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POSSIBLE EFFECT OF VITAMIN D SUPPLEMENTATION ON METHOTREXATE ADVERSE EFFECTS IN PEDIATRIC PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background: *The primary medication used in acute lymphoblastic leukemia is methotrexate, and the problem with this treatment is that it is hazardous to cancer patients. The study aim was evaluation of vitamin D effect on methotrexate toxicity, including oral ulcerations, bone marrow, renal, and hepatic toxicity, besides inflammatory mediators; IL-6 and TNF-α role in this toxicity.* ***Methods:*** *Methotrexate was used to treat 58 patients with acute lymphoblastic leukemia in a clinical trial which was a double-blinded randomized study; (30 in Group A - no vitamin D for 2 weeks after methotrexate treatment) and (28 in Group B - patient received vitamin D3 drops for 2 weeks after methotrexate treatment).* ***Results:*** *Leukocytic count, Hemoglobin and platelet count were significantly lower in patients didn't receive Vitamin D compared to patients received Vitamin D. Serum bilirubin, liver enzymes, urea, creatinine, IL-6 and TNF-α levels were significantly higher in patients didn't receive vitamin D compared to the other group, while there is no significant change in levels of albumin and total proteins in both groups. 24 hours after 2nd cycle of methotrexate, the study group's methotrexate level significantly decreased, while it significantly elevated in the control group. Mild oral mucositis grade (I, II) showed a significant increase in the study group and a decrease in the control group, whereas severe oral mucositis grade (III, IV) showed a significant decrease in the study group and an increase in the control one.* ***Conclusion:*** *Medication with vitamin D may be a significant factor in reducing methotrexate toxicity*

Keywords: *Acute Lymphoblastic Leukemia, Methotrexate, Oral Mucositis, Vitamin D*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a serious condition that resulted from multiplication and malignant transformation of lymphoid progenitor cells in the blood, bone marrow, and extra-medullary locations. Even though children account for 80% of cases of ALL, the disease can be fatal in adults. 1.6 cases of ALL are thought to occur for every 100,000 persons[1]. Leukemia causes for around 33% of pediatric cancers in Egypt. The distribution of ALL is bimodal, with the first peak occurs in youth with the second one reaching approximately 50 years of age[2]. The most prevalent clinical findings and symptoms are typically signs of the underlying thrombocytopenia, neutropenia, and anemia, all of which point to a disruption of healthy hemopoiesis. Fatigue, bone pain, pallor, purpura, bleeding, and fever are among the symptoms[3]. Modern ALL treatment regimens divide therapy into four key therapeutic components: remission induction, CNS preventive therapy, consolidation, and maintenance therapy. As our understanding of

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the disease has progressed, so has our approach to treating it[4].

One crucial drug used in the management of pediatric ALL patients is methotrexate (MTX). This drug is commonly used to treat autoimmune diseases as well as neoplastic conditions such osteogenic sarcoma, lymphoma, choriocarcinoma, leukemia, and head and neck cancer[5]. MTX is referred to as an antimetabolite or antifolate. There is evidence that it affects cancer. Methotrexate inhibits the enzyme dihydrofolate reductase (DHFR) that contributes in the synthesis of tetrahydrofolate, in cancer patients[6]. By blocking the enzymes responsible for nucleotide synthesis, MTX limits cell division and has anti-inflammatory properties[7].

MTX has narrow therapeutic index and serious adverse effects[8]. Oral mucositis, hematological toxicity, and impairment of liver and kidney functioning are examples of serious consequences[9, 10], Thus, "Can we protect pediatric patients from these serious side effects, can we improve these patients' quality of life by simple approaches without interfering with the therapeutic effect of methotrexate?" is the main research question.

Certain general precautions of high dose MTX intake and post-treatment management are fundamental to all regimens in order to prevent MTX toxicity, such as MTX drug monitoring, liver and kidney function tests and complete blood count[11]. Additionally, one crucial factor in reducing the toxicity of MTX is leucoverin rescue[8-11].

Due to its extreme cellular toxicity, methotrexate is advised in cases of high-risk malignancy at doses of up to (5gm/m^2). Close drug monitoring is mandatory[10].

Many studies liked between chronic illnesses and vitamin D insufficiency[12]. A meta-analysis of observational studies investigating the relationship between the risk of major diseases and serum 25-hydroxyvitamin D levels, the most accurate biomarker of an individual's vitamin D status, was one of the studies[13]. Besides, vitamin D affects circulating inflammatory cytokines[14]. Long term usage of low dose vitamin D supplements 10-20 µg/day or 400-800 IU/day has been linked to decrease major adverse events and may reduce mortality in people with poor vitamin D individuals[15].

There was a study in 2019, Oosterom and colleagues observed that children with ALL who experienced severe oral mucositis after HD-MTX therapy had lower vitamin D levels. MTX-induced oral mucositis in ALL children was also linked to vitamin D insufficiency[53].

The rationale of the present research was to improve the quality of life for a group of pediatric patients with ALL and MTX adverse effects by giving them vitamin D supplements. Additionally, the study aimed to determine whether vitamin D could improve any other MTX adverse effects.

The key purpose of the study was investigation of the effects of vitamin D prescription on methotrexate-related side effects, such as bone marrow, hepatic, renal toxicity, and oral mucositis in patients with acute lymphoblastic leukemia. It was also looked into how IL-6 and TNF-α which are important inflammatory biomarkers could influence methotrexate toxicity.

METHODS AND PATIENTS

This work was randomized double blinded randomized clinical trial with digit allocation for each treatment groups. After Assiut Faculty of Medicine ethical committee approval no: 17101228, date: 9/11/2020, this study was conducted on 58 patients using high dose methotrexate (HD-MTX) for treatment of acute lymphoblastic leukemia (ALL) in South Egypt Cancer Institute from September 2021-September 2022. They were selected from 100 patients who recently joined pediatric department receiving chemotherapy MTX.

All patients subjected to comprehensive history and oral ulcer examinations in addition to clinical and laboratory testing (e.g., complete blood count, liver function test, kidney function test, serum level of methotrexate, vitamin D, IL-6, and TNF-α).

Regarding the World Health Organization (WHO), oral mucositis was graded as follows[16]; Grade 0: no mucositis of the mouth, Grade 1: Pain and erythema, Grade 2: ulcer but able to chew solid food, Grade 3: because of mucositis and ulcers, a liquid diet are required, Grade 4: mucositis causes ulcers and prevents alimentation.

Inclusion criteria

Patients in the 1–18 year age range, suffering from acute lymphoblastic leukemia, receiving high doses of methotrexate 5gm/m^2, during consolidation phase and free from other chronic comorbid diseases.

