Mcp-$_1$ in Non-Alcoholic Steatosis in Egyptian Patients

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Abstract: Background: The monocyte chemoattractant protein-$_1$ (MCP-$_1$) is a member of the chemotactic cytokines chemokine family, it may contribute to the formation and maintenance of inflammatory infiltrate observed in chronic liver diseases**.** We aimed in this work to study this protein in non alcoholic fatty liver disease (NAFLD) and to explain its role in pathological progression of simple steatosis to steatohepatitis. **Methods:** This study was done in Zagazig University hospitals between February 2010 and May 2011, and it included **31**control healthy subjects as group **I** (19 males and 12 females) and **55** NAFLD patients (37 males and 18 females) which were classified according to the results of liver biopsy into : simple steatosis (group **II**)and steatohepatitis(group **III) .** All subjects included in the study underwent full history, clinical examination and abdominal ultrasonography **.** For patient groups only, liver biopsy and monocyte chemoattractant protein-$_1$ in serum were determined. **Results:** Our results found significant differences between controls and patients groups and between group II and group III (patient groups) as regard liver enzymes (AST and ALT) and dyslipidemia in form of hypertriglyceridemia **.** As regard MCP-$_1$ levels, our results showed higher levels of MCP-$_1$ in patients groups with a significant differences between control and patients groups and also significant differences between simple steatosis group and steatohepatitis **Conclusion:** Chemokines are important target molecules, especially MCP-1, which affects the pathway of steatosis and its progression into steatohepatitis in NAFLD **.**
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1. Introduction:

Non alcoholic fatty liver disease (NAFLD) is a chronic inflammatory disease involving a wide range of disorders from simple steatosis, passing to steatohepatitis and ended by fibrosis and cirrhosis , even it may be also passing to hepatocellular carcinoma **.** Meanwhile, steatosis has been considered a benign disease. It is necessary to stop the disease at this stage and prevent the progression to stage of steatohepatitis and cirrhosis which followed by hepatocellular carcinoma **.**

Non alcoholic fatty liver (NAFLD) is characterized by liver damage similar to that caused by alcohol but it occurs in individuals that consume toxic quantities of alcohol (1) **.** The prevalence of NAFLD is not well known, but various studies gave range between 3% and 24% (**2,3) .**

Non alcoholic fatty liver disease has been associated with many etiological factors **(1,4,5)** , the most common ones being obesity, type 2 diabetes mellitus and dyslipidemia.

Obesity >10% of normal weight or body mass index (BMI) >30 is the most common cause of NAFLD, approximately 80% of patients are obese, and the opposite is also true, 80% obese people suffer from NAFLD (**6,7,8,9) .**

Hepatic steatosis is characterized by accumulation of lipid droplet in the liver. Although relatively benign, simple steatosis can eventually lead to the development of steatohepatitis which progressed to fibrosis, cirrhosis and eventual liver failure if the underlying cause is not eliminated**.** Two theories explain the progression of disease, the initial one involves fat accumulation in the liver and second hits leads to inflammation and subsequent tissue injury **(10) .**

Over 90% of patients with NAFLD have at least one feature of the metabolic syndrome, with about one-third having the complete syndrome ; which defined as three items of either: central obesity, impaired fasting glucose, hypertriglyceridemia, low high-density lipoprotein–HDL- cholesterol and hypertension. **(11) .** The prevalence of NAFLD in obese individuals is 76% as compared with 16% in non-obese individuals **(12) .**

Body mass index (BMI), triglyceride, total cholesterol and fasting plasma glucose were independently associated with NAFLD in non-diabetic individuals **(13) .**

Obesity increase the risk of hepatocellular carcinoma in the general population, most likely as a consequence of advanced NASH **(14)** .

Chemokines, chemotactic cytokines, are small heparin-binding proteins that constitute a large family of peptides -60-100 acids- structurally related to cytokines, whose play an important function to regulate cell trafficking **(15) .**

Chemokines are secreted in response to signals such as proinflammatory cytokines where they play an important role in recruiting monocytes, neutrophils and lymphocytes and this once stimulated followed by release of this chemokines which directed migration of cells expressing the appropriate chemokine receptors along a chemical liganed gradient known as chemokines gradient, and this allows cells to move toward high local concentration of chemokines **(16) .**

