CLINICAL SIGNIFICANCE OF SERUM MATRIX METALLOPROTEINASE-2 IN PATIENTS WITH CHRONIC HEPATITIS C AND HEPATOCellular CARCINOMA

Thesis
Submitted for Partial Fulfillment of Master Degree of Internal Medicine

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BENHA FACULTY OF MEDICINE
ZAGAZIG UNIVERSITY
2005
Dedication

Dedicated to..
My Father & Mother
My sincere Wife & Son Ahmed
And all my family
And my friends
First and foremost thanks to Allah the most gracious and the most merciful to whom I relate every success in my life.

I’m greatly indebted and greatfull to Dr. Atef Ahmed Ibrahim, Professor of Internal Medicine in Benha Faculty of Medicine, Zagazig University for his continous encouragement and meticulous supervision and great support and for dedicating so much of his precious time to this work, my gratitude to him in beyond the ability of my word to express.

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### Abbreviations

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<tr>
<td>αFP</td>
<td>Alpha-fetoprotein</td>
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<tr>
<td>α2-AP</td>
<td>α2-antiplasmin</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
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<tr>
<td>AFB1</td>
<td>Aflatoxin B1</td>
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<tr>
<td>ALB</td>
<td>Albumin</td>
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
</tr>
<tr>
<td>BFGF</td>
<td>Basic fibroblast growth factor</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
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<tr>
<td>CEA</td>
<td>Carcino-embryonic acid</td>
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<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>CLIP</td>
<td>Cancer of the liver Italian program</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic – T-lymphocytes</td>
</tr>
<tr>
<td>DCP</td>
<td>Des-gamma-carboxy prothrombin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immuno-assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>EMC</td>
<td>Essential mixed cryoglobulinemia</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>HAV</td>
<td>Hepatitis – A – virus</td>
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<tr>
<td>HBV</td>
<td>Hepatitis – B – virus</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepato cellular carcinoma</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis-C virus</td>
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<tr>
<td>HCV-LPs</td>
<td>HCV like particles</td>
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<tr>
<td>IARC</td>
<td>International Agency of Research on Cancer</td>
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<tr>
<td>IFN-alpha</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interlukin-1β</td>
</tr>
<tr>
<td>IMPs</td>
<td>Inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>LIMP</td>
<td>Large inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen activated protein kinases</td>
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<tr>
<td>MMP-2</td>
<td>Matrix metalloproteinase –2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase –9</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MPGN</td>
<td>Membranoproliferative glomerulonephritis</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane dependent matrix metalloproteinase</td>
</tr>
<tr>
<td>NANBH</td>
<td>Non-A non B hepatitis</td>
</tr>
<tr>
<td>NCI</td>
<td>National cancer institute</td>
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<tr>
<td>NCR</td>
<td>Non coding regions</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NHL</td>
<td>Non Hodgkin’s lymphoma</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OLT</td>
<td>Orthotopic liver transplantation</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>Porphyria cutanea tarda</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PEG-IFN</td>
<td>Pegylated interferon</td>
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<tr>
<td>PEI</td>
<td>Percutaneous ethanol injection</td>
</tr>
<tr>
<td>PSI</td>
<td>Percutaneous hot saline injection</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>RIBA</td>
<td>Recombinant immunoblot assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>SMCs</td>
<td>Smooth muscle cells</td>
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<td>TACE</td>
<td>Transarterial chemoembolization</td>
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<td>TGF-α</td>
<td>Tumor growth factor-α</td>
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<td>TGF-B1</td>
<td>Tumor growth factor Beta 1</td>
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<td>TIMPs</td>
<td>Tissue inhibitor of metalloproteinases</td>
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<td>TPA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>UPA</td>
<td>Uroplasminogen activator</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial cell growth factor</td>
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<td>VPF</td>
<td>Vascular permeability factor</td>
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<td>2</td>
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<td>3</td>
<td>Distribution of AST in different study groups</td>
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<td>4</td>
<td>Distribution of ALT in different study groups</td>
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<td>5</td>
<td>Distribution of ALP in different study groups</td>
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<td>6</td>
<td>Distribution of PT in different study groups</td>
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<td>Distribution of ALB in different study groups</td>
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<td>8</td>
<td>Distribution of AFP in different study groups</td>
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HEPATITIS C VIRUS

