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**RESEARCH ARTICLE**

# Platelet Microparticles and Monocyte Platelet Aggregates: Crucial Components of Chronic Hepatitis C Pathway

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## *Abstract*

We aimed to clarify the impact of platelets microparticles (PMP) and monocyte platelet aggregates (MPA) interactions as aetiological factors of liver fibrosis in patients with chronic hepatitis C. Forty five patients were the subjects of the study, classified according to Child Pugh classification into; Child A, B and C groups (15 patients each), in addition to 15 matched controls. Flowcytometry was used for assessment of Platelet activation by quantitative assay of CD62P and CD63 PMPs and percentage of MPA. Liver fibrosis was assessed by both quantitative serum assay of monocyte chemoattractant protein-1 (MCP-1), matrix metaloproteinase-1 (MMP-1) and tissue inhibitor of matrix metaloproteinase-1 (TIMP-1) by ELISA technique, as well as immunohistochemical assay of MMP-1 and TIMP-1 in liver tissue. Enhanced platelet activation was demonstrated by increased PMP and PMA in different patients' groups and was more evident in advanced stages of the disease. Serum fibrosis markers revealed high MMP-1 and low levels of both TIMP-1 and MCP-1. Immunostaining showed increased positivity of MMP-1 with necroinflammtory activity and advancement of fibrosis. Also, immunostaining of TIMP-1 showed decrease in the number of positive cases as well as decrease in the intensity with advancement of fibrosis.

Platelet activation markers showed a significant negative correlation with serum MMP-1 and positive correlation with serum TIMP-1. On the other hand, it showed a positive correlation with tissue immunostaining for both MMP-1 and TIMP-1. The above mentioned data are suggestive of a crucial role of platelet activation in the pathway of liver fibrosis.



## Introduction

Microparticles are submicron vesicles and their roles as participants in coagulation, cellular signalling, vascular injury and homeostasis has been identified (Hargett and Bauer, 2013). They have gained an increasing attention as biomarkers for various diseases (Kornek and Schuppan, 2012). Microparticles derived from various cells, most notably platelets, are present in the circulation of healthy subjects. Shedding of microparticles is amplified in response to cellular activation, high sheer stress as well as cellular apoptosis (Tushuizen et al., 2011). In contrast to their origin, microparticles are now recognized as conveyers of intercellular communication (Hargett and Bauer, 2013, Andrews and Berndt, 2013).

In response to platelet activation several changes occur at the platelet cell surface accompanied by fusion of intrinsic granule membranes with it, thus bringing with them specific membrane glycoproteins which serve as markers for platelet activation. These include P-selectin (CD62P) and lysosomal membrane protein (CD63) that transmit stimulation signals when in contact with other cells (platelets, monocytes and lymphocytes) (Sirolli et al., 2002). Activation of platelets also results in the formation of platelet microparticles (PMPs) which include membrane fragments that are generated by mechanical cell destruction (Ogasawara et al., 2005).

Activated platelets exert a pro-inflammatory action that can be largely ascribed to their ability to interact with leukocytes and modulate their activity (Cerletti et al., 2010). PMPs are not just by-products of cellular activation or apoptosis, but are functional and modulate the activity of other cells (Andrews and Berndt, 2013). Several studies suggested a role of PMPs in inflammatory processes as to enhance adhesiveness of inflammatory target cells, thereby promoting enhanced recruitment of monocytes on inflamed endothelium (Mause et al., 2005). Accordingly, microparticles are not simply surrogate markers of platelet activation or disease activity, but, can also modulate neighbouring cells, through several mechanisms may be by transferring functional cell surface receptors that may trigger novel pathways in the recipient cell, or by exchange of mRNA and/or miRNA , another mechanism could be the transfer of antigen via MCH class II molecules and other surface molecules (Mause et al., 2010).

Platelet interaction with inflammatory and immunological cells is intensified by the products of platelet degranulation and results in the formation of platelet-leucocyte complexes mainly monocyte-platelet aggregates (MPA) in the circulation (Panasiuk et al., 2007; Sayed et al, 2011). The presence of circulating heterotypic monocyte-platelet aggregates is a more sensitive index of platelet activation than is the identification of singlet circulating activated platelets (Pyton et al., 1998). MPA were found to have more adhesive capacity to the endothelium compared to circulating monocytes (da Costa Martins, 2004).

Cirrhotic thrombocytopenia and platelet dysfunction are multifactorial with many simultaneous contributions (Violi et al., 2011), possible mechanisms proposed have been mainly due to portal hypertension that leads to splenic pooling, sequestering platelets from the circulation (Adnolfi et al., 2001). Portosystemic shunting and gut barrier disruption result in endotoxaemia with systemic immune activation , antiplatelet IgG antibody production , aberrant fibrinolysis, and activation of coagulation with platelet consumption (Violi et al., 2011). Moreover, cirrhosis and decreased functional liver mass result in lower thrombopoietin level; in addition to the platelet underproduction by myelosupressive effects of hepatitis C virus on the bone marrow (Espanol et al., 2000).

Although reticulated platelets (RP) are the youngest circulating platelet population newly released by the bone marrow, they represent the assay of stress thrombopoiesis thus provide reproducible indices for timing platelet recovery (Wang et al., 2002), reflect the pathology of thrombocytopenic disorders, and their measurement is useful for the differential diagnosis and analysis of platelet kinetics (Sakakura et al., 2005). It was concluded that, RP determination is a reliable marker of platelet turnover and may be as good as an initial screening test for thrombocytopenic patients (Monteagudo et al., 2008).

Hepatic fibrosis develops in response to chronic injury. It is a multicellular process that is characterized by progressive changes in the extracellular matrix, and in cellular composition and function (Campbell et al., 2005; Luedde and Schwabe, 2011). Blood platelets while activated in inflammatory and immune processes in liver diseases, release active compounds namely platelet factor- 4(PF4), Beta-thromboglobulin (βTG) and platelet derived growth factor (PDGF). These proteins provoke stimulating influence on fibrogenesis and mitogenesis of hepatic stellate cells (HSCs) in the liver (Mannaioni et al., 1997).

