion and its complications can usually affect many different organ systems. During the past few years, focus was diverted to the cytokines and electrolyte status as potential disease severity markers. ELISA was used to identify serum cytokines. The analysis of serum sodium, chloride and potassium was performed using the colorimetric technique. The study included two categories, 50 patients (37.96 years) and 40 controls (40.475 years). The results revealed that thalassemia patients possessed significantly higher serum levels of all studied immunological markers (IL-40, TNF-α, and MCP-1) when compared to the control group (p<0.05). Serum sodium, potassium and chloride levels were also significantly different between the two groups, but serum potassium was not so significant (p<0.05). While there were no significant connections between IL-40 and MCP-1 or IL-40 and TNF-α, there was a significant positive association between MCP-1 and TNF-α. However, there was no significant link between potassium and chloride or between sodium and potassium. The Pearson's correlation analysis also showed a substantial positive association between levels of sodium and chloride. In conclusion, this study showed that thalassemia patients exhibit elevated levels of key immune parameters (IL-40, TNF-α, MCP-1) alongside significant changes in certain electrolyte concentrations, particularly sodium and chloride.

Keywords: Thalassemia, Cytokines, IL-40, TNF, MCP-1, potassium, chloride, sodium.

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Introduction

Thalassemia is a hereditary hemoglobinopathy, which is characterized by impaired production of globin chains, that causes chronic anemia, bone marrow proliferation, and a lifetime need of frequent blood transfusion.[1,2,3] This iron overload is an important cause of systemic complications in which many organs and physiological systems are involved. The interaction between thalassemia and immune functioning is complicated and yet to be fully comprehended, even despite the advances in treatment strategies, especially regular blood transfusion, and iron chelation therapy.[4,5] It is becoming increasingly clear that patients with

β-thalassemia, in particular those who are transfusion-dependent display a very broad spectrum of abnormalities in both humoral and cellular immunity.[6] These immune changes involve disproportions in the T-lymphocyte subsets, low ratios of CD4+/CD8+, compromised neutrophil and macrophage phagocytic activities, reduced oxidative burst capacity, decreased natural killer cell activity, and dysregulated production of cytokines.[7,8] Numerous causes were attributed to these immunological disturbances, such as iron overload, chronic antigenic stimulation as a result of repeated transfusions, splenomegaly or splenectomy, and recurrent infections.[9] Among them, iron overload will be central in the process of interfering with the normal functioning of both the innate and adaptive immune responses.[10,11] High plasma iron levels of labile iron induce oxidative stress, which subsequently affects the signal transduction of the toll-like receptor, and alters the patterns of cytokine release. Iron chelation therapy is reported to decrease iron load and oxidative stress thus partially restoring immune functions.[6] Nevertheless, the therapeutic interventions per se can also have an effect on immunogenicity. An example of this is that repeated blood transfusion exposes the patient to various alloantigens that may result in immune sensitization. Likewise, even though splenectomy could be useful in treating hypersplenism or in reducing the need to transfuse, it can also cause immune dysregulation as a result of the reduction in the levels of immunoglobulins and complement proteins.

Thalassemia is often accompanied by a long-lasting inflammatory process, as observed in increased levels of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL- 6), and an increase in levels of anti-inflammatory cytokine, such as interleukin-10 (IL- 10). Also, lower interleukin-2 (IL-2) was associated with defective T-cell activation and premature immune aging in such patients.[9] The humoral immune parameters such as immunoglobulin (IgG, IgA, IgM) and complement (C3, C4) are vital components that indicate immune competence, especially in people who have undergone splenectomy.[7] Moreover, hematological indices, including neutrophil-to-lymphocyte ratio (NLR), are also considered as indicators of systemic inflammation and may be related to the severity of the disease. Since thalassemia has a multifactorial effect on immune regulation, a detailed analysis that incorporates cytokine profiles, immunoglobulin levels, complement activity and iron related biomarkers is essential.