Exclusion criteria

Patients on low doses of Methotrexate, with other comorbid diseases or refusal of the patient to join the study.

Drugs used in the study

MTX vials: Unitrexate 50 mg/2 mL (United Biotech Pvt Ltd.), solution for injection intravenous (IV). The dose used in ALL in consolidation phase: 5gm/m^2 [17].

Vitamin D drops: Alfacareno (1-alpha-hydroxyvitamin D_3) 2 mcg / mL oral drops (Kahira Pharmaceutical and Chemical industry). For neonates and premature infants, the dosage is 0.05 to 0.1 µg/kg/day, or roughly 5 drops per day; for children under 20 kg of body weight, it is 0.05 µg/kg/day, or roughly 10 drops per day[18].

Deficient (10 ng/mL), insufficient (10 - 30 ng/mL), sufficient (30 - 100 ng/mL), and potentially hazardous (>100 ng/mL) were the four vitamin D concentration levels that were taken into consideration in the investigation. For infants under 12 months, 400 IU per day of vitamin D is the Recommended Dietary Allowance (RDA) and 600 IU for children aged between 1 to 18 year[19].

Chemicals and kits used in the study

Methotrexate assay kit: Siemens Company, Ireland, used for quantitative analysis of methotrexate in human serum. *Vitamin D assay ELISA kit:* Diametra Company, Italy, utilized for determining the amount of 25OH vitamin D in human serum via immunoenzymatic analysis. *TNF alpha (TNF-α) assay ELISA kit:* Thermo Fisher scientific Company, Belgium, used for quantitative immunoenzymatic determination of TNF alpha concentration in human serum. *Interleukin 6 (IL-6) assay ELISA kit:* ThermoFisher scientific Company, Belgium, used for quantitative immunoenzymatic determination of IL-6 in human serum.

Patients groups

Following fulfilling the inclusion criteria, patients who were recruited had been classified into two sectors at random; *Control Group I* (n = 30): patients treated with HD-MTX (5 gm/m^2 once intravenous). *Study Group II* (n = 28): patients treated with HD-MTX (5 gm/m^2 once intravenous) + (vitamin D in the form of oral drops daily for 2 weeks after 1st cycle with HD-MTX until the 2nd cycle).

All patients were subjected to laboratory investigations. The investigations had been taken 3 times*: **1st time***: after hospital admission of the patient before taking chemotherapy, blood picture, renal function, liver function tests and baseline vitamin D level test were done. ***2nd time:*** after 1st cycle with HD-MTX, blood picture, liver function, renal function tests, methotrexate level and serum inflammatory markers; TNF alpha and interleukin 6 were measured. Patients of study group received vitamin D drops for 2 weeks after 1st cycle with HD-MTX and patients < 20 kg received 0.05 µg/kg/day, patients > 20 kg received 1 µg/day until the 2nd cycle. ***3rd time:*** after 2nd cycle (after 2 weeks) of HD-MTX, blood picture, liver function, renal function tests, methotrexate level, TNF alpha, interleukin 6 and vitamin D level were determined.

Sample collection, storage, and handling:

Blood sample: 5 ml of venous blood were collected under complete aseptic condition into EDTA tubes and divided into: 2ml of venous blood for a complete blood count and 3 ml of blood centrifuged at speed of 2000-3000 rpm for 15 minutes and used for liver function, urea, creatinine, vitamin D, IL-6 and TNF-α measurement. Samples were stored at -80°C till use.

Blood picture (complete blood count):

The CELL-DYN Ruby System was used to perform a complete blood count. Using whole blood samples, this automated hematology analyzer counts peripheral blood cells[20].

Liver and renal function tests, Vitamin D, IL-6 and TNF-α levels:

Enzyme-linked immunoassay was utilized for the measurement, and an ELISA reader and washer were used[21].

Methotrexate level test

Methotrexate level was done by using VIVA-E system[22].

RESULTS AND DISCUSSION

Results

I- Effect of treatment with oral vitamin D drops on the serum vitamin D Level

According to the present findings, the study group that received HD-MTX and vitamin D drops experienced a significant elevation in vitamin D concentration after two weeks, while the control group, who received HD-MTX without vitamin D drops, experienced a significant decrease in vitamin D concentration, as shown in **(Table. 1 and Fig. 1)**

II- Effect of treatment with vitamin D on complete blood count

The total leukocytic count, hemoglobin & the count of platelet were significantly lower in the control patients and significantly higher in the study patients indicating a significant impact of the tested medications on the blood constituents. Furthermore, both groups' mean neutrophil counts were significantly lower, but neither group's mean monocyte count changed significantly during the course of the study, as demonstrated by **(Table. 2 and Fig. 2A, 2B, 2C, 2D, 2E)**

Table 1: Effect of Treatment on the Vit.D Level on the two groups (control group, vit.D treated group) of patients with ALL (treated with HD-MTX)

	Control Group (n = 30)	Study Group (n = 28)	P-value*
Vit-D Level (ng/ml)			
• **Baseline**	30.77 ± 3.8#	22.09 ± 2.3#	**= 0.006**
• **After 2-weeks**	17.27 ± 2.1##	51.62 ± 3.4##	**< 0.001**
p-value**	**< 0.001**	**< 0.001**	**< 0.001*****

Two Way RM-ANOVA *between groups **within group ***Interaction analysis.
- Data represent mean ±SD of 58 observations (30 control group and 28 study group).
#significant difference at p <0.05.
##Highly Significant difference at p <0.01.

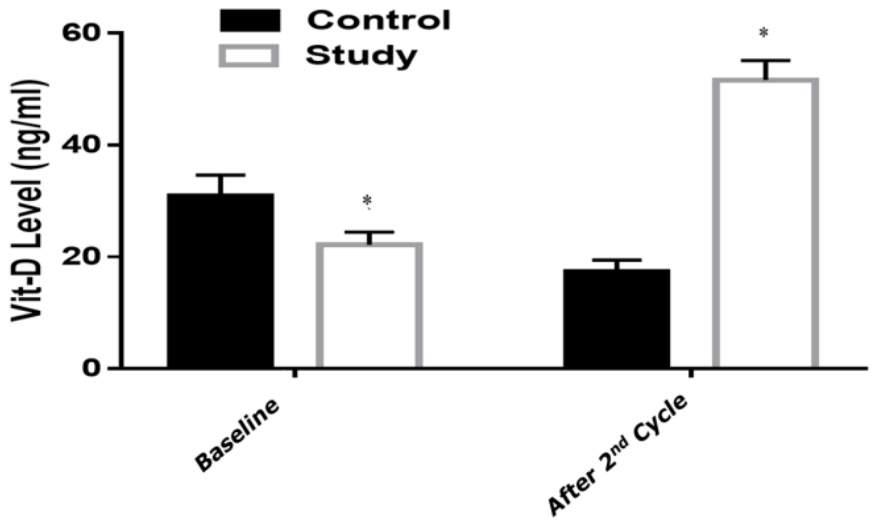

Fig. 1: Change of vitamin D level within control group and vit-D treated group of ALL patients (treated with HD-MTX).
Data represent mean ±SD *Significant difference at p <0.05 vs control group.
Vit-D: vitamin D, ALL: acute lymphoblastic leukaemia, HD-MTX: high dose methotrexate