Panasiuk et al., (2007) and Sayed et al., (2010), concluded that monocyte and platelet activation in liver cirrhosis may contribute to progressive liver injury. Macrophages and infiltrating monocytes participate in the development of fibrosis via several mechanisms, including secretion of cytokines and generation of oxidative stress-related products (Marra et al., 2009). It has been suggested that hybrid microparticles (MP) (which would carry markers for both platelets and, monocytes) could be generated when two different MP classes fuse together, or when, monocyte-derived MP become incorporated into platelets, which then vesiculate in turn. Such MP fusions could explain the conflicting reports on the origin of tissue factor (TF) in circulation. If such fusion MP existed, they could offer a carefully controlled means of providing active TF. Regarding the identification of the cellular origins of MP, the possibility of such fusions presents a novel challenge (Siljander, 2011).

Monocyte chemotactic protein-1 (MCP-1) is a potent chemoatractant and activator for circulating monocytes and T lymphocytes and its hepatic expression is up-regulated during chronic hepatitis C (HCV) infection mainly in activated hepatic stellate cells (HSC) (Mühlbauer et al., 2003). In normal liver, a modest expression of MCP-1 was confined to few perisinusoidal cells and to bile duct epithelial cells. Previous in vitro studies showed that secretion of MCP-1 may contribute to the formation and maintenance of the inflammatory infiltrate observed during chronic liver disease (Marra et al., 1998).

As chronic liver disease progress, an imbalance occurs between synthesis and breakdown of extracellular matrix. Matrix metalloproteinases (MMPs) are involved in degrading extracellular matrix (ECM), while tissue inhibitor of matrix metalloproteinases (TIMPs) prevent their fibrinolytic action (Walsh et al., 1999). Matrix metalloproteinases are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for the tissue remodelling and degradation of the extracellular matrix and are excreted by a variety of connective tissue and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes (Verma and Hansch, 2007). Tissue inhibitor of metalloproteinase-1 (TIMP-1) is present in most human tissues and body fluids that inhibits MMPs, stimulates cell growth, and prevents apoptosis. It has been investigated as a marker of chronic liver disease (CLD). Following liver injury, HSCs become activated and express a combination of MMPs and their specific TIMPs. In the later stages of liver injury and HSCs activation, the cells express a combination of MMPs that have the ability to degrade normal liver matrix, while inhibiting degradation of the fibrillar collagens that accumulate in liver fibrosis (Nagase and Brew, 2003).

**Aim of the work**

The aim of the present study was to assess platelet count, thrombopoiesis, platelet activation markers namely platelet microparticles and monocyte platelet aggregates, and their correlation with serum and tissue markers of liver fibrosis namely monocyte chemoattractant ptotein-1, matrix matalloproteinase-1 and tissue inhibitor of matrix metalloproteinase-1. In an attempt to clarify the impact of PMP and MPA interactions as aetiological factors of liver fibrosis in patients with chronic hepatitis C. Unveiling of this relationship could pave the way for therapeutic pharmacological modulation of these activation markers which might lessen the advancement of fibrosis in chronic hepatitis C patients.

## Material and Methods

Patients Profile:

This study enrolled 45 patients (18 women, 27 men), their ages ranged between 31 and 62 years, with chronic liver disease admitted to Gastroenterology and Hepatology Department, Theodor Bilharz Research Institute (TBRI), Giza, Egypt. All patients were positive for hepatitis C virus, negative for hepatitis B virus. The patients were classified according to modified Child Pugh classification into Child A, B and C groups (15 patients each), in addition to a healthy age and sex matched control group (15 individuals). Diagnosis of patients was based on thorough clinical examination, abdominal ultrasonography, liver function tests and hepatitis markers. Upper gastrointestinal endoscopy and liver biopsy were done when indicated. None of the patients had active variceal bleeding or encephalopathy at time of investigation. Patients with fever, overt infectious disease (septicemia, pneumonia, urinary tract infection) or renal insufficiency were excluded.

The study protocol was approved by Theodor Bilharz Research institutional committee for the protection of human participants and conformed to the guide lines of the 1975 Declaration of Helsinki.

**II- Laboratory works up:**

Five milliliters of venous blood samples were collected under complete aseptic conditions from all patients and control subjects.

Samples were divided into:

- One milliliter EDTA blood was used for complete blood counts using electronic cell counter (ACT differential Beckman Coulter), and flow cytometric assay of MPA.

- For assay of RP and PMP, 1.8 ml of blood was mixed with 0.2 ml (0.1 mol/L) sodium citrate (in a ratio of 9:1) and was separated by double centrifugation. First, platelet rich plasma (PRP) was prepared by centrifugation at 2000 rpm for 10 min and used for counting of RP. Then, platelet poor plasma (PPP) was separated by centrifugation of PRP at 4000 rpm for 1 min and used for PMP counting using flowcytometry.

- Two milliliters of blood was delivered into a clean tube and left to clot. Serum was separated by centrifugation at 3000 rpm for 15 min. Serum samples were used for assay of liver function tests {Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)} using autoanalyzer (Hitachi 736, Hitachim, Japan). Also, serum samples were used for assay of hepatitis markers (HBs-Ag and HCV-ab) using enzyme linked immunosorbent assay (ELISA) technique. In addition serum samples were used for quantitative assay of serum monocyte chemoattractant protein-1 (MCP-1), serum level of marix metalloproteinase-1 (S MMP-1) and serum level of tissue inhibitor of matrix metalloproteinase1 (S TIMP-1).