The objective of the current study was to determine the relationships between the specific immunological parameters of thalassemia patients such as transfusion status, iron load, and the functioning of the spleen, compared to normal controls. The results of such an analytical methodology could give important information regarding immune surveillance and the use of specific interventions in the case of this patient population.[6,11]

Materials and Methods

The study was carried out during the period from February to July 2024 and included 50 patients as a study group, in addition to 40 individuals as a normal control group. The study included women aged 37 to 40 years, who were recruited from Al-Fallujah Teaching Hospital. A blood sample (5 ml) was collected from each patient and control subject in a gel tube, and then centrifuged at 1600 rpm. Then sera were separated and stored at -20°C until the tests performed.

In practice, the quantitative sandwich enzyme immunoassay technique was adopted by immobilizing an antibody specific for one of the studied parameters interleukin-40 (IL 40), tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-I) on a solid surface enzyme-linked immunosorbent assay (ELISA) multi-well plate (96-well). The protocol for each immunological test, IL40, TNF and MCP-I were measured using commercially available ELISA kits (produced by Cusa Bio, USA), following the company's instructions. The amount of serum total potassium, chloride, and sodium was determined by colorimetric methods.

Statistical Analysis

Statistical calculations were carried out using the Minitab (MINI) 13 Statistical Software. ANOVA one-way test (for independent measures) was used. The results were verified as the arithmetic mean ± standard deviation. The level of significance was considered at $p \leq 0.01$.

Results

The mean ages of the two study groups, the 50 patients and the 40 normal controls were compared. The control group's mean age was 40.475± 9.4326 years, while the patient group's mean age was 37.96± 9.3196 years. There was no difference in the average age between the two groups (Figure 1).

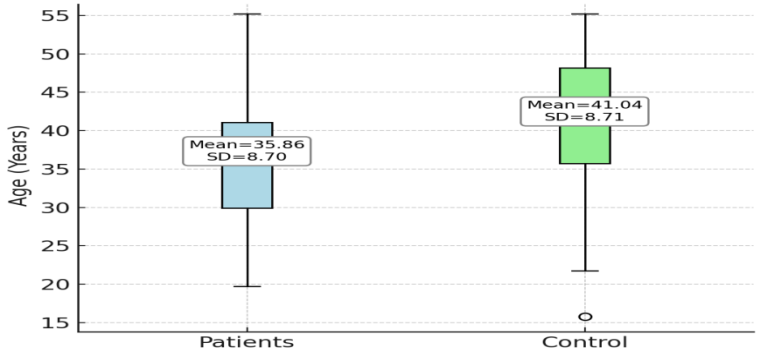

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Figure 1. Differences in age among the patients and control groups.

Immunological study

The research involved a comparison of serum levels of three inflammatory biomarkers IL-40, TNF-a, and MCP-1 in the patient and the control groups. For IL-40, the patient group recorded a mean ± standard deviation of 4390 ± 719.7647, compared to 2432.5±795.6251 in the control group. For TNF-α, the mean serum level was 236.8 ± 52.1203 in patients group, significantly higher than the control group (170.825 ± 44.9135), ($p < 0.05$). For MCP-1, the average concentration in patient was 525.86 ± 52.0988 significantly different than the control group (459.825 ± 44.9135), ($p < 0.05$) (Figure 2).

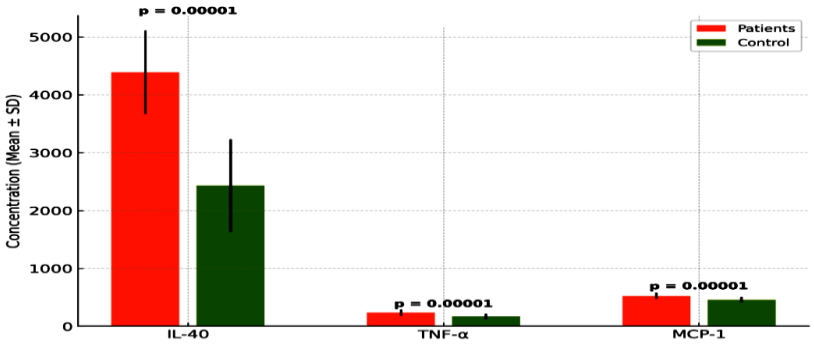

Figure 2. The level of interleukin–40 (IL-40), tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-I) in the patients and control groups.

