Hypothesis

Elucidating the Effects of Methotrexate Therapy on Serum Cytokeratin-18 Levels in Rheumatology

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Abstract: Background: CK-18 is a serological marker of apoptosis that has been widely associated with fibrosis and steatosis in NASH. Many studies have showed association CK-18 levels with NAFLD. To date, there is a profound lack of rheumatology studies on the effect of MTX therapy with regard to CK-18 levels. The relationship between CK-18 levels and cumulative MTX dose, MTX treatment duration, weekly MTX dose is not available in the literature. **Objectives**: The main purpose of this study was to determine the relationship between serum cytokeratin-18 (CK-18) and cumulative methotrexate dose in rheumatology patients on methotrexate (MTX) therapy. Besides, we studied the correlations between CK-18 and clinical parameters including age, disease duration, duration of MTX therapy, cumulative steroid dose and biochemical parameters such as alanine transaminase (ALT) and aspartate aminotransferase AST). **Methods**: We recruited 79 rheumatology patients on methotrexate (MTX) therapy as MTX group and 38 patients not on MTX therapy as non-MTX group. All subjects were tested for their serum CK-18 levels using an enzyme-linked immunosorbent assay (ELISA) test. We identified 20 patients with the highest CK-18 levels and 20 patients with the lowest CK-18 levels and had ultrasound liver performed on them. **Results**: The median serum CK-18 levels were marginally higher among the patients on MTX (1.20 ng/mL [0.19-37.15]) compared to the non-MTX group (1.17 ng/mL [0.37-34.32]). There was a significantly positive relationship between serum CK-18 levels and cumulative MTX dose ($r = 0.329$, $p = 0.003$) and total duration of MTX therapy ($r = 0.284$, $p = 0.011$). Apart from the above-mentioned variables, the CK-18 levels in MTX group significantly correlated with age ($r = 0.265$, $p = 0.018$), ALT ($r = 0.440$, $p = <0.001$) and AST ($r = 0.478$, $p = 0.004$). Ultrasound liver findings showed median CK-18 levels was higher among patients with abnormal liver findings although statistical significance was not reached. **Conclusion**: Among patients on MTX therapy, serum CK-18 levels correlated positively with cumulative MTX dose, total duration of MTX treatment, ALT and AST levels. These findings are preliminary and requires validation by future studies in the field of rheumatology.

Keywords: methotrexate (MTX); rheumatoid arthritis (RA); cytokeratin -18 (CK -18)

Introduction

Methotrexate (MTX) is used as an anchor disease-modifying anti-rheumatic drug (DMARD) in treating rheumatoid arthritis (RA) because of its efficacy and tolerability (1). Besides, it is commonly used for the treatment of several other forms of inflammatory arthritis and autoimmune diseases such as SLE and psoriatic arthritis. MTX can be administered orally or subcutaneously. Initial therapy is generally with a dose of between 5 and 10 mg weekly, thereafter, the dose is increased gradually depending upon the clinical response, tolerability, and factors such as age, size, comorbidities, renal function, and degree of disease activity. The maximum dose we generally use in Malaysia is 25 mg/week.

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The major side effects of MTX include gastrointestinal disorders, hepatic dysregulations, pneumonitis, haematological problems, and infections (2, 3). The frequency of hepatotoxicity can be as high as 70% in the first 2 to 4 years of MTX therapy (4). The liver biopsies of patients on MTX therapy show histological changes include stellate cell hypertrophy, steatosis and hepatic fibrosis (5, 6). Most patients with hepatotoxicity associated with MTX therapy lack symptoms or physical findings. The reported incidence of MTX-induced increases in serum alanine aminotransferase (ALT) is approximately 14% and aspartate aminotransferase (AST) is 8% (7, 8). Reductions in serum albumin may also be seen as a marker of potential liver injury in patients (8).