1- Historical Note:

(Prince et al., 1974) reported that a large proportion of long incubation post-transfusion hepatitis cases were caused neither by HBV nor HAV and suggested the presence of an additional virus or viruses, (Feinstone et al., 1975) made studies on some cases with post transfusion hepatitis and showed antigen negative to HAV, HBV and proposed positive to another viral agent which termed non A-non B hepatitis virus and in (1985, Brodely et al.,) discovered that the NANBH virus is less than 80nm in diameter and sensitive to organic solvent as chloroform which indicates the presence of a lipid envelope and (Choo et al., 1989) isolated a complementary DNA clone from the plasma of an experimentally infected chimpanzee and termed hepatitis C virus (HCV) which proposed to be classified in a new genus of flaviviruses termed Hepacivirus.

2- Structure of HCV:

The genome of HCV consist of a single stranded RNA of about 10,000 nucleotides in length (Purcell., 1997), it contains a single transitional open reading frame (ORF) which contains several genes coding for different structural and non structural viral proteins, the structural proteins include nucleocapsid protein (21-core) and two envelope glycoproteins (E₁, and E₂) which are derived from the 5’ end of the genome and the non-structural proteins include helicase (NS2), protease (NS3), RNA polymerase ) (NS5B), membrane binding protein (NS5A) and other regulatory proteins which are derived from the 3’ end of the genome (Cheney et al., 2000).
Non coding regions (NCR) are present at both ends (5' and 3') of the ORF, the (5’NCR) is 341 nucleotide long and it functions as an internal ribosomal entry site essential for cap-independent translation of the viral RNA and that makes this region the best target for diagnostic PCR (Agnello and Abel, 1999).

The (3’ NCR) region shows variations in both length and in sequence of the nucleotide bases and this sequence heterogeneity is not distributed over the genome but differs between the regions (Agnello and Abel, 1999).

3- HCV Replication :

It appears that HCV-RNA replicates by a direct RNA to RNA mechanism and it's performed by RNA dependent RNA polymerase protein and it's replicated from the 3’ end and generates a negative strand template and from this negative strand a new positive HCV RNA strand will be produced and so HCV has a high replicative capacity with production and clearance of $10^{12}$ virions per day and estimated virion half life of 2.7 hours (Neumann et al., 1999) and this high replicative activity provides the basis for the genetic variability of HCV (Darius et al., 2001).

4- Heterogeneity of HCV :

(Simmonds et al., 1998) classified HCV into six major genotypes according to genetic analysis of the nucleotide sequences derived from part of the gene encoding a non-structural protein:

- Genotype 1 is present in USA, Europe.
- Genotype 2 is present in Japan, south East, Asia and Europe.
- Genotype 3 is present in Europe.
- Genotype 4 is present in Egypt, Zaire, Saudi-Arabia and Yemen.
- Genotype 5 is present in South Africa.
- Genotype 6 is present in Hong-Kong.

**Prevalence of HCV:**

HCV infection occurs among persons of all ages but higher prevalence rates are found among persons aged 30 to 49 years and among males black men who have significant higher seroprevalence of anti-HCV than white men, there are 3 geographical areas with different prevalence.

1- Area with mild prevalence (< 0.5%) North of Europe, Canada and Australia.
2- Area with intermediate prevalence (0.5 -1%) France, Uk, Germany and USA.
3- Area with high prevalence (> 1%) southern and estern Europe, Japan and developing countries as in Egypt *(Denis et al., 1998)*.

In USA about 3.9 million US citizens (1.8%) have been infected with HCV and of those 2.7 million persons are chronically infected with HCV, in Japan the frequency of HCV antibodies among blood donars was found to be 1.4%, in UK prevalence of HCV infection varies between 0.08% and 0.55% *(Williams, 1999)*.

