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Original Article

Biochemistry Section

Quantification of Microsomal Triglyceride Transfer Protein in Non Alcoholic Fatty Liver Disease Patients: A Cross-sectional Study

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ABSTRACT

Introduction: Non Alcoholic Fatty Liver Disease (NAFLD) is one of the common liver disease characterised by fat accumulation in hepatocytes. NAFLD is a broad spectrum of simple steatosis, Non Alcoholic Steatohepatisis (NASH), cirrhosis and hepatocellular carcinoma. Triglycerides (TG) are exported in the form of Very Low Density Lipoprotein (VLDL). VLDL are formed by incorporation of TG into apo-B by Microsomal Triglyceride Transfer Protein (MTP). Therefore, MTP is a key protein for lipid transport. Estimation of MTP protein levels and its correlation with simple steatosis and steatohepatisis can be helpful in understanding its role in NAFLD progression.

Aim: To estimate the serum levels of MTP in simple steatosis and steatohepatisis and also to check its correlation with TG and VLDL in NAFLD patients with and without co-morbidities.

Materials and Methods: The present cross-sectional study was carried out in Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India, between November 2019 to January 2021. Study participants included 60 NAFLD subjects which were divided into simple steatosis (group 1, n=10) and steatohepatisis (group 2, n=50). These subjects were further subdivided into cases with co-morbidities and cases without co-morbidities. Serum levels of MTP, high sensitivity C-Reactive Protein (hs-CRP), Fasting Blood Sugar (FBS), liver enzymes, lipid profile were assessed. Statistical analysis was done by using unpaired Student's t-test and Pearson's correlation.

Results: The mean age was 46.5 years in steatosis group and 48.85 years in steatohepatisis group. In steatosis group, there were 5 (50%) males and 5 (50%) females, whereas in steatohepatisis group 32 (64%) were females and 18 (36%) were males. The serum levels of MTP were significantly decreased in simple steatosis cases with co-morbidities as compared to cases without co-morbidities (p=0.006). A significant negative correlation was observed between MTP vs TG and MTP v/s VLDL (r=-0.665, p=0.036) in simple steatosis cases with and without co-morbidities. Same trend was observed in steatohepatisis cases but the correlation was insignificant (r=-0.08, p=0.563).

Conclusion: The serum levels of MTP decreases as the NAFLD progresses. A significant decrease in serum levels of MTP was also observed in cases with co-morbidities as compared to cases without co-morbidities. Serum levels of MTP showed negative correlation with TG and VLDL in simple steatosis cases.

Keywords: Co-morbidities, Hyperlipidaemia, Lipid transportation, Non alcoholic steatohepatisis, Very low density lipoprotein

INTRODUCTION

The NAFLD is the most common liver disease with a prevalence of 9-32% in India and 25% of global adult population [1]. NAFLD is characterised by the accumulation of excessive fat in the liver in absence of alcohol intake [2]. NAFLD includes different stages, where simple fat accumulation without any inflammation of hepatocytes is termed as simple steatosis or Non Alcoholic Fatty Liver (NAFL). Simple steatosis if left untreated further progresses to inflammation of hepatocytes and this stage is termed as NASH, followed by fibrosis and hepatocellular carcinoma [3].

Fat accumulation in liver can be a result of either lack of breakdown of fatty acids or lack of fatty acid export from liver to peripheral tissues [4,5]. TG are exported from liver in the form of VLDL particles. TG rich VLDL particles represent the mechanism by which fatty acids are exported from the liver and delivered to muscle for oxidation and adipose tissue for storage, respectively [6].

The VLDL are formed by incorporation of TG into apolipoprotein B (apoB) by MTP [7-9]. MTP was identified as a major cellular protein capable of transferring neutral lipids and is localised to endoplasmic reticulum in hepatocytes. MTP is a heterodimer with two distinct subunits. A unique large α-subunit (97kDa) and a multifunctional Protein Disulfide Isomerase (PDI) β-subunit (55kDa). The 97kDa subunit confers all of the lipid transfer activity to the heterodimer [10]. VLDL assembly is a two-step process that begins in the endoplasmic reticulum lumen. In the first step, MTP acts to incorporate a small amount of TG in apoB100 as it is being translated by ribosomes and translocated across the endoplasmic reticulum membrane [6]. In the second step, additional TG is packaged into the nascent apoB100-containing particles as they traverse from the endoplasmic reticulum to the golgi apparatus to form VLDL particles [11].