Although in clinical practice patients on MTX therapy are routinely tested for liver function test, non-alcoholic steatohepatitis (NASH) can be missed as it may occur even with normal levels of ALT and AST. Various radiologic methods can detect non-alcoholic fatty liver disease (NAFLD), but no imaging modality is routinely used to differentiate between the histologic subtypes of NAFLD and NASH (9). NAFLD is divided into non-alcoholic fatty liver (NAFL) and NASH, with the former having a relatively good prognosis. However, NASH may progress to cirrhosis, and in some cases, hepatocellular carcinoma (HCC). Although liver biopsies are the current gold standard for diagnosing NASH, the procedure is invasive and not routinely performed in all NAFLD patients (10). Recent studies have reported that serum cytokeratin (CK)- 18 is useful for diagnosing NASH (11-16). The current American guidelines for the diagnosis and management of NAFLD indicate that serum CK-18 levels may be useful for distinguishing NASH from NAFLD (17).

CK-18 is a serological marker of apoptosis that has been widely associated with fibrosis and steatosis in NASH (16, 18, 19). CK-18 is an intermediate filament protein present in large quantities in the liver, accounting for 5% of liver proteins. During cellular apoptosis, CK-18 is cleaved at Asp396, and the CK-18 fragments are recognized by an M30 antibody specific for the liberated C-terminus. Thus, CK-18 is a marker of hepatocyte apoptosis. In a study by Kawanaka et al., serum CK-18 levels correlated with the fibrosis stage, lobular inflammation, portal inflammation, steatosis, hepatocellular ballooning, and the presence of Mallory bodies (10).

To date, there are no rheumatology studies on the correlation of cumulative MTX dose with CK-18 levels. This prompted us to conduct this study to determine the aforementioned relationship. Besides, we wanted to determine the the relationship between serum CK-18 levels and biochemical parameters such as ALT and AST and clinical parameters including age, disease duration, duration of MTX therapy and cumulative steroid and MTX dose.

Methodology

Study design and study population

This was a monocentric cross-sectional study conducted among the outpatients of the rheumatology clinic of Hospital Canselor Tuanku Muhriz (HCTM) between May 2022 to March 2023. This study was approved by the Research Ethics Committee of University Kebangsaan Malaysia with the research code of FF-2021-163. Through convenient sampling, rheumatology patients on MTX therapy were recruited into this study. Written consent was obtained from all patients prior to recruitment.

We recruited a total of 117 subjects including 79 in MTX group and 38 in non-MTX group. The inclusion criteria for the subjects were rheumatology patients aged more than 18 years and on methotrexate therapy for a minimum of 6 months duration. We excluded all patients with viral hepatitis (chronic hepatitis B and C), alcoholic liver disease, and underlying chronic liver diseases such as autoimmune hepatitis, primary biliary cirrhosis. The non-MTX group patients were rheumatology patients never or not on MTX therapy for at least 6 months.

Sample Size Estimation

This is an observational pilot study. Teare et al recommended population size (N) of 70 in pilot studies (20). The sample size calculation was performed using the using Krejcie and Morgan formula (21). The calculated sample size was 66 patients using the below equation:

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$s = x^2NP(1 - P) \div d^2 (N - 1) + x^2P(1 - P)$

$66 = [(3.841)^2 (70)(0.50)] \div [(0.05)^2(70-1) + (3.841)^2 (0.50)(1-0.50)]$

s = required sample size

x^2 = the table value of chi-square for 1 degree of freedom at the desired confidence level (3.841)

N = the population size (70)

P = the population proportion (assumed to be .50 since this would provide a maximum sample size)

d = the degree of accuracy expressed as a proportion (.05)

Data collection

The medical records were reviewed to collect data on diagnoses, disease duration, MTX duration, MTX dose, comorbidities and medication history including other DMARDS such as sulfasalazine, leflunomide, hydroxychloroquine, biologics and steroids. Doubts were clarified by interviewing the subjects. Subjects with RA were assessed for their disease activity using DAS 28. As part of routine care, all patients had their bloods tested for liver function test, lipid profile and HbA1c.

Cytokeratin-18

For serum CK-18, 3mls of blood was withdrawn into plain tubes and stored at -70 degrees Celsius until testing. The concentration of CK-18 in the patients' serum was determined by an enzyme-linked immunosorbent assay (ELISA) commercial kit. In this study, we used RayBio®Human CK-18 ELISA kit (RayBiotech, Norcross, Georgia, United States). The antibody specific for human CK-18 was coated on a 96-well plate. The standards were prepared according to the manufacturer's manual which included zero standard (0 ng/ml), 0.164 ng/ml, 0.41 ng/ml, 1,024 ng/ml, 2.56 ng/ml, 6.4 ng/ml, 16 ng/ml and 40 ng/ml.