**Prevalence of HCV in Egypt:**

The prevalence of HCV infection is high in Egypt *(Wassim et al., 1998)* and overall prevalence of HCV antibodies in the general population is around 15-20% and highly prevalent among Egyptian blood donors *(El Gohary et al., 1999)*, also he found that HCV gene type II/ Ib was the most prevalent genotype as a single infectious agent or in association with other genotypes while *(El Zayadi et al., 2001)* stated that HCV type
4 is the predominant genotype found in Egypt and the reasons of high prevalence rate of HCV in Egypt is the past practice of unsterile parenteral therapy (Tartaremetic) for shistosomiasis (Sherlock and Dooley, 2002).

1- Among the general population:

Seroprevalence of anti-HCV was reported to be 12.1% among rural primary school children, 18.1% residents of rural villages and in 22% of army recruits (Abdel-Wahab et al., 1998).

2- Among blood donors:

Seroprevalence of anti-HCV among Egyptian blood donors was found to be 19.2% (Saeed et al., 1997) and 10.9% by (Kamel et al., 1998) but Hoballah and Sabry, 2000) stated that the correct prevalence of anti-HCV Egyptian blood donors was 6.7% by using the 2nd generation Recombinant Assay test system.

3- Among liver disease patients:

Anti-HCV was detected in 8% of male military recruits with acute hepatitis (Zakaria et al., 1998), (Abdel Wahab et al., 1998) described seropervalence of anti-HCV positivity to be 16.4% among children with hepatomegaly and 47.7% of adults with chronic liver disease or hepatocellular carcinoma.

4- Among special risk groups:

A) Schistosomiasis infections:

Analysis of risk factors for acquiring HCV infections showed a strong association between the parenteral therapy of shistosomiasis and anti-HCV seropositivity (Kamel et al., 1998) and (El Gohary et al., 1999). However (El Sahly et al., 1999) described frequency rate of anti-HCV of 14.3% in patients with active simple shistosomiasis and 66.6% among patients with hepatosplenomegaly.
B) Hemodialysis patients:

Seroprevalence of anti-HCV among hemodialysis patients was reported to be 45.2% by (Abdel-Wahab et al., 1998) and 70.45% by (El Gohary et al., 1999) and 74% by (Hoballah and Sabry, 2000).

C) Transfused children:

Prevalence of anti-HCV among transfused children was reported to be 55% among those with hematological disorders (Khalifa et al., 1997) and 54.9% among hospitalized multi-transfusion children (Abdel-Wahab et al., 1998) and 75.6% in thalassemic children (El Gohary et al., 1999).

Routes of transmission:

HCV is a blood borne virus and its transmission is through the transfusion of blood or blood products or through organ transplantation and through sharing of contaminated needles among drug abusers, also perinatal, sexual and occupational transmission could occur (Alter, 1997).

1- Parenteral transmission:

(1) Blood and blood products recipients:

In countries where all blood donors are screened for hepatitis B surface antigen, HCV may account for more than 90% of all cases of post transfusion hepatitis (Dana et al., 1997), the prevalence of HCV among haemophiliacs correlates with the amount and type of product transfused (Mauser et al., 1998), seroprevalence of anti-HCV among Egyptian blood donors was found to be 19.2% by using the 2nd generation RIBA and ELISA (Saeed et al., 1997).

(2) Haemodialysis patients:

Chronic HCV infection is a common problem in patients on maintenance hemo-dialysis due to multiple blood transfusion, persistence of viable virus in the dialysis equipment despite sterilization (Allander et
al., 1997), seroprevalence of anti-HCV among haemodialysis patients was reported to be 74% by (Hoballah and Sabry, 2000).

(3) Intravenous drug abusers.
The prevalence of HCV in intravenous drug abusers is high due to shared contaminated needles

(4) Health care workers :
Increased risk of HCV infection among doctors and nurses due to needle stick injury (Dusheiko and Kiyosawa, 1999).

Non parenteral transmission :
(1) Perinatal transmission :
The risk of transmission depends on the trimester at exposure, no perinatal transmission has been reported after maternal infection in 2\textsuperscript{nd} trimester while few reported cases after maternal infection in 3\textsuperscript{rd} trimester and recently the risk of transmission is correlated with HCV RNA levels in mother, no transmission occur when the level is below 106 genome per milliliter and mothers infected by both HCV and HIV are more likely to transmit HCV to their infants than those infected by HCV only (Pol et al., 1998).