In-vitro studies have shown that lipid transfer by the MTP is most efficient with TG and cholesterol although MTP transfers polar lipids also along with the neutral lipids [8,12]. According to several lines of evidence, MTP polymorphisms have been linked to lipid homeostasis and, in turn, to a higher risk of NAFLD [13,14]. Lower hepatic expression of MTP plays a crucial role in NAFLD development and is one of the potential gene found to be associated with NAFLD susceptibility [15]. The most common and widely investigated polymorphism in MTP gene is -493 G>T (rs1800591) [16,17].

Therefore, aim was to study MTP in simple steatosis and steatohepatisis, which can be helpful in assessing the disease progression. Simple steatosis is asymptomatic and steatohepatisis is an advanced stage which further progresses to irreversible conditions like fibrosis and cirrhosis. Usually many patients are diagnosed only at advanced stage of NAFLD [18]. Therefore, assessment of NAFLD is essential at early stages to prevent the further complications and progression of the disease. Hence, simple steatosis and steatohepatisis were selected in this study, to stratify before NAFLD progresses to advanced stages. However, to the best of our knowledge, till date no study measured the serum levels of MTP. Thus, this study was aimed to measure the serum levels of

MTP and also to check its correlation with the stages of NAFLD in patients with or without co-morbidities in order to understand its role in disease progression.

MATERIALS AND METHODS

The present cross-sectional study was carried out in Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India between November 2019 to January 2021. Study was approved by the Institutional Ethics Committee (IEC) of Sri Devaraj Urs Medical College (DMC/KLR/IEC/657/2021-22). The written informed consent was obtained prior to the recruitment of the subjects. Total 60 patients were enrolled in present study by convenient sampling.

Inclusion criteria: The NAFLD patients, aged between 25-60 years and with or without complications (diagnosed by laboratory tests, Ultrasonography) such as obesity, hyperlipidaemia, hypertension, diabetes and cardiovascular diseases, were included in the study.

Exclusion criteria: Subjects with other chronic liver diseases like hepatitis, chronic obstructive pulmonary disease, alcohol consumption, smoking history were excluded from the study.

Subjects and Study Design

The study participants included 60 NAFLD patients. The patients were broadly categorised into two groups; group 1 (simple steatosis) and group 2 (steatohepatisis). National Cholesterol Education Program Adult Treatment Panel (NCEP ATPIII) guidelines [19] and Ultrasonography were followed to differentiate the stages. Cases with high serum levels of liver enzymes like Alanine transaminase (ALT) (<35 mg/dL), Aspartate aminotransferase (AST) (<45 mg/dL) and TG (>150 mg/dL) along with impression of hepatomegaly with fatty infiltration were considered as steatohepatisis or NASH. In addition to above, cases without inflammation (hs-CRP within the normal range: <10 mg/L) were considered as simple steatosis and with inflammation (hs-CRP >10 mg/L) were considered as steatohepatisis in the present study [19].

Simple steatosis (Group 1): Characterised by increased fat accumulation without inflammation included 10 patients. Further patients with simple steatosis were subdivided based on co-morbidities into- 1A (cases with co-morbidities- five patients) and 1B (cases without co-morbidities-five patients).

Steatohepatisis (Group 2): Characterised by increased fat accumulation with inflammation included 50 patients. Patients with steatohepatisis were subdivided based on co-morbidities into- 2A (cases with co-morbidities- 25 patients) and 2B (cases without co-morbidities- 25 patients).

The co-morbidities like obesity, hyperlipidaemia, hypertension, diabetes and cardiovascular diseases were considered. Body Mass Index (BMI) was calculated to confirm obesity, FBS was measured for diabetic patients, and blood pressure for hypertension. Patients with any three of the above mentioned co-morbidities were considered in cases with co-morbidities group.

Sample Collection

Five mL venous blood was collected from all the subjects in tubes containing sodium fluoride for FBS estimation. For investigations like hs-CRP, Liver Function Tests (LFT) and lipid profile blood was collected in tube without anticoagulant. Blood samples were centrifuged at 3000 rpm for 10 minutes at room temperature within 2 hours of collection. Basic parameters were analysed immediately and serum for MTP estimation was aliquoted and stored at -70°C until further analysis.

Biochemical Evaluation

The biochemical parameters were analysed by standard methods using Vitros 5.1 FS clinical chemistry analyser [Table/Fig-1]. VLDL was calculated using Friedwald's formula. Serum levels of MTP were estimated by Enzyme Linked Immunosorbent Assay (ELISA) using a commercial kit. (Catalogue no. SEC641Hu, Cloud clone corp., USA).