Biochemical study

The mean sodium level in the patients group was 144±3.24 mmol/l which was not significantly different than the control group (140.88±5.90 mmol/l). The mean potassium level in the patients group (4.266±0.4856 mmol\l), was not significantly different than the control group (4.2975± 0.5894 mmol\l) ($p<0.05$).

The men chloride level in the patients group was 99.5± 1.6194, which was not significantly different than the control group was (100.4±1.5157) (p<0.05) (Figure 3).

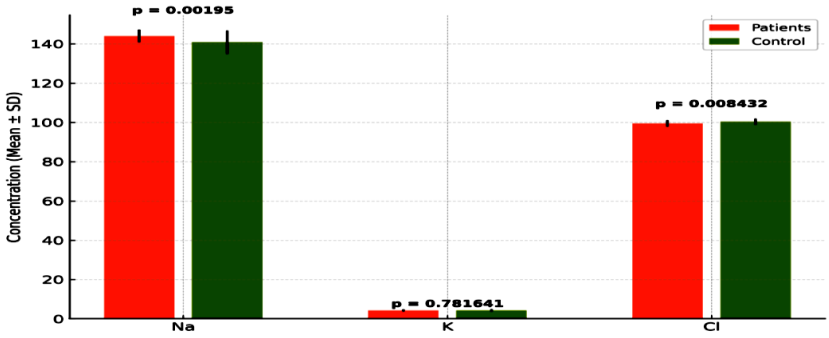

Figure 3. Level of the studied biochemical parameters (Na, K and CL) in the patients and control groups.

Correlations between MCP-I, IL 40, and TNF-α in serum of the patients and controls group

MCP-1, IL-40 and TNF-a levels were compared between the patients and controls groups. The level of MCP-1 in patients was significantly greater than in controls (p<0.01), which showed that the inflammatory activities were higher in patients. On the same note, the level of TNF-α was significantly increased in the patients (p<0.01), implying its role in the disease process. Conversely, IL-40 in all cases was statistically the same in the two groups (p>0.01) and thus could not be associated unequivocally to disease state in this group. In the control group, results of the Pearson correlation indicated that there was a significant large positive relationship between MCP-1 and TNF-α, (r (38) = 1, p <0.001, results of the Pearson correlation indicated that there is a non-significantly small positive relationship between IL-40 and TNF-α, (r(38) =0.2504, p = 0.119). (Table1).

Table 1. Correlations between MCP-I, IL-40, and TNF-α in serum of the patients and controls groups.

group	parameters		IL-40	MCP-I	TNF-α
patients	MCP-I	r	**0.08987**	1	1
		p	NS	0	0
	IL-40	r	1	**0.08987**	**0.08688**
		p	0	NS	NS
	TNF-α	r	**0.08688**	1	1
		p	NS	0	0
group	parameters		IL-40	MCP-I	TNF-α
control	MCP-I	r	**0.2504**	1	1
		p	NS	0	0
	IL-40	r	1	**0.2504**	**0.2504**
		p	0	NS	NS
	TNF-α	r	**0.2504**	1	1
		p	NS	0	0

r: Pearson Correlation, *p*: *P*-value, p > 0.05 is not significant (NS).

Correlations between Na, K, and Cl in serum in the patients and the control groups

The Pearson correlation analysis showed that there was no significant relationship between sodium and potassium (r=0.082, *p*> 0.01), or between potassium and chloride (r=0.082, *p*>0.05. In contrast, there was a perfect positive correlation observed between sodium and chloride (r=1.000, *p*<0.01). This result, however, must be interpreted with caution, as perfect correlations are extremely rare in biological data, and may reflect methodological or data-related constraints, as opposed to an actual physiological association. In the control group, the results of the Pearson correlation indicated that there was a non-significant small positive relationship between Na and K, (*r* (38) = 0.251, *p* = 0.118). Also, the results of the Pearson correlation indicated a significant large negative relationship between Na and Cl, (*r* (38) = 0.593, *p* < 0.001). And, the results of the Pearson correlation indicated that there was a non-significant small negative relationship between K and Cl (Table 2).