Chemokines are classified into four subfamilies on the basis of the numbers and location of the cysteine residues at the N-terminus of the molecule and their names are CXC, CC, CX_3C and C **(17) .** The structure of chemokines comprises three distinct domain: (**1**) highly flexible N-terminal domain, which is attached by disulfide bonding between the N-terminal cysteine (s); (**2**) a long loop that leads into three antiparallel B-pleated sheets; and (**3**) an - helix that overlies in sheets **(18) .**

Adipose tissue secretes bioactive proteins termed adipokines, in obesity an increased production of various adipokines leads to multiple pathological disturbance**(19) .**

The monocyte chemoattractant protein-1 (MCP-$_1$/CCL_2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes, it is the first discovered human CC chemokine, it is composed of 76 amino acids and is 13 KDa in size**.** On the other hand, it also named CCL_2 (cysteine =cysteine motif chemokine liganed) **(20).**The chemokine monocyte chemoattractant protein-1 (MCP-$_1$), has been recently been added to the growing list of adipokines **(21,22) .** On the other hand, MCP-$_1$ expression has been shown to be increased in number of pathological conditions including atherosclerosis(**23)**, pulmonary fibrosis**(24)** kidney diseases **(25)**, tumors **(26)**, rheumatoid arthritis **(27**), insulin resistant diabetes **(28**)and multiple sclerosis (**29) .**

Previous research stated that secretion of MPC-$_1$ may contribute to the formation and maintenance of inflammatory infiltrate observed in chronic liver disease (**30) .**

This work was designed to study monocyte chemoattractant protein one- MAP-$_1$in NAFLD and to explain its role in pathological progression of simple steatosis to steatohepatitis**.**

2. Materials and Methods

Research Design:

This prospective case-control study was performed from February, 2010 till May, 2011in Zagazig University hospitals, and included **55** NAFLD patients (37 males and 18 females- mean age 47± 7.5) and **31** healthy control subjects (19 males and 12 females- mean age 48±6.5) **.** Patients group was divided according to liver biopsy into simple steatosis patients (group **II**) and steatohepatitis patients (group **III**) **.**

A consents from all subjects participated in the study were taken **.** All subjects underwent full history (asymptomatic-fatigue-right hypochondrial pain and dyspepsia), clinical examination (general examination, especially BMI- waist circumference-blood pressure) and local abdominal examination (hepatomegaly , splenomegaly and ascites) **.**

Abdominal ultrasonography to asses degree of hepatic steatosis and exclude other hepatic disorders was performed **.**

Investigations were done for patients and control and includes **:** liver and kidney function tests, viral markers (HCV Ab and HB s antigen), ANA ,AMA , fasting and 2hour post prandial blood glucose and lipid profile(total triglyceride – HDL cholesterol) **.**

Special investigations (for patients only)**:** Liver biopsy and monocyte chemoattractant protein-$_1$ (MCP-$_1$)in serum.

Inclusion criteria:

1-Obese and overweight (patients and control)

2-Diagnosis of NAFLD according to the following criteria **:**

- Normal or elevated transaminases(>2times of upper normal value ,for 6 months)**.**
- No other cause for liver disease **.**
- Bright echopattern by abdominal sonography **.**
- Liver biopsy **:** NAFLD patients group will divided according to liver biopsy into simple steatosis patients (group **II**) and steatohepatitis patients (group **III**) **.**

Exclusion criteria :

- History of alcohol intake **.**
- Evidence of viral hepatitis (HCV and HBV markers), drug induced hepatitis or autoimmune hepatitis **.**
- Liver cirrhosis, cholestasis ,or hepatic focal lesion **.**
- History of drugs causing hepatic steatosis as corticosteroids, amiodarone, calcium channel blockers or methotrexate **.**
- Other diseases associated with increasing level of MCP-$_1$ as **:** pulmonary Fibrosis, kidney diseases, rheumatoid arthritis, atherosclerosis , tumors and multiple sclerosis **.**

Study steps:

1- Thirty one healthy subjects were included as control group (19 males and 12 females- mean age 48±6.5). They had normal liver function tests, negative viral hepatitis markers, normal liver echogeneicity by ultrasonography and no evidence of chronic liver diseases (CLD). They also were matched to patients as regard age, sex and BMI **.**

2- Fifty five NAFLD patients (37 males and18 females - mean age 47±7.5) **.** The followings were applied **:**

1) Full history taking and clinical examination with stress on manifestations of CLD.

2) Estimation of BMI using the formula weight (kg) /height (m2). Subjects with BMI 25-29.9 and >30 were considered over weight and obese respectively .