Parameters	Test	Normal range
hs-CRP	Immunoturbidimetry	0.5-10 mg/L
FBS	Glucose-oxidase/ peroxidase	<100 mg/dL
AST	Oxaloacetate Decarboxylase, Pyruvate oxidase/Peroxidase	<35 U/L
ALT	Multipoint enzymatic by using LDH	<45 U/L
ALP	P-Nitrophenyl phosphate as substrate	42-128 U/L
Albumin	Bromocresol Green	3.5-5.2 g/dL
GGT	l-gamma-glutamyl-p-nitroanilide as substrate	<48 U/L
Serum cholesterol	Cholesterol oxidase peroxidase method	<200 mg/dL
Triglycerides	Lipase glycerol kinase peroxidase method	<150 mg/dL
HDL	Direct precipitation method	>60 mg/dL
VLDL	Friedwald's formula	<30 mg/dL

[Table/Fig-1]: Test and normal range of the biochemical parameters measured. hsCRP: High sensitivity C-reactive protein; FBS: Fasting blood sugar; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; HDL: High density lipoprorein; VLDL: Very low density lipoprotein

STATISTICAL ANALYSIS

Statistical analysis was carried out using Graph Pad Prism V.9. Shapiro-Wilk test was used to assess the normality of data. Statistical difference between the two groups were analysed using unpaired t-test. Results are represented as Mean±Standard Deviation (SD). Pearson's correlation was used to determine the relationship between variables. The p-value <0.05 was considered statistically significant and p-value <0.0001 as highly significant.

RESULTS

Simple steatosis had 5 (50%) females and 5 (50%) males and there were 32 (64%) females and 18 (36%) males in steatohepatisis. No statistical differences (p<0.05) were observed between Simple steatosis and steatohepatisis, with respect to age, BMI, FBS and Lipid profile. The hs-CRP levels showed a significant increase in steatohepatisis (group 2) as compared to Simple steatosis (group 1) (p<0.0001). Steatohepatisis subjects had higher serum levels of TG and VLDL (217.71±97.16) (43.54±19.43), respectively as compared to serum levels of triglyceride and VLDL Simple steatosis subjects (193.2±80.30) (38.64±16.06), respectively, but did not show any statistically significant difference (p=0.071).Serum MTP levels were measured and analysed in simple steatosis (1.93±0.61) and steatohepatisis (1.48±1.02) patients of NAFLD subjects and no statistical significant difference was observed (p=0.995) [Table/Fig-2].

Mean VLDL and TG levels were significantly higher in NAFLD patient with co-morbidities as compared to NAFLD without co-morbidities in steatohepatisis patients and this difference was significant (p<0.0001), but non significant in simple steatosis patients (p=0.107).

Parameters	Group 1 (n=10)	Group 2 (n=50)	p-value
Age (years)	46.5±10.18	48.85±10.43	1.02
Gender (F/M)	5/5(50%/50%)	32/18 (64%/36%)	0.41
BMI	27.56±7.82	27.69±7.17	0.65
hs-CRP	7.136±2.24	32.08±36.36	<0.0001**
FBS	184.5±105.022	169.67±80.86	0.24
LFT			
ALT	50.4±35.38	43.71±38.41	0.85
AST	40.7±19.18	49.21±45.45	0.008*
ALP	125.2±85.42	98.49±50.13	0.02*
Albumin	3.42±0.66	3.38±0.79	0.59
GGT	26.3±10.39	26.38±10.19	0.85

Lipid profile			
SC	128.5±58.62	158.67±57.71	0.86
TG	193.2±80.30	217.71±97.16	0.07
HDL	24.6±6.619	27.30±9.24	0.28
VLDL	38.64±16.06	43.54±19.43	0.07
MTP	1.93±0.61	1.48±1.02	0.99

[Table/Fig-2]: Demographic and biochemical characteristics of simple steatosis and steatohepatisis subjects.
Values are expressed as Mean±SD. *p<0.05 statistically significant and **p<0.0001 as highly significant. Unpaired Student t-test was used.
BMI: Body mass index; hs-CRP: High-sensitivity C-reactive protein; FBS: Fasting blood sugar; ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transferase; SC: Serum cholesterol; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein

Mean MTP levels were significantly lower in NAFLD cases with co-morbidities as compared to NAFLD cases without co-morbidities in simple steatosis patients (p=0.006), but non significant in steatohepatisis patients (p=0.532) [Table/Fig-3].