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Table 2: Change of Laboratory Findings within the two groups (control group, vit.D treated group) of ALL patients (treated with HD-MTX) regarding the CBC parameters: TLC, neutrophils, monocytes, haemoglobin and platelets.

(Mean ± SD)	**Control (n = 30)**	**P-value****	**Study (n = 28)**	**P-value****	**P-value*****
TLC*10^3 /ml					
• **Baseline (1)**	5.50 ± 0.5		4.09 ± 0.4		**= 0.026**
• **After 1st MTX(2)**	3.56 ± 0.3		2.88 ± 0.3		= 0.095
• **After 2nd MTX(3)**	4.06 ± 0.7$^{\#}$	**1 vs. 3=0.006**	4.56 ± 0.3$^{\#\#}$	1 vs. 3=0.117	= 0.528
P-value*	**= 0.029**		**= 0.001**		**= 0.010***
Neutrophil%					
• **Baseline (1)**	51.99 ± 3.7		49.50 ± 2.6		= 0.582
• **After 1st MTX (2)**	38.63 ± 3.5		32.09 ± 2.5		= 0.141
• **After 2nd MTX(3)**	42.77 ± 3.8$^{\#}$	**1 vs. 3=0.017**	46.3 ± 3.8$^{\#\#}$	1 vs. 3 =0.364	= 0.571
P-value*	**= 0.017**		**< 0.001**		= 0.153*
Monocytes%					
• **Baseline (1)**	12.56 ± 1.8		14.47 ± 1.2		= 0.370
• **After 1st MTX (2)**	9.60 ± 1.1		13.43 ± 1.1		**= 0.018**
• **After 2nd MTX(3)**	13.12 ± 2.1	1 vs. 3=0.790	13.82 ± 1.6	1 vs. 3=0.744	= 0.795
P-value*	= 0.181		= 0.823		= 0.541*
(Mean ± SD)	**Control (n = 30)**	**P-value****	**Study (n = 28)**	**P-value****	**P-value*****
HGB (g/dl)					
Baseline (1)	11.02 ± 1.5		10.80 ± 1.4		= 0.605
After 1st MTX (2)	10.14 ± 1.3		9.90 ± 1.1		= 0.523
After 2nd MTX(3)	9.78 ± 1.2$^{\#\#}$	**1 vs. 3=0.001**	11.3 ± 1.3$^{\#\#}$	**1 vs. 3=0.031**	**< 0.001**
P-value*	**< 0.001**		**< 0.001**		**< 0.001***
Platelet*10^3 /ml					
Baseline (1)	308.4 ± 21.7		320.4 ± 15.9		= 0.663
After 1st MTX (2)	240.9 ± 21.9		245.1 ± 14.7		= 0.873
After 2nd MTX(3)	260.3 ± 22$^{\#}$	**1 vs. 3=0.016**	317.2 ± 19$^{\#\#}$	1 vs. 3 =0.836	= 0.061
P-value*	**= 0.003**		**< 0.001**		= 0.126*

***Two-Way Repeated Measure ANOVA was used to compare the mean differences over time.**
****Post-hoc test with Bonferroni Correction for Pairwise comparison.**
*****Independent t-test was used to compare the mean differences.**

- Data represent mean ±SD of 58 observations (30 control group and 28 study group)
#significant difference at p <0.05
##Highly Significant difference at p <0.01
-CBC: complete blood count
-TLC: total leukocytic count
-HGB: haemoglobin
-vit.D: vitamin D
-ALL: acute lymphoblastic leukaemia
-HD-MTX: high dose methotrexate

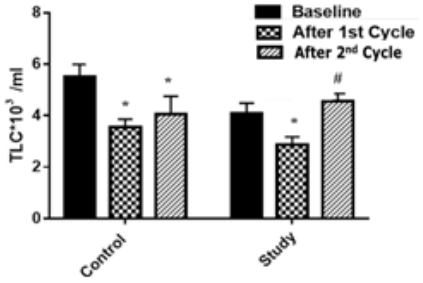

Fig. 2A: Change of total leukocytic count within control group and vit-D treated group of ALL patients (treated with HD-MTX).

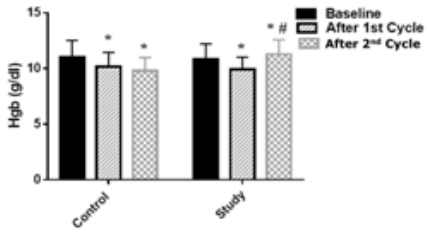

Fig. 2B: Change of hemoglobin level within control group and vit-D treated group of ALL patients (treated with HD-MTX).

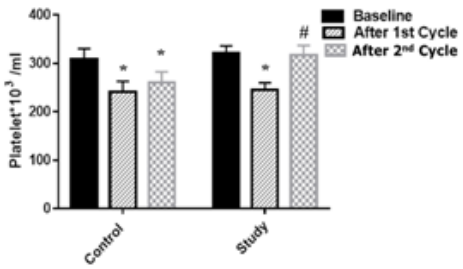

Fig. 2C: Change of platelet count within control group and vit-D treated group of ALL patients (treated with HD-MTX).

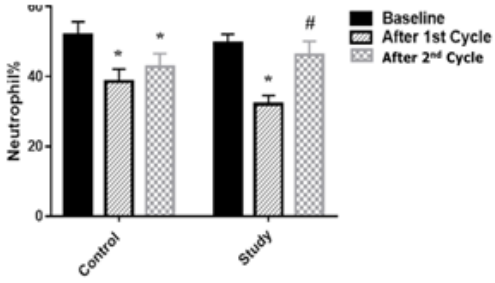

Fig. 2D: Change of monocytes count within control group and vit-D treated group of ALL patients (treated with HD-MTX).