As per the general protocol for ELISA testing, first the patients' samples were diluted 2- fold with the sample diluent. The prepared standards and samples were pipetted into the wells and CK-18 that was available in standards and samples were bound to the wells by the immobilized antibody. Subsequently, after the washing process, biotinylated anti-human CK-18 was added. Next, after another washing process that removed the unbound biotinylated antibody, the horseradishperoxidase (HRP)-conjugated streptavidin was pipetted to the wells. The 3,3', 5,5''-tetramethylbenzidine (TMB) substrate solution was added after another washing step. Finally, the stop solution was added and the absorbance was measured at 450 nm spectrophotometry. The intra-assay co-efficient variability was between 2.6% to 8.3%. The minimum detection limit for this assay was 0.16 ng/ml.

Ultrasound of the Liver

After obtaining the serum CK-18 results, 20 patients with the lowest CK- 18 levels and 20 patients with the highest CK-18 levels were identified and had ultrasound of the liver performed. Due to budget constraints, ultrasound was not ordered for all subjects. The liver ultrasound was performed by a single radiologist who was blinded to the cases.

Statistical Analysis

Data analyses was performed using the Statistical Product and Service Solutions (SPSS) version 27.0. Continuous data were expressed as mean ± standard deviation when normally distributed while the rest were expressed as median (interquartile range). The categorical variables were presented as number (percentages). Correlation between serum CK18 and other parameters were performed using the Spearman correlation test. A p-value <0.05 was considered as statistically significant.

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Results

Demographic characteristics of subjects

A total of 117 subjects (79 in MTX group and 38 in non-MTX group) were recruited in this study. The demographic features of the subjects were presented in Table 1. The MTX group and non-MTX group were age and gender matched. Majority of the patients were females. For the MTX group, majority had RA (81%). The non-RA subjects included psoriatic arthritis and systemic lupus erythematosus. All the patients in non-MTX group had RA. The MTX group and non-MTX group had comparable disease duration, disease activity and frequencies of diabetes mellitus and dyslipidemia. The use of DMARDs ($p < 0.001$), cumulative prednisolone dose ($p = 0.025$) and HbA1c levels ($p = 0.041$) were significantly different between both groups. However, the use of leflunomide did not differ significantly between both groups. We compared leflunomide between two groups in order to reduce confounding factor effect. The remaining laboratory parameters (ALT, AST, total cholesterol, triglycerides, fasting blood sugar) including CK-18 did not differ significantly between the 2 groups. The median CK-18 level was marginally higher among the patients on MTX.

Table 1. Demographic data and clinical parameters of the subjects.

Variables	MTX Group N = 79	Non-MTX Group N = 38	p Value
Age (years)*	60.05 ± 10.76	61.26 ± 16.18	0.631
Gender			0.783
Male	9 (11.4%)	5 (13.2%)	
Female	70 (88.6%)	33 (86.8%)	
Disease			**0.004***
RA	64 (81.0%)	38 (100%)	
Non-RA	15 (19.0%)		
Disease duration (years)*	11.65± 8.42	10.08 ± 7.36	0.329
Comorbidities			0.131
Diabetes	18 (22.8%)	3 (7.9%)	
Dyslipidemia	26 (32.9%)	12 (31.6%)	
Seropositive RA patients	52 (81.0%)	29 (76.3%)	0.551
DAS 28 score *	3.32 ± 0.94	3.34 ± 1.29	0.927
MTX dose (mg) **	10.00 (5.0- 20.0)		
MTX duration (years)*	10.03 ± 7.59		0.129
MTX cumulative dose (mg)**	3840.00 (150.00-14420.00)		0.259
Prednisolone cumulative dose (mg)**	3320.00 (160.00-33375.00)	7750.00 (1800.00-26190.00)	**0.025***
Other DMARDS			**<0.001***
Sulfasalazine	15 (19.0%)	10 (26.3%)	