3) Waist/ hip ratio was measured at the level midway between the lowest rib margin and the iliac crest and was plotted on American percentile for waist circumference**(31).** Hip circumference was measured at the widest level over the greater trochanter in a standing position, by the same examiner, after that calculation of W/P ratio was done . Abnormal W/H ratio was considered if it was > 0.89 **.**

4) Laboratory measurement including fasting, post prandial blood glucose level (impaired glucose tolerance if fasting glucose<126and 2 hour post prandial glucose >140 **(32)**, liver function tests and total cholesterol and triglyceride, using Hitachi 912 autoanalyzer system (Roche-Diagnostic GmbH, Mannheim, Germany) **.**

5) Abdominal ultrasonography **.**

6) Special investigations **:**

A. **Liver biopsy :** Histopathological examination of specimens for grading and staging of steatosis, microinflammation and fibrosis was done according to criteria at the 45th Annual Meeting of the JSH in June 2009, it was agreed that a diagnosis of NASH should be based on the following three features; (**1):** Hepatic steatosis (>5%-10% of hepatocyte affected); (**2):**Lobular inflammation with mononuclear cells and/or neutrophils; and (**3):** Ballooning degeneration of hepatocytes **(33) .**

B. **Determination of MCP-1 protein :** MCP-$_1$ protein levels in serum obtained from study participants were measured using a solid –phase sandwich enzyme-linked immunosorbent (MCP-$_1$ Quntikine ELISA kit, R&D systems, Abingdon, UK) **.**

Briefly,100 uL of duplicated samples or standards (reeom-binant human MCP-$_1$) were incubated (2 hours at room temperature) in wells pre-coated with primary antihuman MCP-$_1$ antibody. After incubation, wells were washed three times, horseradish peroxidase- conjugated polyconal antibodies against MCP-$_1$ were add (for 2 hours at room temperature) **.**

Finally, tetramethylbenzidine substrate solution was applied for 30 minutes in dark , and after stopping the reactions by 2M sulfuric acid, the absorbance was measured at 450 nm (with correction at 540 nm). The data were evaluated with KIM-E software (USOL, Prague ,Czech Republic); the detection limit of the MCP-$_1$ was 5.0 pg/ml.

3. Results

As regard age and sex, our study showed no significant difference between control and patients groups ; simple steatosis and steatohepatitis groups .

Also, our results showed a significant differences between controls group and NAFLD groups as regard BMI and W/H ratio(expression about the degree of obesity) .

Our results found significant differences between controls and patients groups and between group II and group III (patient groups) as regard liver enzymes, AST and ALT and dyslipidemia in form of hypertriglyceridemia **.**

As regard MCP-$_1$ levels, our results showed higher levels of MCP-$_1$ in patients groups with a significant differences between control and patients groups and also significant differences between simple steatosis group and steatohepatitis .

Table (1): Demographic data for controls(group I) and NAFLD groups (group II and III) .

Groups / Data	Control Group(I)N=31	Simple steatosis Group (II) N=34	Steatohepatitis Group (III) N=21	Value P
Age	45.3±3.5	45.1±3.3	45.4±3.5	P =0.905
Sex				
Male	19	20	12	
Female	12	14	9	P=0.954
BMI(Kg/m)	24.7±3.6	28.9±1.9	33.4±2.5	P<0.0001
Waist/hip ratio cm (not more than 0.89)	0.96±0.2	1.2±0.1	1.3±0.3	0.0001< P

Table(2) :Specific laboratory finding of controls group I and patients groups II & III.

Groups / Parameters	Control Group(I) N=31	Simple steatosis Group(II) N=34	Steatohepatitis Group(III) N=21	Value P
Liver enzymes				
ALT(n=5-41)	17.9±5.8	34.5±3.7	53.7±14. 5	0.0001<
AST(n=5-37)	20±2.8	30±2.1	44.9±5.5	0.0001<
FBG(mg/dl) n=65-110)	86.9±8.3	100.4±3.1	108.7±6.3	0.0001<
Cholesterol T(mg/dl) N=100-200)	127.4±16.3	126.1±10.8	126.9±16.4	0.935<
Triglyceride (mg/dl) N=35-160)	114.7±8.2	162.1±20.9	183.6±8	0.0001<
HDL (mg/dl) N=(30-70)	42.1±2.3	37.4±3.15	37.5±1.4	0.0001<
MCA-P_1(pg/ml)	309±78.1	365.9±148.4	482.9±132.1	0.0001<

Table (3): Clinical and Laboratory differences between simple steatosis (group II)and steatohepatitis (group III) .