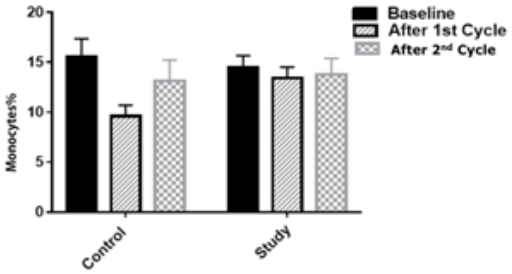

Fig. 2E: Change of neutrophils count within control group and vit-D treated group of ALL patients (treated with HD-MTX).

Data represent mean ±SD *Significant difference at p <0.05 vs group baseline, #Significant difference at p<0.05 vs group after 1st cycle,TLC: total leukocytic count, Hgb: haemoglobin, Vit-D: vitamin D, ALL: acute lymphoblastic leukaemia, HD-MTX: high dose methotrexate.

III- The changes in liver function tests after vitamin D treatment

According to the current study, there was no significant difference in total proteins and albumin in the two groups, but serum bilirubin and liver enzyme levels increased dramatically in the control patients and reduced significantly in the study one as shown in **(Table.3 and Fig. 3A, 3B, 3C, 3D, 3E)**

IV- The changes in kidney function tests after vitamin D treatment:

The current investigation revealed that during the course of the study, serum urea and creatinine considerably dropped in the study patients and increased in the control one as shown in **(Table 4 and Fig. 4A, 4B).**

Table 3 : Change of liver function test within the two groups (control group, vit.D treated group) of ALL patients (treated with HD-MTX) regarding level of total bilirubin, total protein, ALT, AST and albumin**.**

(Mean ± SD)	**Control (n = 30)**	**P-value****	**Study (n = 28)**	**P-value****	**P-value*****
T. Bilirubin (mg/dl)					
• **Baseline (1)**	0.37 ± 0.3		0.52 ± 0.6		= 0.290
• **After 1st MTX (2)**	0.60 ± 0.4		0.88 ± 0.2		= 0.204
• **After 2nd MTX(3)**	0.84 ± 0.5##	**1 vs. 3 <0.001**	0.42 ± 0.3≠	**1 vs. 3=0.043**	**< 0.001**
P-value*	**< 0.001**		**= 0.004**		**= 0.003***
T. Protein (g/l)					
• **Baseline (1)**	64.80 ± 6.1		64.93 ± 6.2		= 0.937
• **After 1st MTX (2)**	64.43 ± 5.9		64.21 ± 5.3		= 0.884
• **After 2nd MTX(3)**	64.67 ± 6.9	1 vs. 3=0.913	64.01 ± 6.1	1 vs. 3 =0.494	= 0.535
P-value*	= 0.905		= 0.735		= 0.647*
ALT (µ/L)					
• **Baseline (1)**	24.97 ± 1.9		23.68 ± 1.6		= 0.619
• **After MTX (2)**	57.43 ± 5.8		74.71 ± 13.2		= 0.318
• **After 2nd MTX(3)**	70.5 ± 4.1##	**1 vs. 3 <0.001**	36.57 ± 4.1≠	**1 vs. 3 =0.002**	**= 0.003**
P-value*	**< 0.001**		**= 0.001**		**= 0.001***
AST (µ/L)					
• **Baseline (1)**	25.90 ± 2.1		25.39 ± 1.9		= 0.858
• **After MTX (2)**	44.01 ± 6.3		57.61 ± 7.8		= 0.181
• **After 2nd MTX(3)**	60.4 ± 7.7##	**1 vs. 3 <0.001**	31.29 ± 3.1≠	**1 vs. 3 =0.025**	**= 0.001**
P-value*	**< 0.001**		**< 0.001**		**= 0.001***
Albumin (g/L)					
• **Baseline (1)**	42.23 ± 5.8		41.79 ± 5.9		= 0.774
• **After 1st MTX(2)**	41.97 ± 5.1		41.96 ± 6.1		= 0.999
• **After 2nd MTX(3)**	42.10 ± 5.1	1 vs. 3 =0.914	44.46 ± 3.8≠	**1 vs. 3 =0.031**	**= 0.049**
P-value*	= 0.791		**= 0.017**		= 0.105*

***Two-Way Repeated Measure ANOVA was used to compare the mean differences over time.**
****Post-hoc test with Bonferroni Correction for Pairwise comparison.**
*****Independent t-test was used to compare the mean differences.**

- Data represent mean ±SD of 58 observations (30 control group and 28 study group)
#significant difference at p <0.05
##Highly Significant difference at p <0.01
-T.bilirubin: total bilirubin
-T.protien: total protien
-ALT: alanine transaminase
-AST: aspartate aminotransferase
-vit.D: vitamin D
-ALL: acute lymphoblastic leukaemia
-HD-MTX: high dose methotrexate

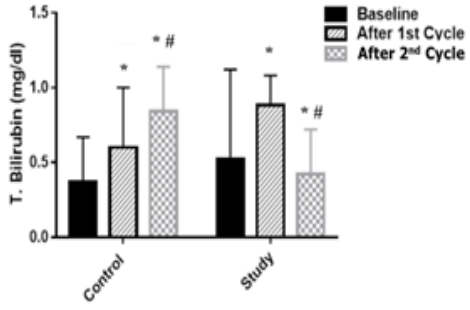

Fig. 3A: Change in mean total bilirubin within control group and vit-D treated group of ALL patients (treated with HD-MTX).

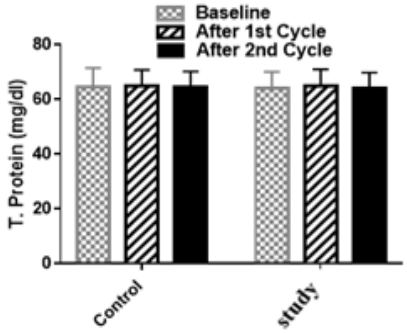

Fig. 3B: Change in mean total protien within control group and vit-D treated group of ALL patients (treated with HD-MTX).

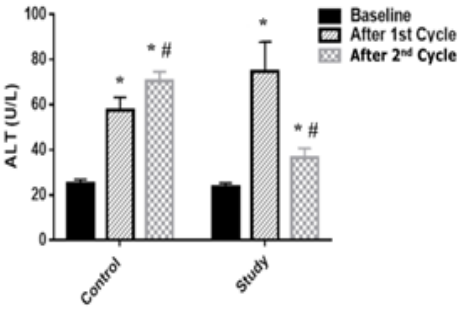

Fig. 3C: Change in mean ALT within control group and vit-D treated group of ALL patients (treated with HD-MTX).