**Assay of reticulated platelet:**

Five µl of PRP was added to one ml of thiazole orange and incubated for 30 min at room temperature. The percentage of circulating thiazole orange positive platelets was determined by flowcytometry

**Assay of monocyte-platelet aggregates ($CD14^+$, $CD41^+$)**:

EDTA blood samples were fixed with 1% paraformaldehye for 10 min. Two ml of lysing solution (EasyLyseTM: erythrocyte-lysing reagent, Dakocytomation), was added followed by centrifugation. Then 5µl of each sample was incubated with 5µl of CD14 monoclonal antibody labelled with fluorescin isothiocyanate (FITC) and 5µl of CD41 monoclonal antibody labelled with phycoerythrin (PE) (Bekhman Coulter, USA) for 30 min. For each sample, matched control tube (without CD14 and CD41) was used to correct for non specific binding. Monocytes were identified by gating on CD14 positive. The percentage of double positive cells for CD14 and CD41 represented monocyte platelet aggregates.

**Assay of platelet microparticles ( CD 62p+, CD 63+)**:

Five µl of PPP was incubated with 5 µl anti-CD62P monoclonal antibody labelled with fluorescin isothiocyanate (FITC) (Bekhman Coulter, USA) and 5 µl anti-CD63 monoclonal antibody labelled with phycoerythrin (PE) (Bekhman Coulter, USA) for 30 min. One ml of phosphate buffered (PBS) saline was added. Then 100 µl of flow count flurospheres (Flow beads of standard size, Beckman Coulter, USA) were added and the samples were analyzed by flow-cytometry. For each sample, matched control tubes (lacking the corresponding monoclonal antibody) were used to correct for non specific binding. PMPs were characterized by size <1µm

and bound to CD62P and CD63. Data acquisition was stopped after 1000 fluorospheres were counted. To count PMPs, the following calculation was used according to Baran et al., (2010):

Microparticles =

$$\frac{\text{No. of events in region containing MP}}{\text{No. of events in absolute count bead region}} \times \frac{\text{No. of beads per test}}{\text{Test volume (µl)}}$$

**Assay of Fibrotic Markers**

Enzyme linked immunosorbent assay (ELISA) was used for quantitative determination of serum MCP-1 (using BMS281 and BMS281TEN kit, Bender MedSystem, Austria, Europe), serum MMP-1 (using Quantikine kit, R&D Systems, USA) and serum TIMP-1 (using BMS2018 and BMS2018TEN kit, Bender Med System, Austria, Europe).

**Histological and Immunohistochemical study**

Formalin-fixed and paraffin embedded tissues from liver biopsy samples were used for immunohistochemical analysis of MMP-1 and TIMP-1. Liver biopsies were analyzed according to a histological METAVIR scoring system (The French METIVIR cooperative study group). Using two separate scores, one for necro-inflammatory grade (A for activity), where A1: minimal activity, A2: moderate activity, A3: severe activity and another for the stage of fibrosis (F), which scores fibrosis from F0-F4. A score of F1 to F2 signifies significant fibrosis, while a score of F3 and F4 signifies advanced fibrosis.

**Immunohistochemical analysis**

Immunostaining reaction was performed using an avidin biotin complex (ABC) immunoperoxidase technique according to Hsu et al, (1981), using anti human MMP-1 and TIMP-1 on paraffin sections; dewaxed in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in 100% methanol for 20 min. Antigen retrieval was performed by microwaving the sections in citrate buffer (PH 6.0) for 15 min at 700 Watt. Sections were incubated overnight at 4°C with the anti-human primary antibodies against MMP-1 and TIMP-1 (purchased from Santa Cruz Biotechnology Inc.; Santa Cruz, USA) monoclonal antibody, diluted at 1:100, 1:150 respectively in phosphate buffer saline (PBS). Next day, after thorough washing in PBS, the sections were incubated with streptavidin-biotin-peroxidase preformed complex and using a peroxidase/DAB (3, 3'-diaminobenzidine) enzymatic reaction. Staining was completed within 5-10 minutes, resulted in a brown-colored precipitate at the antigen site. The cell nuclei were counterstained with Mayer's haematoxylin. The cover slips were mounted using Dpx. Positive and negative control slides for each marker were included within each session. As a negative control, liver tissue section was processed in the above mentioned sequences but with the omission of the primary antibody which was replaced by PBS.

*The scoring of MMP-1 and TIMP-1 in liver tissue was based on intensity and extensiveness (by percentage population) of the positively stained cells. Both parameters were scored on a scale of 0-3 as follows:*

- Intensity according to Chen et al., (2003):

0 = negative staining (-), 1= weakly positive staining (+), 2= moderately positive staining (++), and 3= strongly positive staining (+++).

- Range according to Itoi et al., (2004):

0 = negative, 1 = positive staining in < 10% of cells, 2 = 10%-50%, and 3 = > 50%.

Liver sections were examined by Zeiss light microscopy at power X400 for both markers; the number of positively stained cells with the highest expression was semi-quantitatively recorded within ten successive fields were counted / section and the final value represents the mean. Zero percentage was given to unstained sections.

**Statistical Analysis**:

All statistical analyses were performed using the SPSS for Windows, version 11(software). Results were expressed as means ± standard deviation. Comparing means was performed by one-way ANOVA test. To evaluate correlations among the variables, a Pearson correlation test or Spearman correlation test was used as appropriate.

Stepwise multiple linear regression analysis was employed to evaluate any association between platelet activation markers and markers of liver fibrosis. A $p < 0.05$ was considered statistically significant.

## Results

Assessment of thrombopoiesis, platelet activation markers and serum liver fibrosis markers in different studied groups are illustrated in table (1). Tissue expressions of MMP-1 and TIMP-1 immunostain are illustrated in Tables (2, 3).

Study of platelet count together with reticulated platelets demonstrated increased platelet turn over as well as the presence of active thrombopoiesis with progression of liver disease. Enhanced platelet activation was demonstrated by increase in percentage of MPA, as well as PMP count measured by CD62P and CD63 in all patients` groups compared to controls. Significant inter-group difference, fulminating with disease progress in chronic hepatitis C patients, suggesting enhanced platelet activation associated with progress of disease.

Low serum MMP-1 levels was evident in patients groups compared to control and showed significant progressive decrease with advancement of disease **,** while serum concentration of TIMP-1 and MCP-1 values increased significantly in Child B and C, patients compared to controls with significant inter group differences (table: 1).