Groups		Triglyceride		VLDL		MTP	
		Mean±SD	p-value	Mean±SD	p-value	Mean±SD	p-value
Group 1	1A	241.8±80.7	0.107	48.4±16.1	0.107	0.8±0.4	0.006*
	1B	144.6±19.3		28.9±3.9		3.1±0.6	
Group 2	2A	291.7±87.5	<0.0001**	58.4±17.5	<0.0001**	1.4±1.0	0.532
	2B	146.3±20.9		29.3±4.2		1.6±1.0	

[Table/Fig-3]: Serum levels of triglycerides, VLDL and MTP between Group 1 and Group 2 in NAFLD patients.
**p<0.0001 as highly significant. Unpaired Student t test was used.
1- Simple steatosis, 2- Steatohepatisis, A- cases with co-morbidities, B- Cases without co-morbidities

Correlation analysis was done between serum levels of MTP versus TG and MTP versus VLDL. A negative correlation was observed in both the cases with co-morbidities (group 1A and 2A), suggesting inverse relation between MTP and TG as well as VLDL. However, a significant negative correlation was observed between MTP and TG, VLDL in simple steatosis cases with and without co-morbidities (1A+1B) (r=-0.665, p=0.036) [Table/Fig-4].

Groups	Triglyceride vs MTP		VLDL vs MTP	
	r	p-value	r	p-value
1A + 1B + 2A + 2B	-0.177	0.16	-0.177	0.16
1A + 2A	-0.022	0.906	-0.022	0.906
1B + 2B	0.041	0.822	0.041	0.822
1A	-0.448	0.449	-0.448	0.449
2A	-0.047	0.815	-0.047	0.815
1B	-0.134	0.83	-0.134	0.83
2B	0.083	0.673	0.083	0.673
1A + 1B	-0.665	**0.036***	-0.665	**0.036***
2A + 2B	-0.08	0.563	-0.08	0.563

[Table/Fig-4]: Correlation between Group 1 and Group 2 of MTP with Triglyceride and VLDL in NAFLD cases with and without co-morbidities.
Pearson's correlation was used. *p<0.05 statistically significant.
1- Simple steatosis, 2- Steatohepatisis, A- Cases with co-morbidities, B- Cases without co-morbidities

DISCUSSION

The MTP as the name suggests is involved in transfer of TG from hepatocytes to the peripheral tissues. Therefore, if MTP is dysfunctional it may contribute to TG accumulation in hepatocytes which eventually results in fatty liver [20,21]. Present study measured and compared the serum levels of MTP in NAFLD subjects with simple steatosis (Group 1) and steatohepatisis (Group 2). Present study results showed that levels of MTP were decreased in steatohepatisis as compared to simple steatosis. As steatohepatisis of NAFLD is associated with inflammation and is the advanced stage of NAFLD, decreased levels of MTP in steatohepatisis indicates impact of its association with NAFLD and its stages.

Studies have associated NAFLD with co-morbidities like obesity, diabetes mellitus, cardiovascular disease and hyperlipidaemia [22-26]. These co-morbidities with NAFLD carries an increased risk of liver related morbidity and mortality [27]. Therefore, in this study authors wanted to investigate whether MTP levels are affected by the presence of co-morbidities in NAFLD patients. In the present study, when MTP levels were compared between cases with co-morbidities and cases without co-morbidities in both the stages, subjects with co-morbidities had decreased levels of MTP as compare to subjects without co-morbidities. This further supports the role of MTP in pathogenesis and progression of NAFLD stages.

The NAFLD is a multifactorial disease that results from complicated interactions among dietary components, lifestyle choices and genetic determinants [28]. Several research have been looked into gene-expression based NAFLD staging in order to better understand disease progression and find the effective treatment [29-31]. An interaction between MTP and NAFLD is also been identified with the help of genetic studies. A polymorphism at promoter region (-493G/T) of MTP is associated in NASH with type 2 Diabetes Mellitus. The G allele is associated with decreased MTP transcription and is prone to increased TG content in hepatocytes [30-33]. Transversion of base guanine to thymine at 493 position in the promoter region of MTP is found to be associated with decreased transcription level of MTP and failure in TG secretion from hepatocytes which increases the susceptibility to NAFLD [17,20]. The -493 allele is also considered as a risk factor in pathogenesis of metabolic syndromes [34]. The polymorphism in MTP gene is also been suggested to be a useful and practical biomarker for early diagnosis of NAFLD [35]. Therefore, decreased serum levels of MTP observed in advanced stage of NAFLD in present study thus could be linked to altered gene expression.

Limitation(s)

The study was limited firstly by small sample size due to inadequate number of patients who met the inclusion criteria and secondly due to lack of genetic analysis.

CONCLUSION(S)

The decreased serum levels of MTP in advanced NAFLD stage suggests that MTP can be a complementary marker in differentiating simple steatosis from steatohepatisis. As MTP also shows a negative correlation with TG and VLDL when compared between cases with co-morbidities and cases without co-morbidities, therefore MTP can be considered as complementary marker in subjects with co-morbidities as well. For further prospects, studies involving all stages of NAFLD in a larger population are required. The genetic analysis and increased sample size might show significant results which can be helpful in identifying MTP as a complementary marker for differentiating the simple steatosis from steatohepatisis and assessing the disease progression.

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