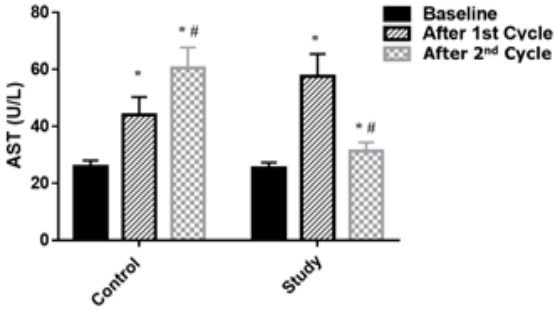

Fig. 3D: Change in mean AST within control group and vit-D treated group of ALL patients (treated with HD-MTX).

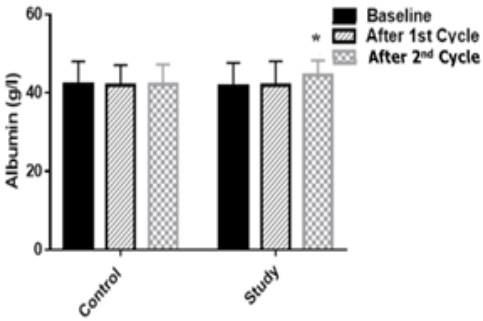

Fig. 3E: Change in mean Albumin within control group and vit-D treated group of ALL patients (treated with HD-MTX).

Data represent mean ±SD, *Significant difference at p <0.05 vs group baseline, #Significant difference at p<0.05 vs group after 1st cycle, T.bilirubin : total bilirubin, T.protien: total protein, ALT: alanine transaminase, AST: aspartate aminotransferase, Vit-D: vitamin D, ALL: acute lymphoblastic leukaemia, HD-MTX: high dose methotrexate.

Table 4: Change of kidney function test within the two groups (control group, vit.D treated group) of ALL patients (treated with HD-MTX) regarding level of blood urea and creatinine.

(Mean ± SD)	Control (n = 30)	P-value**	Study (n = 28)	P-value**	P-value***
B. Urea (mg/dl)					
• **Baseline (1)**	12.87 ± 1.3		12.04 ± 1.3		= 0.640
• **After 1st MTX (2)**	19.13 ± 1.2		20.61 ± 1.7		= 0.491
• **After 2nd MTX(3)**	27.2 ± 2.6##	**1 vs. 3 <0.001**	11.82 ± 1.2	1 vs. 3=0.836	**< 0.001**
P-value*	**< 0.001**		**< 0.001**		**< 0.001***
S. Creatinine (mg/dl)					
• **Baseline (1)**	0.31 ± 0.1		0.32 ± 0.1		= 0.909
• **After 1st MTX (2)**	0.46 ± 0.1		0.60 ± 0.2		**= 0.006**
• **After 2nd MTX(3)**	0.76 ± 0.2##	**1 vs. 3 <0.001**	0.31 ± 0.1	1 vs. 3=0.676	**< 0.001**
P-value*	**< 0.001**		**< 0.001**		**< 0.001***

***Two-Way Repeated Measure ANOVA was used to compare the mean differences over time.**
****Post-hoc test with Bonferroni Correction for Pairwise comparison.**
*****Independent t-test was used to compare the mean differences.**
- Data represent mean ±SD of 58 observations (30 control group and 28 study group)
#Significant difference at p <0.05
##Highly Significant difference at p <0.01
-B.urea : blood urea
-S.creatinine: serum creatinine
-vit.D: vitamin D
-ALL: acute lymphoblastic leukaemia
-HD-MTX: high dose methotrexate

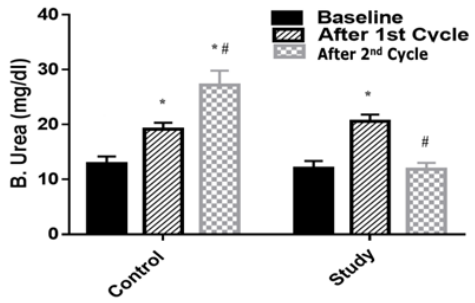

Fig. 4A: Change in blood urea within control group and vit-D treated group of ALL patients (treated with HD-MTX).

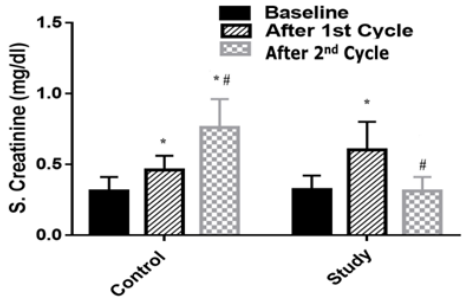

Fig. 4B: Change in mean serum creatinine within control group and vit-D treated group of ALL patients (treated with HD-MTX).

Data represent mean ±SD, *Significant difference p <0.05 vs group baseline, #Significant difference p<0.05 vs group after 1st cycle, B.urea: blood urea, S.creatinine: serum creatinine, Vit-D: vitamin D, ALL: acute lymphoblastic leukaemia, HD-MTX: high dose methotrexate.

V- Effect of treatment with vitamin D on Methotrexate level

According to the results of the current investigation, the mean methotrexate level after 24 hours increased significantly in the control patients while it significantly decreased in the study one during the study period as shown in (**Fig. 5A, 5B)**

VI- The changes in the inflammatory markers after vitamin D treatment:

During the study period, the study group experienced a considerable decrease in concentration of serum IL-6 & TNF-α while the control group experienced a small decrease, as shown in **(Table 6 and Fig. 6A, 6B).**

VII- The effect of treatment with vitamin D drops on the oral mucositis:

According to the study's findings, oral mucositis grades I and II significantly increased in the study patients and decreased in the control one; in contrast, oral mucositis grades III and IV significantly decreased in the study patients and increased in the control one during the study's duration **(Fig. 7A, 7B, 7C, 7D)**

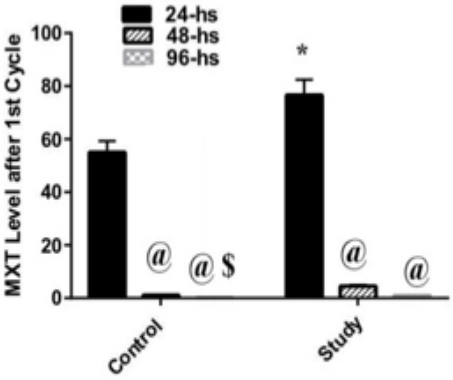

Fig. 5A: Mean MTX Level difference within control group and vit-D treated group of ALL patients (treated with HD-MTX) after 1st Cycle with HD-MTX.