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Hydroxychroloquine	19 (24.1%)	13 (34.2%)	
Biologics	3 (34.2%)	9 (23.7%)	
Leflunomide	17 (21.5%)	12 (31.6%)	0.238
History of transaminitis	19 (24.1%)	10 (26.3%)	0.790
Liver function test**			
ALT (U/L)	21.00 (6 -83)	17.00 (7 -81)	0.105
AST (U/L)	21.50 (15- 60)	24.50 (14- 61)	0.633
Other laboratory parameters *			
Triglycerides (mmol/L)	1.18 ± 0.66	1.18 ± 0.75	0.969
Total cholesterol (mmol/L)	4.87 ± 0.86	5.07 ± 1.04	0.280
FBS (mmol/L)	5.82 ± 2.42	5.28 ± 1.05	0.196
HbA1c (%)	6.11 ± 1.30	5.62 ± 0.80	**0.041***
CK-18 (ng/ml)**	1.20 (0.19-37.15)	1.17 (0.37-34.32)	0.608

RA: rheumatoid arthritis, MTX: methotrexate, ALT: alanine transaminase, AST: aspartate aminotransferase, DAS 28 score: Disease Activity Score-28, FBS: fasting blood sugar, CK-18: cytokeratin- 18. Data expressed as either counts (percentages), mean ± SD * or median (range)**.

Serum CK-18 levels and its correlation with clinical and laboratory parameters

For both study arms, the vast majority of the subjects had CK-18 levels ranging from 0-10 ng/ml (97.4% and 91.14% respectively) (Figure 1). Table 2 summarises the correlation analyses between CK-18 and the other study parameters. Among the MTX group, CK18 was positively correlated with age ($r = 0.265$, $p = 0.018$), total duration of MTX ($r = 0.284$, $p = 0.011$), cumulative MTX dose ($r = 0.329$, $p = 0.003$), ALT level ($r = 0.440$, $p < 0.001$), and AST level ($r = 0.478$, $p = 0.004$). Among the non-MTX group, CK-18 showed positive correlations with HbA1c ($r = 0.339$, $p = 0.040$) and ALT ($r = 0.367$, $p = 0.024$). The strength of the above-mentioned relationships with CK-18 levels were generally weak except for ALT and AST among the MTX group. The Spearman rho (r) of above 0.4 indicates strong relationship. On multivariate correlation analysis, cumulative MTX dose ($p = 0.042$) was the only variable found to have an independently significant association with CK-18. Figure 2 shows the relationship between CK-18 and cumulative MTX dose on linear regression analysis.

Our CK-18 levels were measured in ng/ml. There was paucity of data on normal CK-18 values using the same unit of measurement. Hence, with reference to a study by Darweesh et al which studied the association between CK-18 and liver fibrosis among patients with NAFLD, we used 19 ng/ml as the cut off value of CK-18 in NAFLD (22). The receiver-operator characteristic (ROC) curve analysis revealed that the optimal cut-off value of cumulative methotrexate dose to predict CK-18 level more than 19 ng/ml is 2680 mg. ROC curve analysis revealed that the sensitivity of this prediction was 0.7 and the specificity of the prediction was 0.8.

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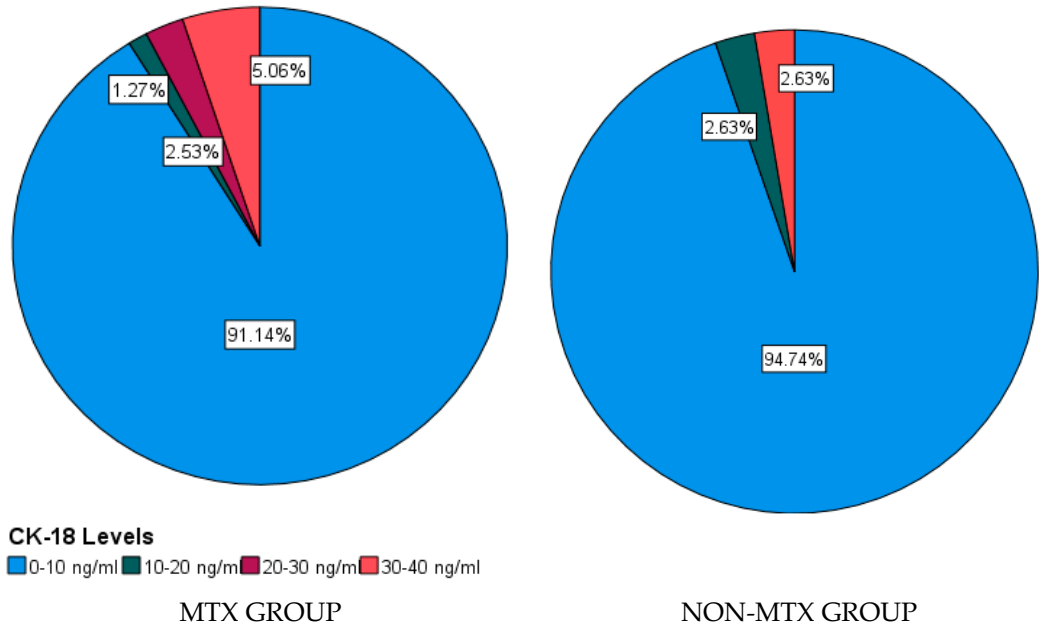