Table 2. Correlations between Na, K, and Cl in serum of the patients and control groups.

group	parameters		Na	K	CL
patients	Na	r	1	**0.08176**	1
		p	0	NS	0
	K	r	0.08176	1	**0.08176**
		p	NS	0	NS
	CL	r	1	0.08176	1
		p	0	NS	0
group	parameters		Na	K	CL
control	Na	r	1	**0.2513**	**-0.5933**
		p	0	NS	0.00005
	K	r	**0.2513**	1	**-0.2887**
		p	NS	0	**NS**
	CL	r	**-0.5933**	**-0.2887**	1
		p	0.00005	**NS**	0

r: Pearson Correlation, *p*: *P*-value, *p* > 0.05 is not significant (NS).

Discussion

Demographically, variables as age must be similar in the study groups to exclude possible confounding factors.[13] Under the umbrella of immunosenescence, age is a vital determinant of immune system function and age-related changes in both innate and adaptive immunity are well established.[14,15] Among these changes are reduced productions of naive T-cells, conversion to memory phenotypes and altered patterns of cytokine production.[14] Thus, a significant age disparity between groups can result in biased interpretations in case of immunological parameter comparison. Lack of statistically significant difference in age between the groups increased internal validity of subsequent analysis of immune markers, as the probability of group differences being due to age related changes in immune status. Also, the relatively close standard deviations suggested the similarity in the age distribution

between the two groups, another indication of balanced sampling. However, it should be stressed that statistical non-significance does not mean that there was no difference between the two variables; instead, it should be understood that there was no substantial evidence to show the difference between the two variables. Future research can also take advantage of formal equivalence testing to ascertain that demographic matching is more robust.[16]

Moreover, the average ages of both groups are within adult age range where the immune senescence is generally more robust in the adult age range as opposed to elderly population.[17] Nevertheless, it is still preferable to include age as a covariate in multivariate analyses, especially when assessing immunological parameters, which could be affected by age. In general, the age matching between control and patient groups can be considered as a methodological strength of the current study, as the results could be interpreted more reliably without focusing so much on the age-related bias in the results.

IL-40 is a relatively newer cytokine linked with the regulation of B-cells, immune homeostasis, and chronic inflammatory diseases.[18] The high increase in IL-40 in patients could be connected to the role of IL-40 in sustaining immune dysregulation. Increased IL-40 expression in autoimmune and inflammatory diseases was reported in a previous study.[19] These findings showed significantly high levels of IL-40 in patients, indicating that it may have a role in the pathogenesis of the disease. TNF-α is an established pro-inflammatory cytokine that centrally mediates the process of systemic inflammation, endothelial activation, and subsequent cascades of downstream cytokines.[20] The much higher levels of TNF-α in patients compared to controls is in line with earlier results that found a positive association between high levels of TNF-α and greater oxidative stress, immune activation and the severity of anemia in chronic hematological disorders, such as thalassemia.[21,22] TNF-α has also been implicated in an increase in the levels of MCP-1 which contributes to vascular inflammation, tissue remodeling and oxidative damage in chronic conditions related to iron overload.[23]

In the framework of this study, the continued recruitment of monocytes mediated by high levels of MCP-1 may be a contributing factor to tissue damage and amplification of the effects of inflammatory signaling. IL-40 could be an upstream regulator of adaptive immune responses, whereas TNF-α could be a central effector of systemic inflammation, and MCP-1 could be an upstream regulator of systemic inflammation. Taken together, these biomarkers could be an integrated network of inflammation that leads to the severity and progression of diseases.

The clinical implications of these findings are that it may be targeted towards developing anti-inflammatory therapeutic interventions. Nevertheless, more longitudinal and mechanistic research is needed to gain a better understanding of causal relationships and whether the modulation of these inflammatory pathways can be therapeutically beneficial, especially in patients with high levels of serum sodium.[24]

The higher level of sodium detected in the patients may indicate minor changes in the mechanism of electrolyte regulation or the balance of fluids related to the disease process. High sodium levels could be due to increased secretion of aldosterone, altered regulation of antidiuretic hormone or chronic inflammatory processes that influence renal sodium handling.[25] Other contributory factors might be repeated blood transfusion, iron chelation therapy, and endocrine complications, which are commonly linked to thalassemia.[26] By contrast, potassium levels did not differ significantly when the study groups were compared and thus the potassium homeostasis appears to be preserved despite the underlying disease. The balance of potassium is highly regulated by renal excretion, intracellular-extracellular movement and the lack of any significant differences proves that the mechanisms of potassium regulation are not altered significantly in the study cohort.[27]