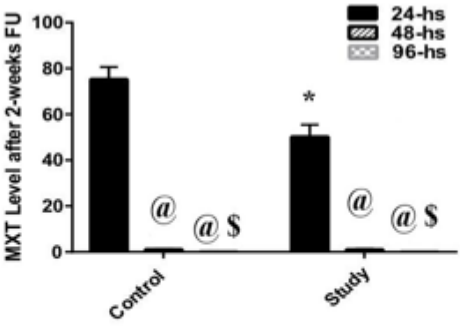

Fig. 5B: Mean MTX Level difference within control group and vit-D treated group of ALL patients (treated with HD-MTX) after 2nd Cycle with HD-MTX.

Data represent mean ±SD, * Significant difference at p< 0.05 vs control group after 24 hours, @ Significant difference at p <0.05 vs same group after 24 hours, $ Significant difference at p<0.05 vs same group after 48 hours, Vit-D: vitamin D, ALL: acute lymphoblastic leukaemia, HD-MTX: high dose methotrexate.

Table 6: Change of the Inflammatory Biomarkers within the two groups (control group, vit-D treated group) of ALL patients (treated with HD-MTX).

	Control Group (n = 30)	**Study Group (n = 28)**	**P-value***
TNF-α (ng/ml)			
• **After 1st MTX**	74.84 ± 4.9	80.68 ± 8.3	**= 0.004**
• **After 2nd MTX**	63.59 ± 3.9	51.73 ± 6.4#	**= 0.006**
p-value**	**< 0.001**	**< 0.001**	**< 0.001*****
IL-6 (pg/ml)			
• **After 1st MTX**	88.05 ± 5.6##	77.50 ± 8.5	= 0.164
• **After 2nd MTX**	73.71 ± 5.2	42.39 ± 5.9##	**< 0.001**
p-value**	**< 0.001**	**< 0.001**	**< 0.001*****

Two Way RM-ANOVA *between groups **within group ***Interaction analysis
- Data represent mean ±SD of 58 observations (30 control group and 28 study group)
#significant difference at p <0.05
##Highly Significant difference at p <0.01

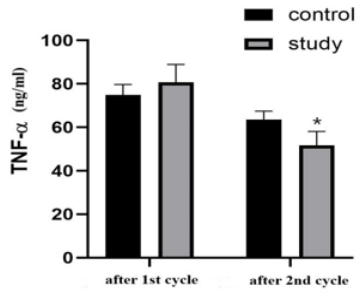

Fig. 6A: Change in mean TNF-α difference within control group and vit-D treated group of ALL patients (treated with HD-MTX).

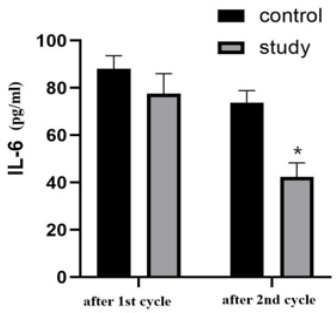

Fig. 6B: Change in mean IL-6 difference within control group and vit.D treated group of ALL patients (treated with HD-MTX).

Data represent mean ±SD, *significant difference at p <0.05 vs same group after 1st cycle, TNF-α: Tumor necrosis factor alpha, IL-6: Interleukin 6.

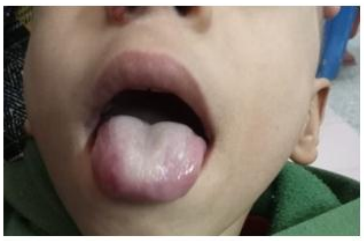

Fig. 7A: A patient with grade I oral mucositis after 1st cycle HD-MTX..

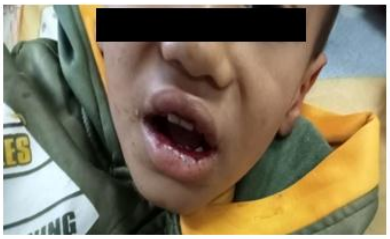

Fig. 7B : same patient with grade III oral mucositis after 2nd cycle HD-MTX not receiving vitamin D drops.

Fig. 7C: A patient with grade II oral mucositis after 1st cycle HD-MTX.

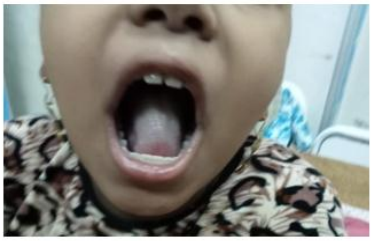

Fig. 7D: Same patient with grade I oral mucositis after 2nd cycle HD-MTX receiving vitamin D drops.

HD-MTX: high dose methotrexate

Discussion

For management of Egyptian pediatric acute lymphoblastic leukemia, high dosage methotrexate is now utilized[23]. For improvement of treatment, quality of life, and decrease mortality in MTX therapy, toxicity must be reduced and side effects must be understood. Although higher doses of MTX are often more beneficial, they can cause toxicity and side effects such as hematologic toxicity, nephrotoxicity, hepatotoxicity and an increased risk of infections[9].

Regarding the question of how the tested medications affected the complete blood count, this study reported that the study patients who received vitamin D along with methotrexate experienced a highly significant increase in total leukocytic count, while the control group, which received methotrexate alone, showed a significant decrease in this count. On the other hand, our study revealed no significant relationship between the number of monocytes or neutrophils and vitamin D during the study period.

Studies have explained the effects of methotrexate on blood elements as methotrexate inhibits folic acid pathway enzymes, preventing purine and pyrimidine production. This results in decreased DNA replication and cell proliferation. Methotrexate is most effective as a chemotherapeutic agent in tissues with high cellular turnover. However, it can also cause mucositis and hair loss[24].

Hamed and his group have reported that most common hematological toxicity with methotrexate is myelosupression and pancytopenia, which may include leukopenia, anemia and/or thrombocytopenia. When other risk factors such folic acid deficiency, renal and/or liver impairment are present, the likelihood of these major side effects may increase significantly[9].

Additionally, studies have shown how vitamin D affects blood components. After converting 25-OH-D to 1, 25-OH-D, the active form of the vitamin binds to the nuclear vitamin D receptor, which is expressed in a variety of organs and controls the functioning of many different types of cells, including blood cells[25].

On the other hand, it was observed that there was no significant link between total leukocytic count in patients with deficiency of vitamin D and normal individuals[26]. This proposal was supported by a team of researchers who found no correlation between the numbers of leukocytes, neutrophils, monocytes, or lymphocytes in the several groups divided based on vitamin D status[27].