Figure 1. CK-18 levels among subjects.

Table 2. Correlation analyses between serum CK-18 and clinical parameters.

Variables	**MTX Group N = 79**		**Non-MTX Group N= 38**	
	r value	p value	r value	p value
Age	0.265	**0.018***	-0.017	0.918
Disease duration	0.159	0.160	0.127	0.448
Total duration of MTX	0.284	**0.011***		
Current MTX dose	0.028	0.806		
Cumulative MTX dose	0.329	**0.003***		
Cumulative prednisolone dose	0.100	0.484	0.069	0.822
ALT level	0.440	**<0.001***	0.367	**0.024***
AST level	0.478	**0.004***	0.339	0.235
Total cholesterol	0.069	0.547	0.174	0.304
HbA1c	0.131	0.252	0.339	**0.040***
Triglycerides	0.164	0.150	0.204	0.227
DAS 28 score	0.014	0.911	0.248	0.133

MTX: methotrexate, ALT: alanine transaminase, AST: aspartate aminotransferase, DAS 28 score: Disease Activity Score-28.

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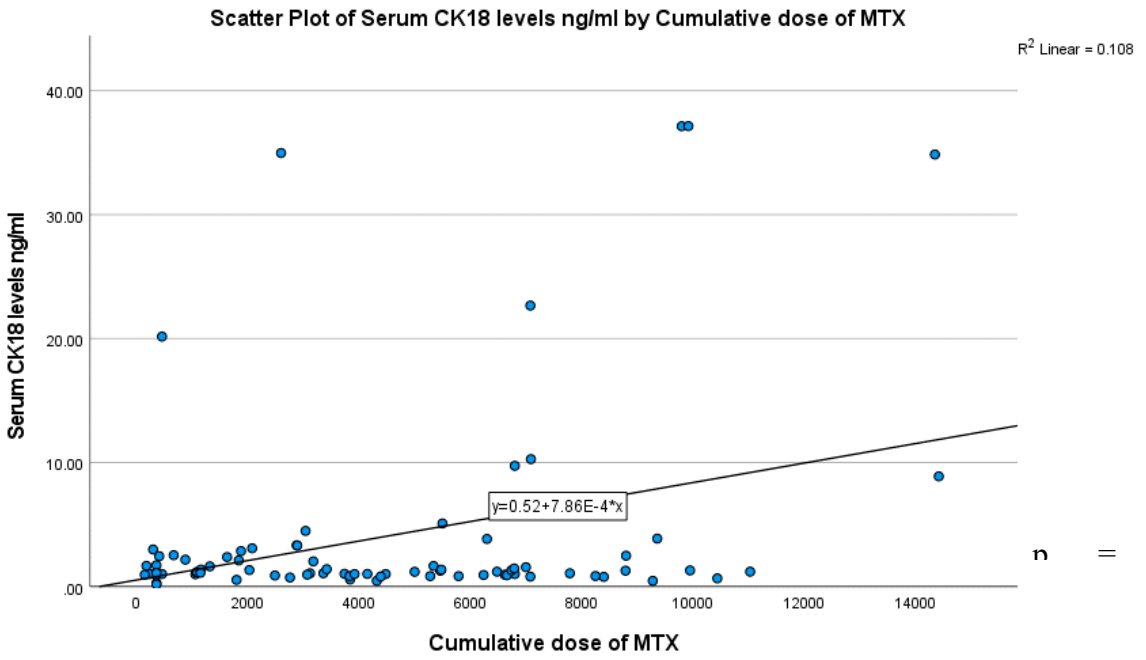

Figure 2. The relationship between serum CK-18 and cumulative dose of MTX.