Immunohistochemical staining of MMP-1 and TIMP-1 proteins were observed in the cytoplasm of hepatocytes , blood vessels endothelium, hepatic sinusoids and biliary endothelial cells (Figure: 1, 2). In the current study, normal liver specimens showed faint MMP-1 and TIMP-1 protein expression in 53.5% and 33.3% of cases respectively (table 2, 3) (Figure: 1, 2).

Tissue protein MMP-1 showed increased expression and intensity of positivity with the necroinflammatory activity and advancement of fibrosis according to METVIR scoring system (table: 4, 5). On the other hand immunostaining of TIMP-1 showed significant decrease in the number of positive cases as well as decrease in the intensity with the necroinflammatory activity and advancement of fibrosis according to METVIR scoring system (Table: 4, 5) (Figure: 1, 2).

**Correlation analysis:**

Platelet activation markers (PMP expressing CD62P, CD63 and MPA) showed a highly significant positive correlation ($P<0.01$) with the markers of liver cell damage (serum AST and ALT) (table: 6), suggesting that platelet activation might have a great impact on the progression liver injury in CHC patient. Although, MCP-1 was significantly high in patients' groups compared to control, and the high serum level is more obvious with progress of the disease (table: 1), no statistical significant correlation could be detected between MCP-1 and markers of liver cell damage ($P>0.05$).

Low platelet count was encountered in all patients' groups compared to the control, with evident low counts in advanced stage of the disease. Statistical analysis revealed a significant negative correlation between platelet activation markers and platelet count ($P<0.05$), which clarify that platelet turnover is an important mechanism of thrombocytopenia in CHC patients.

In addition, all studied parameters of platelet activation showed a significant negative correlation with serum MMP-1 ($P<0.05$) and positive correlation with serum TIMP-1 ($P<0.05$). On the other hand, platelet activation markers showed a significant positive correlation with tissue immunostaining for both MMP-1 ($P<0.05$) and TIMP-1 ($P<0.05$). The above mentioned data are suggestive of a crucial role of platelet activation in the pathophysiological events associated with liver fibrosis.

**Table (1): The results of studied parameters in different groups**

| | Control Group (15) | Child A Group (15) | Child B Group (15) | Child C Group (15) |
|---|---|---|---|---|
| **AST** (IU/L) | 25.86±6.26 | 57.93±27.94$^{aa}$ | 56.0±26.8$^{aa}$ | 74.33±26.58$^{aa,b}$ |
| **ALT** (IU/L) | 27.6±4.89 | 56.06±28.94$^{aa}$ | 63.4±20.92$^{aa}$ | 77.73±25.34$^{aa,bb}$ |
| **Platelet Count** ($x10^3$/µl) | 267.46±49.15 | 217.26±30.55$^{a}$ | 151.8±32.27$^{aa,bb}$ | 81.0±22.67$^{aa,bb,cc}$ |
| **RP** (%) | 4.79±1.01 | 7.26±1.11$^{aa}$ | 10.29±1.56$^{aa,bb}$ | 19.07±2.35$^{aa,bb,cc}$ |
| **CD62P PMP** ($x10^3$/ml) | 1.98±1.01 | 6.76±1.11$^{aa}$ | 11.23±2.00$^{aa,bb}$ | 19.11±2.59$^{aa,bb,cc}$ |
| **CD63 PMP** ($x10^3$/ml) | 2.37±0.89 | 6.88±1.29$^{aa}$ | 11.61±2.01$^{aa,b}$ | 19.28±2.71$^{aa,bb,cc}$ |
| **MPA** (%) | 3.79±2.36 | 49.71±12.17$^{aa}$ | 57.39±12.92$^{aa}$ | 76.62±16.28$^{aa,bb,cc}$ |
| **Serum MMP-1** (pg/ml) | 16.46±4.19 | 8.52±2.76$^{aa}$ | 4.38±1.93$^{aa,bb}$ | 3.68±2.01$^{aa,bb}$ |
| **SerumTIMP-1** (pg/ml) | 395.86±107.98 | 445.2±212.03 | 611.2±223.5$^{aa,b}$ | 776.6±180.03$^{aa,bb,c}$ |
| **MCP-1** (pg/ml) | 32.41±8.86 | 29.52±19.33 | 39.29±26.74$^{b}$ | 46.18±30.98$^{aa,b}$ |
| **Tissue MMP-1,** within 10 successive microscopic fields (× 400)/ section (mean percentage) +ve cells ± S.D | 5.2 ± 0.82 | 13.46 ± 5.91$^{aa}$ | 38.43 ± 6.20$^{aa,bb}$ | 64.32± 6.41$^{aa,bb,cc}$ |
| **Tissue TIMP-1,** within 10 successive microscopic fields (× 400)/ section (mean percentage) +ve cells ± S.D | 9.8 ± 0.8 | 22.0 ± 4.73$^{aa}$ | 36.31 ±2.69$^{aa,b}$ | 52.3±4.5$^{aa,bb,c}$ |

$^{a}$: P<0.05 significant difference compared to Control Group.
$^{aa}$: P<0.01 significant difference compared to Control Group.
$^{b}$: P<0.05 significant difference compared to Child A Group.
$^{bb}$: P<0.01 significant difference compared to Child A Group.
$^{c}$: P<0.05 significant difference compared to Child B Group.
$^{cc}$: P<0.01 significant difference compared to Child B Group.