Likewise, the current study indicated that the study group's hemoglobin level significantly increased from baseline to the end of the second cycle of methotrexate; in contrast, the hemoglobin level was decreased significantly in the control patients.

Numerous studies have confirmed up the earlier recommendations and shown that giving vitamin D to critically ill individuals raised their hemoglobin levels and improved their anemia[28, 29].

The present study reported significant increase in platelet count in study group received vitamin D over the study period, oppositely the level of platelet count showed significant decrease in control group over the study period. Accordingly, research had been shown an association between vitamin D and platelet count. The administration of vitamin D increased platelet counts. In medical situations where platelet counts are below normal, this could be helpful[30, 31].

In terms of how the drugs under test affected the liver function test, the current study demonstrated that the group receiving methotrexate treatment alone had a significant increase in bilirubin levels & the liver enzyme, whereas the group receiving methotrexate and vitamin D concurrently had a significant decrease in these parameters.

In addition to that, the mean albumin level in the vitamin D-treated group increased significantly while the mean albumin level in the control group did not significantly change However, regarding the effect on total protein there was no significant difference between the two patient groups.

As revealed *by Saskia and her group*, methotrexate has been known to promote chronic drug-induced liver damage in the form of fibrosis. They identified oxidative stress caused by glutathione depletion and disturbance in cellular respiration as the two main mechanisms underlying methotrexate-induced toxicity in a glutathione-independent way[32].

Moreover, a number of investigations on both humans and animals have been carried out to look into the mechanism underlying methotrexate's hepatocellular toxicity[24]. Elevation of bilirubin and liver enzymes had been demonstrated to be caused by depletion of folic acid and accumulation of methotrexate polyglutamates[33].

There had been a connection between serum vitamin D3 levels and liver enzymes, according to several cross-sectional studies. According to reports, low levels of vitamin D were linked to elevated hepatic enzyme levels[34-36].

Consistent with current findings, a pediatric investigation discovered that children exhibiting elevated serum bilirubin levels were more prone to have either inadequate or deficient levels of vitamin D[37].

Regarding the impact of the drugs under evaluation on the kidney function test, the current study found that the group receiving methotrexate only showed a significant increase in mean blood urea and serum creatinine, while the group receiving methotrexate and vitamin D concurrently showed a significant decrease in mean blood urea and serum creatinine over the course of the study.

Many studies revealed the toxic effects of methotrexate on the kidneys[24]. Although the precise mechanism of renal toxicity remains unclear, prior research has indicated that a direct harmful effect on renal tubules or precipitation in the tubules may be responsible for methotrexate nephrotoxicity[38].

Elisa and her team had reported biomarkers that indicate injury to the kidneys. Oncogene 24p3, often referred to as neutrophil gelatinase-associated lipocalin (NGAL), is expressed in the kidney, prostate, and respiratory and gastrointestinal tract epithelia. NGAL had become a crucial biomarker for acute kidney damage[38].

Numerous research focused on the impact of vitamin D use on the general public, particularly in individuals with renal disorders. This is because vitamin D has an important role in many physiological processes; blood pressure regulation through control aldosterone, renin, and angiotensin systems[39]. Furthermore, because vitamin D can lower urine protein-creatinine ratios, it is important for people with proteinuric renal disorders[40].

Accordingly, vitamin D supplementation increases proteinuria and inflammatory indicators but not blood urea, creatinine, or glomerular filtration rate, according to *a 2019 study by Wang and colleagues*[41].

Regarding the impact of vitamin D on the serum level of methotrexate, the current study showed that the study group who receiving vitamin D caused a decrease in methotrexate level after 24 hours, while the control group, experienced a significant elevation in the methotrexate level after 24 hours (after the second cycle of high dose methotrexate) during the study period.

This could be explained as methotrexate enters malignant cells and performs antimetabolite activity, while the remainder of the drug exits the cell and is eliminated by circulation, a decrease in the amount of medication in circulation indicates that more medication penetrated the cancerous cells and had an effect.

Considering the Leucoverin rescue protocol and MTX toxicity according to Children's Cancer Hospital Egypt (CCHE57357): Leucoverin rescue protocol: 5mg/m^2 orally or IV will be given at 24 hours after methotrexate, then 23 hours after high dose MTX 5gm/m^2 (targeted up to 65 μM): More than 95 μM check hydration and alkalization are adequate, check the nephrotoxic drugs, and close monitor the urinary output. More than 150 μM all of the above plus obtain another blood sample for assay consider possible early leucoverin rescue.

Studies on the relationship between methotrexate and vitamin D demonstrated that the latter greatly reverses the cytotoxicities induced by methotrexate. This suggests that vitamin D supplements may be used in clinical settings to shield tissues from the toxic effects of chemotherapy, which are primarily brought on by methotrexate therapy[42].

In the context of how tested medications affected inflammatory markers, the current study revealed that the study group receiving vitamin D along with methotrexate therapy had a significant decrease in the IL-6 and TNF-α levels, whereas the control group experienced a mild decrease in the same measures.

Consistent with this finding, it was proposed that methotrexate may reduce the amount of inflammatory markers because it possesses anti-inflammatory properties, as evidenced by multiple studies[43].

The essential ingredient for methotrexate's anti-inflammatory effect is adenosine. Adenosine's interaction with adenosine receptors produces the anti-inflammatory effect, which prevents leukocyte chemotaxis, neutrophils and monocytes oxidative inflammation, and the production of cytokines[44]. Furthermore, it was found that methotrexate had an extra anti-inflammatory effect by scavenging free radicals and lowering markers of oxidative stress; protein-adduct of malondialdehyde and acetaldehyde[45].

Added to that, *a 2022 study by Alsufiani and colleagues* found a correlation between low levels of pro-inflammatory cytokines such as IL-6, and IL-8 & TNF-α and high serum vitamin D concentrations[14]. This conclusion supported by other studies[46-48]. Dendritic cells and monocytes has been affected by vitamin D as well. It prevents monocytes from producing inflammatory cytokines such TNF-α, IL-1, IL-6, IL-8, and IL-12[49]. Besides, it prevents dendritic cell development and differentiation while maintaining immature subtypes[50].

Moreover, *Baeke and his colleagues in 2010* pointed to the effect of vitamin D on the immune system and inflammatory processes[51], *Feldman and his team* also proposed *in 2014* that vitamin D supplementation could improve

outcomes and lower the risk and progression of cancer[12].

In addition, *Pludowski and colleagues in 2013* found that one of the most important aspects of prophylaxis for a range of inflammatory illnesses was getting enough vitamin D from supplements and appropriate sun exposure to achieve optimal vitamin D status[52].