Groups / Parameters	Simple steatosis Group(II) n=34	Steatohepatitis Group (III)n=21	Value P
BMI	28.9±1.9	33.4±2.5	0.000<
W/P ratio	1.2±0.1	1.3±0.3	< 0.049
Liver enzymes			
ALT(n=5-41)	34.5±3.7	53.7±14. 5	0.000<
AST(n=5-37)	30±2.1	44.9±5.5	0.000<
Cholesterol T(mg/dl) N=100-200)	126.1±10.8	126.9±16.4	< 0.828
Triglyceride (mg/dl) N=35-160)	162.1±20.9	183.6±8	< 0.000
HDL (mg/dl) N=(30-70)	37.4±3.15	37.5±1.4	< 0.879
MCA-P_1(pg/ml)	365.9±148.4	482.9±132.1	<0.005

4. Discussion

Non alcoholic fatty liver (NAFLD) is the most common cause of chronic liver disease . The histological spectrum of NAFLD includes, simple steatosis which has a benign prognosis, and nonalcoholic steatohepatitis which is a more aggressive form of liver injury that may progress to cirrhosis and its complications.

Many researches are deal with studying the inflammatory markers responsible for the pathological progression in NAFLD. As the most important cause in NAFLD is obesity so our research is containing obese patients depending on BMI and W/P ratio..

As regard BMI and W/H ratio in our study, the significant differences between hepatic steatosis and hepatic steatohepatitis groups is agreed with **Finucane et al., (2008)**[34] , which can be explained by the fact that high dyslipidemia in obese patients can contribute to more hepatic injury and fatty liver pathological changes

On the other hand, a multivariate analysis showed that BMI was only predictor of pathological progression in children with NASH **(35)**, but this research including only child group of patients .

As regard liver enzymes and dyslipidemia our results are in agreement with study reported by **Assy et al. (2000)**[36] who found serum AST values, hypertriglyceridemia independently predict the presence of fatty infiltration

EL-Karaksy et al. (2011)[37] supported our results as they found a significant differences between simple steatosis group and steatohepatitis group (resemble our patients groups)as regard higher liver enzymes , ALT, AST, cholesterol and triglycerides , and a lower HDL.

This results can be explained by the more pathological progression from just a simple steatosis to more pathological progression causing more hepatic injury, and this reflecting in the form of elevated liver enzymes, more dyslipidemia in the form of hypercholesterolemia and

hypertriglyceridemia and lower HDL, causing more pathological injury of the liver and progression from simple steatosis to steatohepatitis .

Studies dealing with several inflammatory markers responsible for pathological progress in NAFLD reported that MCP-$_1$ is an important one of these markers . The main receptor in vivo for MCP-$_1$ is CCR2 **(38)** .

As regard MCP-$_1$ our results showed significant higher levels of MCP-1 in patients groups(more in steatohepatitis group) compared to controls. This was agreed with study reported by **Haukeland et al.,(2006)[39]** The results, as CCL$_2$/MCP-**1** is not only of major importance for monocyte recruitment in inflamed tissue but also a potent activator of leukocyte and other cell types at the site of inflammation through its ability to induce oxidative stress and matrix degradation **(40,41)**.

Other explanation assumed that obesity activate expression liganed 2 which is CCL$_2$/MCP-$_1$ leading to hepatic recruitment of myeloid cell that promote hepatosteatosis, especially if the diet containing high fat content, on the other hand these cells activate hepatic transcription of genes responsible for fatty acid esterifications and steatosis **(42)** .

Finally and in this respect, chemokines are important target molecules, especially MCP-1, which affects the pathway of steatosis and its progression into steatohepatitis in NAFLD. So, presence of oral chemokines receptor antagonist starting with MCP-1 may help in reverse of steatohepatitis and stopping the bad pathological progression of NAFLD to cirrhosis and liver failure.

So, we recommended a more studies to give enough explanation of the pathogenesis of NAFLD, larger studies and analysis of liver specimens are mandatory. Further elucidation of the pathophysiology of the disease and role of other inflammatory markers in pathogenisis of NFLD in order to identify cellular and molecular pathways for monocyte and macrophage differentiation and interaction with hepatic cell which may represent novel targets for future therapeutic approaches in liver fibrosis.

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