**Table (2): Tissue expression of MMP-1 immunostain in liver tissue of different studied cases.**

| Clinical Scoring | Positive Cases | | Range % | | | Intensity% | | |
|---|---|---|---|---|---|---|---|---|
| | No | % | <10% | 10%-50% | >50% | + | ++ | +++ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Controls (15)** | 8 | 53.5 | 7 | 1 | - | - | - | - |
| **Child A (15)** | 10 | 66.6 | 4 | 6 | - | 3 | 7 | - |
| **Child B (15)** | 13 | 86.6 [ab] | 5 | 6 | 2 | 4 | 8 | 1 |
| **Child C (15)** | 15 | 100[abc] | 1 | 7 | 7 | - | 7 | 8 |

[a]*p* Statistically significant from control group (*p*<0. 05).
[b]*p* Statistically significant from Child A group (*p* <0. 05).
[c]*p* Statistically significant from Child B (*p* <0.05)

**Table (3): Tissue expression of TIMP -1 immunostain in liver tissue of different studied cases.**

| **Clinical Scoring** | **Positive Cases** | | **Range %** | | | **Intensity%** | | |
|---|---|---|---|---|---|---|---|---|
| | **No** | **%** | **<10%** | **10%-50%** | **>50%** | **+** | **++** | **+++** |
| **Controls (15)** | 5 | 33.3 | 5 | - | - | 2 | - | - |
| **Child A (15)** | 15 | 53.3[a] | - | 8 | 7 | 6 | 9 | - |
| **Child B (15)** | 14 | 93.3[ab] | 3 | 10 | 1 | 3 | 9 | 2 |
| **Child C (15)** | 8 | 100 [ab] | 1 | 5 | 2 | 2 | 5 | 1 |

[a]*p Statistically significant from control group (p<0. 05).*
[b]*p* Statistically significant from Child A group (*p* <0. 05).
[c]*p* Statistically significant from Child B (*p* <0.05)

**Table (4): Mean ± SD of The studied parameters according to METAVIR activity scoring system in CHC patients (necro-inflammatory).**

| **Parameters** | **A1 (n=20)** | **A2 (n=15)** | **A3 (n=10)** |
|---|---|---|---|
| **Tissue MMP-1** within 10 successive microscopic fields (× 400)/ section (mean percentage) +ve cells ± S.D | 16.24 ± 4.23 | 38.00 ± 5.32 [a] | 62.4 ± 5.42[ab] |
| **Tissue TIMP-1** within 10 successive microscopic fields (× 400)/ section (mean percentage) +ve cells ± S.D | 28.38 ± 3.33 | 31.500 ± 4.54 | 45.12 ± 5.8[ab] |

[a]*p* Statistically significant from A1 group (*p*<0. 05).
[b]*p* Statistically significant from A2 group (*p*<0.05).

**Table (5): Mean ± SD of The studied parameters according to METAVIR fibrosis scoring system in CHC patients (Fibrosis)**

| **Parameters** | **F1 (n=15)** | **F2 (n=13)** | **F3 (n=10)** | **F4 (n=7)** |
|---|---|---|---|---|
| **Tissue MMP-1** within 10 successive microscopic fields | 10.12 ± 53.02 | 19.35 ± 1.30 | 27.44 ±1.28[ab] | 43.9 ± 2.01[abc] |

| | | | | |
|---|---|---|---|---|
| (× 400)/ section (mean percentage) +ve cells ± S.D | | | | |
| **Tissue TIMP-1** within 10 successive microscopic fields (× 400)/ section (mean percentage) +ve cells ± S.D | 15.20 ± 3.2 | 16.9 ± 7.706 | 23.04 ± 2.08 [a] | 44.47 ± 2.16[abc] |

[a]*p* Statistically significant from F1 group ($p$<0. 05).
[b]*p* Statistically significant from F2 group ($p$<0.05).
[c]*p* Statistically significant from F3 group ($p$<0.05).

**Table (6): Correlations between monocyte-platelet aggregates and platelet micro-particles and some studied variables**

| | **MPA (%)** | **Platelets Microparticles** | |
|---|---|---|---|
| | | **CD62P PMPs** ($x10^3$/ml) | **CD63 PMPs** ($x10^3$/ml) |
| **AST** (IU/L) | 0.633* | 0.627* | 0.630** |
| **ALT** (IU/L) | 0.660* | 0.665** | 0.669** |
| **platelet Count** ($x10^3$/µl) | -0.913** | -0.976** | -0.967** |
| **RP (%)** | 0.916** | 0.985** | 0.981** |
| **Serum MMP-1** (pg/ml) | -0.974** | -0.926** | -0.918** |
| **Tissue MMP-1** (T. Intensity) | 0.788** | 0.823** | 0.817** |
| **Tissue MMP1** (%) | 0.807** | 0.870** | 0.866** |
| **Serum TIMP-1** (pg/ml) | 0.822** | 0.745** | 0.765** |
| **Tissue TIMP-1** (T. Intensity) | 0.363** | 0.488* | 0.473* |
| **Tissue TIMP-1** (%) | 0.318* | 0.381* | 0.363* |

* Significant :( $p$<0.05)
**Highly Significant :( $p$<0.01)