Regarding the influence of the tested medications on oral mucositis and ulcerations, the current findings showed that moderate and severe oral mucositis significantly increased in the control group during the follow-up of patients with acute lymphoblastic leukemia receiving high doses of methotrexate; in contrast, the co-administration of vitamin D with methotrexate revealed a significant decrease in moderate and severe grades of oral mucositis, which were subsequently replaced by milder degrees over the course of the trial.

In agreement to that, Oosterom and his colleagues reported in 2019 that vitamin D insufficiency was associated with MTX-induced oral mucositis in ALL children and that children with ALL who experienced severe oral mucositis following HD-MTX therapy had lower vitamin D levels[53].

In relationship to mucositis in children with cancer, a meta-analysis examined genes implicated in the pharmacokinetics and pharmacodynamics of high dosage methotrexate. Apart from genetic variations, the investigation evaluated alterations in DNA methylation concerning methotrexate-induced oral mucositis[54].

While some researches indicated a connection between low vitamin D levels (or receptor expression) and the emergence of an enhanced inflammatory response in the mucosa, other investigations found no connection between vitamin D levels and the onset of oral mucositis[55].

Ultimately, there was disagreement about the optimal vitamin D level to produce immunomodulatory and anti-inflammatory effects; this disagreement may have resulted from variations in populations, sample sizes, or methods of measurement. Consequently, further research was needed to elucidate the function of vitamin D in humans and generate firm conclusions [56].

Conclusion

In conclusion, treating severe vitamin D insufficiency is still crucial, as vitamin D supplements may improve overall health.

It's critical to identify MTX side effects because they can be fatal and should be treated with implementation of the rescue measures. Several of these adverse effects are preventable with careful management.

The study suggests assessing both the effects of vitamin D therapy and a patient's current vitamin D status. We recommend giving vitamin D to patients with acute lymphoblastic leukemia who are receiving large doses of methotrexate under the guidance of oncologists in order to treat the adverse reactions of the medication.

Statement of Ethics

Prior to the study, the Institutional Review Board of Assiut University's Faculty of Medicine granted approval for it. All research subjects provided written informed consent. The study's duration as well as the participants' unrestricted right to withdraw from it at any moment were clearly stated in their informed consent. Additionally, by giving each participant a code number, the confidentiality and anonymity of the participants were guaranteed. None of the participants received any gifts or rewards.

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تأثير إعطاء فيتامين د علي الأثار السلبية لدواء الميثوتركسات في علاج الأطفال المرضي بسرطان الدم الليمفاوي الحاد

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المعلومات الاساسية. يستخدم دواء الميثوتركسات كعلاج اساسي لمرضي سرطان الدم الليمفاوي الحاد ، و لكن يوجد العديد من الأثار السلبية لهذا الدواء و لهذا كان الهدف من هذه الدراسة هو اعطاء فيتامين د مع الميثوتركسات للمرضي و تقييم مدي تأثير اعطاء فيتامين د علي الاثار السلبية المترتبة علي علاج الميثوتركسات بما في ذلك تقرحات الفم ، فشل نخاع العظام ،التأثير السلبي و ارتفاع وظائف الكبد و الكليتين وايضا تأثيره علي وسطاء الالتهاب TNF-α و IL-6.

منهج البحث. الدراسة عبارة عن تجربة سريرية عشوائية شملت ٥٨ طفلا مريضًا يعاني من سرطان الدم الليمفاوي الحاد الذين تلقوا العلاج بالميثوتريكسات (٣٠ في المجموعة أ - لا يعطي فيتامين د لمدة أسبوعين بعد العلاج بالميثوتريكسات) و (٢٨ في المجموعة ب - تلقى المريض فيتامين د لمدة أسبوعين بعد العلاج بالميثوتركسات).

النتائج: أظهرت نتائج الدراسة أن مستويات إجمالي عدد خلايا الدم البيضاء ونسبة الهيموجلوبين وعدد الصفائح الدموية كانت أقل بشكل ملحوظ في المجموعة الضابطة ، ولكنها أعلى في مجموعة الدراسة خلال فترة الدراسة ؛ بالإضافة إلى ذلك ، كان متوسط عدد خلايا النيتروفيل أقل بشكل ملحوظ في كلا المجموعتين ، ولكن لم يكن هناك تغيير كبير في متوسط عدد خلايا المونوسايت في أي من المجموعتين خلال فترة الدراسة. علاوة على ذلك ، كشفت النتائج التي توصلنا إليها أن مستويات البيليروبين في الدم وأنزيمات الكبد واليوريا والكرياتينين زادت بشكل ملحوظ في المجموعة الضابطة ولكنها انخفضت بشكل ملحوظ في مجموعة الدراسة خلال فترة الدراسة ، بينما لا يوجد تغيير كبير في الالبيومين و البروتينات الكلية في كلا المجموعتين.

بالإضافة إلى ذلك ، أوضحت نتائج دراستنا كذلك أن هناك زيادة في متوسط مستوى الميثوتريكسات بعد ٢٤ ساعة في المجموعة الضابطة، بينما كان هناك انخفاض في متوسط مستوى الميثوتريكسات بعد ٢٤ ساعة في مجموعة الدراسة و اما عن مستوي TNF-αو IL-6 اوضحت النتائج

ان هناك انخفاض بسيط فيهما في المجموعة الضابطة و انخفاض بشكل ملحوظ في مجموعة الدراسة التي تلقت دواء فيتامين(د).

يضاف إلى ذلك ، أن نتائج دراستنا أظهرت أنه من حيث التهاب الغشاء المخاطي للفم الخفيف و المتوسط الدرجة الاولي و الثانية تزداد في مجموعة الدراسة وتنخفض في المجموعة الضابطة خلال فترة الدراسة ، في حين أظهر التهاب الغشاء المخاطي الشديد الدرجة الثالثة و الرابعة انخفاضًا ملحوظًا في مجموعة الدراسة وزيادة في المجموعة الضابطة على مدار الدراسة.

الاستنتاج. توصلنا إلى الاستنتاج من خلال تقديم المشورة بشأن قياس مستويات فيتامين (د) في المرضى المصابين بأمراض خطيرة ، وعلاج فيتامين (د) الموصى به في المرضى الذين يعانون من نقص فيتامين (د) ، و توصي الدراسة بضرورة اعطاء فيتامين(د) لمرضي سرطان الدم الليمفاوي الحاد الذين يتلقون جرعات كبيرة من الميثوتركسات و ذلك لتقليل اثاره الجانبية.