Ultrasound liver findings and CK-18 levels

Based on the ultrasound findings, the patients on MTX had higher incidence of fatty liver (41.4%) and cirrhosis (10.3%) compared to the non-MTX group (27.3% and 18.2%, respectively). However, the difference was not statistically significant (p=0.686). The median CK-18 level in individuals with abnormal liver findings (3.04ng/ml [0.37-34.48]) was higher than those with normal liver (0.92ng/ml [0.19-37.14]) (Figure 3).

Table 3. Ultrasound liver findings among the MTX group and non-MTX group.

Findings	MTX Group N = 29	Non-MTX Group N = 11	p Value
Normal liver	14 (48.3%)	6 (54.5%)	0.686
Fatty liver	12 (41.4%)	3 (27.3%)	
Cirrhosis	3 (10.3%)	2 (18.2%)	
Echogenicity			
Normal	25 (86.2%)	9 (81.8%)	0.671
Heterogenicity	4 (13.8%)	2 (18.2%)	
Liver fibrosis			
Mild fibrosis	3 (10.3%)	2 (18.2%)	0.597
No fibrosis	26 (89.7%)	9 (81.8%)	
Liver size (cm)*	13.20 ± 0.83	12.99 ± 1.05	0.520

Data presented as either counts (percentages) or mean ± SD *.

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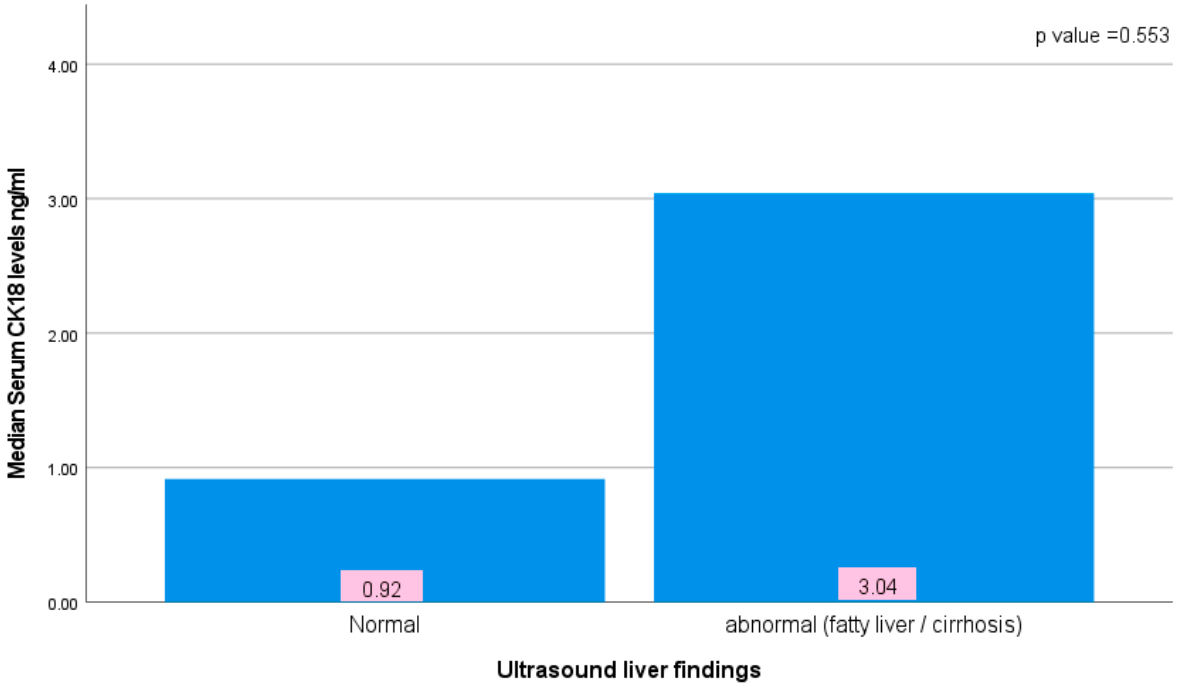

Figure 3. Comparison of CK-18 levels between subjects with normal and abnormal ultrasound liver findings.