The statistically significant but small (1 unit) change in the chloride level of patients might have some clinical implications, since the

chloride level is an important factor in acid-base balance and renal tubular functions.[28] Lower chloride levels could be indicative of more subtle changes in the acid-base homeostasis or the effects of medical interventions like diuretics, but the magnitude of the change observed in this study was small. A recent study indicated that mild hypochloremia can be linked with negative effect in the chronic diseases, possibly due to the fact that sodium and chloride are more prone to disease-related changes than potassium.[29] The close relationship that exists between MCP-1 and TNF--α may be an indication that they share some control mechanisms or are involved in an overlapping role in the processes of inflammation. This is supported by clinical evidence that MCP-1 expression can be directly regulated by TNF-α activity in inflammatory diseases, such as rheumatoid arthritis and ankylosing spondylitis. Even though, it is related to B-cell activity, mucosal immunity and humoral immune responses. In this study, IL-40 failed to demonstrate significant correlations with MCP-1, or TNF-α. This implies that IL-40 could be an independent or context-dependent controller in the immune system.

It is important to note that the ideal correlation (r = 1.000) seen between MCP-1 and TNF-α is not typical in biological data, and should be interpreted with caution. Although it might be a strong biological co-regulation, it might also be caused by methodological constraints, such as a small sample size or methodological constraints. However, with its biological plausibility and correspondence to clinical phenomena, this correlation can still be said to be meaningful.

Physiologically, sodium is the major extracellular cation, and potassium is the major intracellular cation and their gradients are closely regulated by the N+/K+-ATPase pump.[30,31] Though these ions are functionally interdependent with each maintaining membrane potential and cellular homeostasis, this relationship does not always translate into a strong linear correlation in serum levels because of tight mechanisms of homeostasis.

Correspondingly, weak and statistically non-significant correlation between chloride and potassium (r = 0.08176, p = 0.5725) suggested that the level of chloride in the serum has no strong correlation with the amount of potassium in the serum. Chloride is closely related to sodium in restoring osmotic balance and electro-neutrality, and potassium regulation is largely regulated by hormonal processes such as aldosterone-mediated secretion.[32,33]

Conversely, the positive correlation between sodium and chloride (r=1.000, p<0.01) is strong and expected to follow the physiological principles. These ions usually co-regulate and occur as primarily sodium chloride in extracellular fluids. The regulation of their coordinating functions is also necessary to ensure the volume of extracellular fluid, the osmotic pressure, and acid-base balance.[33,34] A previous research indicated that variations in sodium levels are usually accompanied by similar changes in chloride, especially in situations like dehydration, fluid overload or impaired renal sodium handling.[35]

In conclusion, the current research supports convincing evidence that patients with thalassemia have strong immunological and biochemical changes in contrast to normal controls. High concentrations of IL-40, TNF-α and MCP-1 indicated a persistent immune activation and inflammatory process, which might have some role in the pathophysiology of the disease. Major differences in serum sodium and chloride concentrations as well as the high positive correlation between these electrolytes are evidence of dislocation in the electrolyte homeostasis that can be associated with disease progression, treatment impact, or chronic hemolysis. Little change in the concentration of potassium might indicate more stable regulation of this electrolyte or compensatory processes in the thalassemia patients. In addition, the positive correlation between MCP-1 to TNF-α, points to a potential synergistic effect in the facilitation of inflammatory mechanisms.

Author Contributions

HFS, TAA, DJH, AM collected the data and wrote the draft of the manuscript. RTM, proposed the topic of this research and designed the study, and revised draft of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol of this study was reviewed and approved by the Research Ethics Committee of the Ministry of Higher Education and Scientific Research of the University of Baghdad, (approval dated December, 2023).

Informed consent

All study subjects provided a verbal consent before being included in the study. To achieve confidentiality and anonymity of the participants, no picture of patients, controls and other recognizable body parts were taken in this study.

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