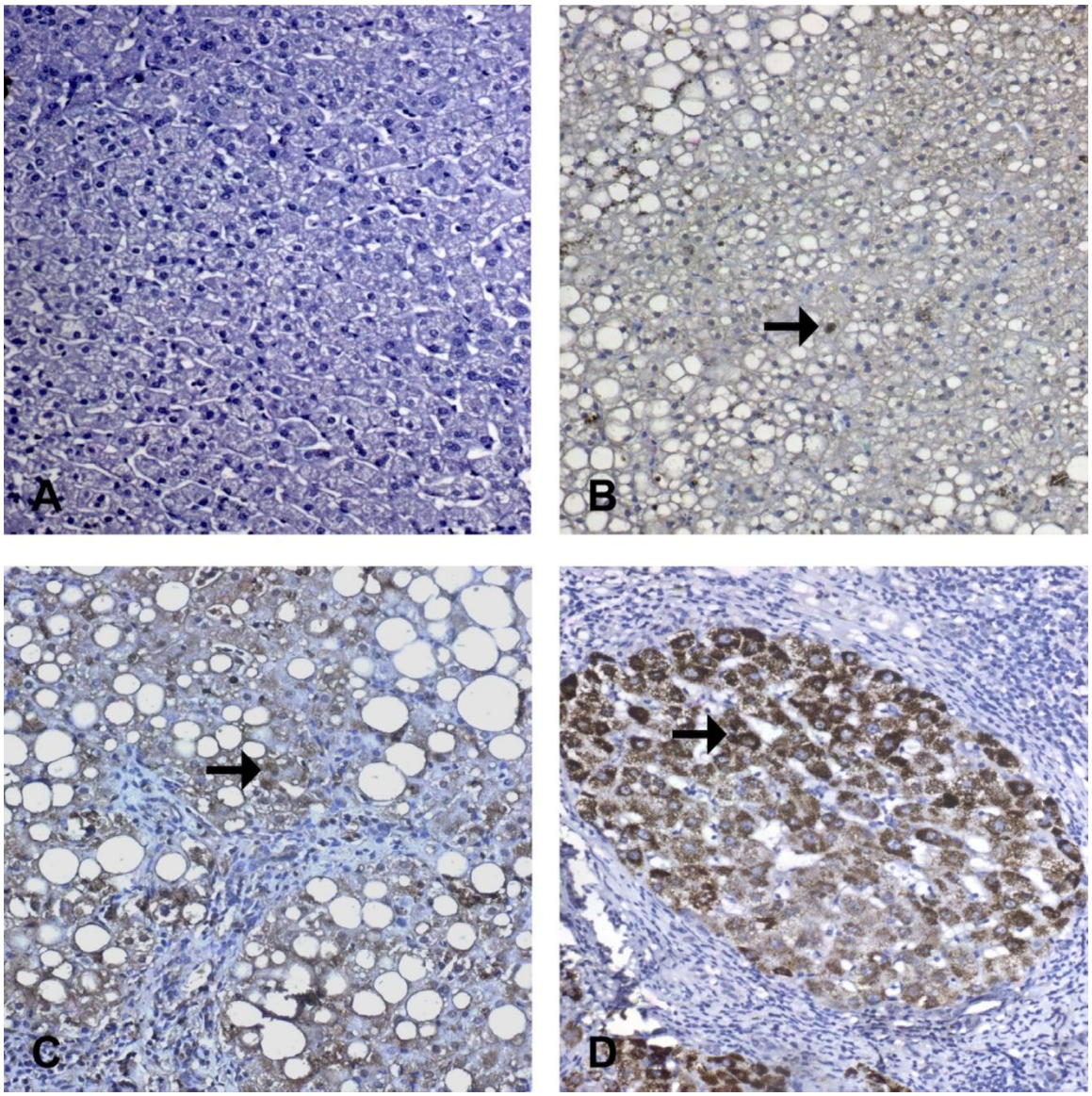


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**Figure (1): A) normal hepatocyte from a control case, showing mild expression of MMP1 (IHC, DAB, X200). B) A case of CHC without cirrhosis, A1F1, showing moderate expression of MMP1 as cytoplasmic stain in the hepatocytes (IHC, DAB, X200). C) A case of CHC with cirrhosis, A2F3, showing cirrhotic nodule with moderate expression of MMP1 as cytoplasmic stain in the hepatocytes, at periportal area (arrow)( IHC, DAB, X 200). D) A case of cirrhosis, A2F4, showing cirrhotic nodule with moderate expression of MMP1 as cytoplasmic (arrow)( IHC, DAB, X 200).**