Discussion

The novel findings of this study are the significant correlations between serum CK-18 levels and the cumulative dose as well as the total duration of MTX therapy. There are hardly any rheumatology studies on CK-18 in the literature and this study is most probably the first. The few available studies were on antibodies against CK-18 and on serum caspase-cleaved CK-18 (23, 24). CK-18 is a relatively new biomarker of drug-induced liver injury. According to Church et al, the level of CK-18 in the serum reflects the degree of necrotic hepatocellular injury and/or apoptosis (25). During hepatocellular injury, necrotic cells passively release CK-18 into the circulation due to the loss of cell membrane integrity. Long-term MTX therapy leads to accumulation of intracellular MTX metabolites in the liver, which eventually leads to oxidative stress, inflammation, and fibrosis (26). When MTX enters hepatocytes, folylpolyglutamate synthetase (FPGS) converts MTX into MTX polyglutamates (MTX-PGs). At the same time, gamma-glutamyl hydrolase is responsible for the removal of glutamate from MTX-PGs and converts them back to MTX. Thus, MTX-PG stays a long time in target cells and eventually triggers various intracellular pathological events leading to oxidative stress, apoptosis of the hepatocytes (27). Several articles have stated that methotrexate-induced hepatotoxicity is related to the cumulative dose and duration of the therapy (28-30).

For the MTX and non-MTX group, there was a significant relationship between the level of ALT and CK-18 levels. Many previous studies have published similar findings (31-33). Altaf et al reported that ALT levels correlated with CK-18 in a study comprising 148 NAFLD subjects in Pakistan (33). Similarly, Alt et al disclosed a significant correlation between ALT and CK-18 in German non-infectious chronic liver disease patients (34). The median CK-18 levels did not differ significantly between the MTX group and non-MTX group. This could be due to the relatively low median dose of MTX (10mg weekly) of the MTX group which reduced the likelihood of MTX-induced liver injury.

The mean HbA1c levels among both groups were within non-diabetic range i.e., <6.3%. We found that there was a significant relationship between the level of serum CK-18 and HbA1c in the non-MTX group. Of note, only a small percentage (7.9%) of the non-MTX group were diabetics. Maher et al revealed significant positive correlation between CK-18 and HbA1c (35). Some authors consistently reported the same despite ethnic variations across the studies (36-38). Intriguingly, these

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studies did not find a difference in the CK-18 levels between the diabetics and the non-diabetics. The hepatocytes play a pivotal role in glucose homeostasis by regulating various pathways of glucose metabolism, including glycogenesis, glycogenolysis, glycolysis and gluconeogenesis (39). Other than hepatocytes, CK-18 is one of the main keratins in the pancreatic islets and islet cell apoptosis may lead to the development diabetes mellitus (40).

In our study, median CK-18 levels though higher among subjects with abnormal ultrasound findings, statistical significance was not reached. In contrast, patients with ultrasound-proven fatty liver and liver cirrhosis had much higher CK-18 levels in previous studies (18, 41, 42). Our findings should be interpreted with caution as the number of subjects who had ultrasound performed was small. Furthermore, in a histological study by Tsutsui et al, total CK-18 levels had positive correlations with NAS (NAFLD histologic activity score) components, such as steatosis, lobular inflammation, and ballooning. They demonstrated CK-18 levels closely correlated with the individual NAS components (43).

This study has several limitations. CK-18 levels as with transaminases may fluctuate in the blood circulation. Thus, one stop sampling may not be accurately reflective of the levels of CK-18. Besides, the cumulative MTX dose was calculated based on the assumption that the subjects were compliant with the medication. The subjects of this study were on a relatively low median dose of MTX (10mg/week) leading to lower average concentrations of CK-18. We did not include body mass index (BMI) which is important factor in diagnosis of NAFLD.

Conclusion

In conclusion, this study highlighted that in patients on MTX therapy, CK-18 levels correlated with cumulative MTX dose, duration of MTX therapy, age, ALT and AST. These findings are preliminary and requires validation by future studies in the field of rheumatology.

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