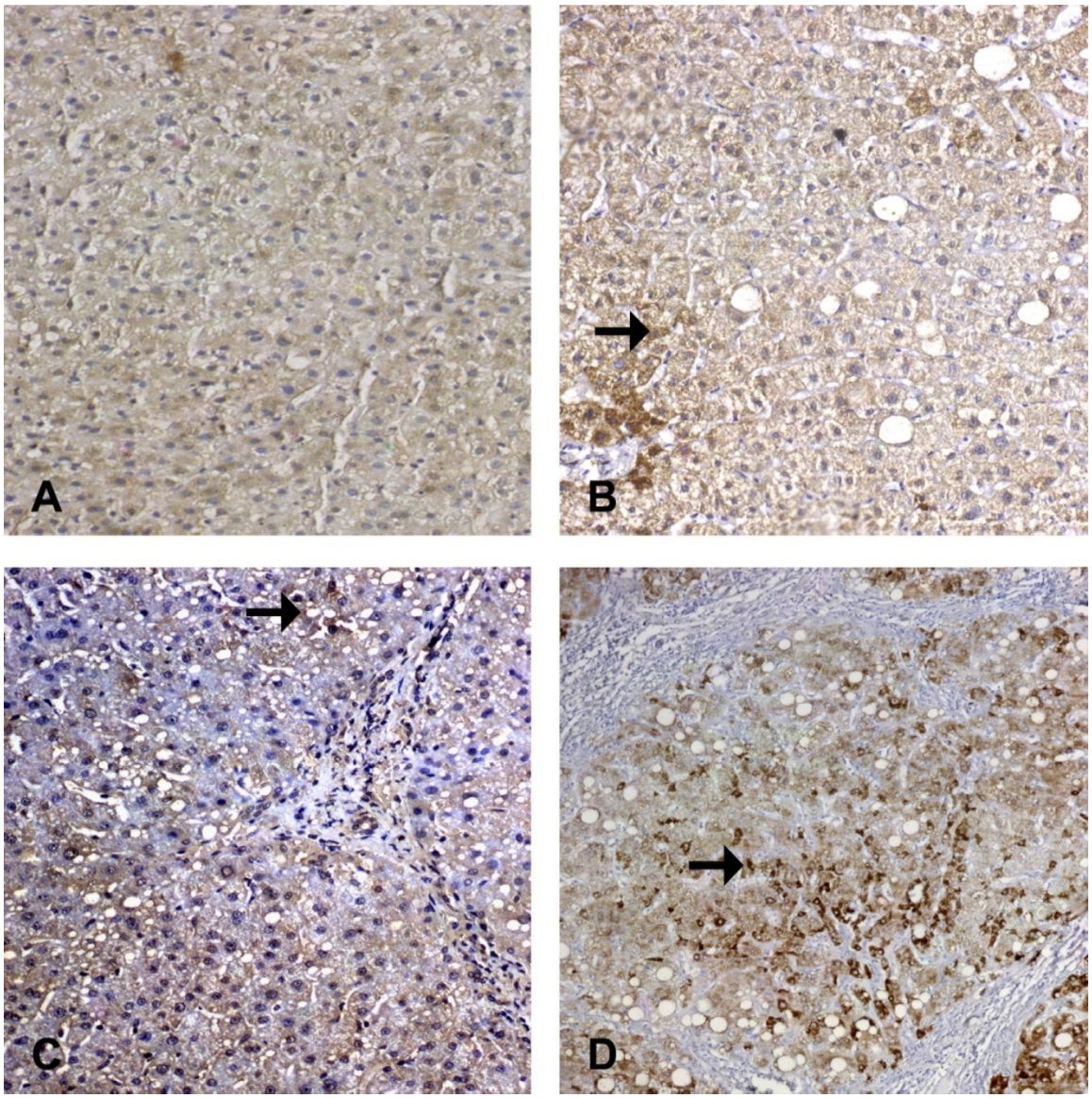


**Figure (2): A) normal hepatocyte from a control case, showing faint expression of TIMP1 as cytoplasmic stain in the hepatocyte (IHC, DAB, X200). B) A case of CHC without cirrhosis, A1F1,showing mild expression of TIMP1 cytoplasmic stain in the hepatocytes, bile ducts, and lymphocytes at periportal area (IHC, DAB, X200). C) A case of CHC with cirrhosis, A2F2, showing moderate expression of TIMP1 as cytoplasmic stain in the hepatocytes lymphocytes at periportal area (arrow)( IHC, DAB, X 200). D) A case of cirrhosis, A3F4, showing cirrhotic nodule with moderate expression of MMP1 as cytoplasmic (arrow)( IHC, DAB, X 200).**

**Discussion**

Liver disease is a heavily burdened clinical condition and evident platelet and monocyte activation are one of many conflicting events. Blood platelets, by connecting haemostatic and inflammatory processes, participate in the pathogenesis of CLD. Blood platelet while activated in inflammatory and immune processes and in the haemostatic disorders in the liver, release active compounds that have stimulating influence on fibrinogenesis and mitogenesis of Ito cells in the liver (Panasiuk et al., 2005).

Circulating platelet counts gradually decrease in parallel with progression of CLD. In thrombocytopenic conditions, quantization of reticulated platelets in the peripheral blood by flowcytometry has been shown to differentiate increased platelet turnover from insufficient platelet production (Thomas-Kaskel et al., 2007). Our study demonstrated increased percentage of reticulated platelets, which was more evident in advanced stages of the disease. On the other hand, platelet count showed a significant negative correlation with platelet activation markers suggesting that thrombocytopenia is likely to be caused by platelet activation, destruction and consumption. Kopke-Aguiar et al., (2009), reported that cirrhotic patients with portal hypertension have increased reticulated platelets associated with normal thrombopoietin serum levels. However, Peck Radosavijevic, (2000) and Panasiuk et al., concluded that decreased thrombopoietin production in the cirrhotic liver is an important etiologic factor for thrombocytopenia in liver disease. However, Kajihara et al., (2007), reported that thrombopoietin deficiency is unlikely to be the primary contributor of cirrhotic thrombocytopenia.

In the current study, high levels of PMP subpopulations exposing CD62P and CD63 were detected in different patients' groups compared to controls and became more evident with progress of the disease. Similar result was previously detected in alcoholic liver cirrhosis (LC) (Ogasawara et al., 2005) and post-hepatitic LC (Sayed et al., 2011). PMPs are generally considered a marker of platelet activation (Ogasawara et al. 2005), however PMP subpopulations exposing CD62P or CD63 reflect platelet activation more closely than overall numbers of PMPs (Nomura and Fukuhara, 2004). The causes of platelet activation in liver cirrhosis are complex, increased cytokines (IL-2, IL-6, TNF-α) and hyperdynamic portal circulation with retention in the splenic microcirculation may act to stimulate platelets. Also, immunological and inflammatory phenomena in liver tissues and their influence may contribute to platelet activation in these patients (van der Zee et al., 2006 and Panasiuk et al., 2007). Moreover, Zahran et al., (2007), reported that serum hepatitis C virus RNA might be directly responsible for the in vivo platelet activation in patients with CHC.

Circulating MPA was found to be a sensitive marker of in vivo platelet activation ((Ferroni et al., 2001). Results of the present study revealed significant increase in MPA percentage in all the diseased groups compared to the controls, in addition the increase in MPA percentage matched the severity of the disease. The elevated percentages of activated MPA in this study are confirmatory to the previous studies reported by Panasiuk et al., (2007) and Sayed et al., (2010). Circulating PMPs may form complexes with monocytes in vivo and thereby enhance monocyte arrest on endothelium (Theilmeier et al., 1999). Activated platelets are bound to leukocytes with the involvement of P-selectin and activated β2-integrin CD11b/CD18 (Evangelista et al., 1999). An array of platelet-derived adhesion and chemokine receptors, such as P-selectin, platelet glycoprotein IIb/IIIa (GPIIb/IIIa), GPIb, and CXCR4 are present on the surface of activated platelets and PMPs which mediates adhesion to leukocytes (Steiner et al., 2003). Binding via P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1) on monocytes, induce up-regulation and activation of β1 and β2 integrins and increased adhesion of monocytes to activated endothelium. Alternatively, PMPs may transfer platelet-derived adhesion receptors (eg, GPIb) to monocytes and increase their endothelial homing (Janowaska-Wieczorek et al., (2001). Another mechanism by which PMPs enhance adhesiveness of inflammatory target cells is based on the transfer of arachidonic acid, resulting in an up regulation of integrins and adhesion molecules on monocytes or endothelial cells (Barry et al., 1998). Hence, monocytes within MPA are in a higher state of activation and have more adhesive capacity (Forlow et al., 2000 and de Costa Martins et al., 2006). PMPs may also activate endothelial cells via interleukin-1β (Lindemann et al., 2001), induce cytokine production by monocytes and endothelium (Nomura et al., 2001).

Panasiuk et al., (2005), concluded that monocyte and platelet activation in LC may contribute to progressive liver injury. In inflammatory processes, activated platelets can module functions of cells that participate in inflammation (von Hundelshausen et al., 2001). Accumulating experimental evidence recently emphasized that the infiltration of inflammatory monocyte is a key factor for the progression of hepatic inflammation and fibrosis in injured murine liver (Tacke, 2012). During fibrosis progression, monocyte-derived macrophages can release several cytokines including interleukin-1 (IL-1) and tumor necrosis factor (TNF), which in turn can activate other macrophages, this produce a continuous or chronic condition and a cascade of secondary fibrogenic and inflammatory substances (e.g., PDGF, IL-8 and prostaglandins) perpetuating chronic inflammation as well as directly activate HSC, resulting in their proliferation and transdifferentiation into collagen-producing myofibroblasts (Karlmark et al., 2009). Furthermore, IL-1decreases hepatic cytochrome P-450 and related enzyme activities and can directly increase transcription of type I, III and IV collagen (Peterson and Isbrucker, 1992).

MCP-1 has been implicated in the process of hepatic inflammation, recruiting monocytes and lymphocytes during liver injury. Also, it activates directly hepatic stellate cells, which play a major role in hepatic fibrosis (Tsuruta et al., 2004). Elevated MCP-1 levels that originated from Mesenchymal stem cells (MSC) attract transendothelial migration of mononuclear leukocytes from blood to the tissue (Cieslik et al., 2014) and might

participate in the fibrotic process by inducing the secretion of extracellular matrix components (Eddy, 2000). Result of the present study revealed a significant increase in MCP-1 specially in child B and C groups compared to both child A and control groups. However, statistical analysis didn't show a significant correlation between MCP-1 and platelet activation markers. Moreover, no significant correlation could be detected between MCP-1 and both serum and tissue markers of hepatic fibrosis which might suggest that MCP-1 doesn't play a pivotal role in the process of liver inflammation and fibrogenesis.

In various fibrotic diseases, MMPs and their natural inhibitors, TIMPs, play an important role in inducing and preventing the degradation of the extracellular matrix (ECM), respectively (Kessenbrock et al., 2010). Our results showed decreased serum level of MMP-1 and increased serum level of TIMP-1 in patients' groups compared to controls. Our results were in accordance with Murawaki et al., (1997) and (1999), and Badra et al., (2010), who reported inverse relation between MMP-1 and histological grading of chronic hepatitis. TIMP-1 has been suggested to be a serum marker for liver fibrosis, and the expression is induced during liver injury (Nobili et al., 2009). In addition, TIMP-1 also plays an important role in promoting liver fibrosis (Wang et al., 2011). The profibrogenic effects of TIMP-1 are thought to be mediated via preventing collagen degradation through inhibition of MMPs and protecting against activated hepatic stellate cell (HSC) death (Friedman, 2008 and Gieling et al., 2008). The altered balance between circulating MMP-1 and TIMP-1 may play an important role in aggravating liver injury progression in CHC patients.

There was a significant correlation between the percentage of PMP and the levels of liver fibrosis markers, such as MMP-1, and TIMP-1 in CHC, suggesting the relationship between platelet activation and liver fibrosis. Platelet activation was markedly enhanced in CHC patients with high histological scores of liver fibrosis. Moreover, our data can provide evidence that with LC development, thrombocytopenia may be the result of platelet activation and consumption in platelet-monocyte aggregates.

Immunohistochemical study showed augmented hepatic expression of MMP-1 in CHC patients' groups compared to controls which became more pronounced with the progression of necro-inflammatory activity and the advancement of fibrosis. On the other hand, hepatic expression of TIMP-1 was significantly decreased in patients' specimens compared to controls and impaired expression is more evident with the progression of necro-inflammatory activity and the advancement of fibrosis. These results suggest that hepatic fibroproliferation is associated with alterations of hepatic TIMP and MMP expression

The results of our investigations showed that platelet activation markers and development of MPA were strongly correlated with both serum and tissue markers of liver fibrosis (S.MMP-1, S.TIMP-1, T.MMP-1 and T.TIMP-1). The continuous flow of leukocytes and platelets through inflamed and fibrotic liver tissues affects the activation of these cells. Moreover, the microcirculation in sigmoid sinuses slows cell flow and exposes the cells to a long exposure of active proinflammatory factors (Fusegawa et al., 2002). Zhang and Yang, (2012), hypothesized that microparticles are the basic storage units for different proteins in platelet granules and demonstrated that fibrinogen, platelet factor 4, β thromboglobulin and platelet derived growth factor are all stored in and released from PMPs. Biological significance of these proteins has stimulating influence on fibrinogenesis and mitogenesis of Ito cells in the liver (Panasiuk et al., 2005). On stimulation of platelets, substantial amounts of regulated on activation normal T cell expressed and secreted (RANTES) are redistributed to PMPs. Circulating PMPs may serve as a finely tuned transcellular delivery system for RANTES, triggering monocyte arrest to inflamed endothelium, introducing a novel mechanism for platelet-dependent monocyte recruitment in inflammation (Mause et al., 2005). Furthermore, incorporated in MPA seems to be relevant for the monocyte extravasation into the liver upon acute or chronic liver injury (de Costa Martins et al., 2006).

In conclusion, the proceeding data might extend our understanding of the role of PMPs in inflammatory and fibrotic process in CHC. Formation of a complex with monocytes may represent a cross talk mechanism by which PMPs participate in these processes. A selective targeting of adhesive events involved in this mechanism (eg, by blocking P-selectin) might be useful in launching new therapeutic modalities to interfere with inflammatory and fibrotic disorders accompanied by platelet activation and enhanced MPA generation.

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