(2) Sexual transmission and role of saliva :
The prevalence of anti-HCV is higher in patients with multiple hetero-sexual contacts and in homosexual populations, the prevalence of anti-HCV in sexual partners is about 5% of HCV infected patients (Booth et al., 1997) also anti-HCV antibodies and HCV RNA have been detected in both urine and saliva and transmission by human bite has been documented (Constantin et al., 1999).
3) **Community acquired hepatitis-C infection (Nosocomial transmission):**

   It's likely if disinfection procedures are inadequate and contaminated equipment is shared between patients and it may account for a substantial proportion of HCV infection (*Quer and Esteban, 1998*).

**Pathogenesis of HCV:**

   HCV is a single stranded RNA virus which doesn't integrate into the host's genetic material but it replicates to maintain its presence in human cells, HCV replication is detected in hepatocytes and in peripheral lymphocytes (*Chang et al., 1998*).

   The mechanism by which HCV causes liver damage isn't known but mainly it is immunologically mediated mechanism evidenced by:

   - In primary HCV, liver cell damage correlates with development of host immune response.
   - Immunosuppression of patients with HCV results in transient improvement in liver function tests (*Cerny et al., 1999*).

**Pathology of HCV:**

**Acute Hepatitis C:**

   It's dominated by a pattern of cell injury as shown histologically by ballooning degeneration, spotty necrosis and hepatocellular apoptosis and these changes are paired with changes of regeneration including mitotic activity, widened liver cell plates and disorganization of the reticulin pattern and in severe cases small areas of confluent necrosis may be seen and the necrosis may span the acinus giving rise to bridging necrosis and the hepatocellular injury is paired with an inflammatory
response characterized by lobular infiltrate of lymphocytes and activated kupffer cells (Liang et al., 1999).

**Chronic Hepatitis C :**

The histological picture of chronic hepatitis C is necro-inflammatory hepatocellular injury coupled with progressive fibrosis, first there is piecemeal necrosis or interface hepatitis (Khakoo et al., 1999) then as the disease progresses periportal collagen deposition replaces the destroyed hepatocytes giving a stellate appearance to the portal areas on connective tissue stains which is called periportal fibrotic expansion (Kleiner, 2000).

Lymphoid aggregates are seen frequently in hepatitis C and they have a structure and composition similar to primary follicles of lymph nodes (Danque et al., 1997).

Steatosis or fatty change is another parenchymal change associated with hepatitis C and it tends to be mild and macrovesicular and it may be zonally distributed or random (Miham et al., 1998).

**The immune response to HCV :**

**Immune -Mediated Mechanisms :**

(1) **Humoral Immune Response :**

Which consist of activated B cells in lymphoid aggregates, peripheral expansion of B cells and antibodies to structural and nonstructural peptides of virus which have two effects which are viral neutralization and antibody dependent cellular cytotoxicity (ADCC) (Ray et al., 1997), antibodies against envelope proteins have neutralizing ability and antibodies against conserved epitopes of HCV envelope proteins (E1 and E2 HVR1 domain) give resistance against infection with
homologous HCV but not heterogenous strains and assessment of neutralizing antibody response has been done by the ability of a serum sample to neutralize the binding of HCV E2 to human cells (Cheney et al., 2000).

2- Cellular Immune Response:

The cellular immune response is triggered by viral peptides expressed on cell membranes in conjunction with Major Histocompatibility Complex, CD4+ T cell response has been related to clearance of acute HCV infection because it occurs relatively early, augments antibody production by B-cells and stimulates CD8+ T cell response (Lechmann et al., 1998). The CD8+ Cytotoxic T lymphocytes (CTL) have been isolated from both liver and peripheral blood in a significant proportion of chronic hepatitis C patients, the CTL reduce viral replications and mediate liver damage and fibrosis by the local production of lymphokines such as INF gamma and tumor necrosis factor alpha (TNF- alpha) (Koziel et al., 1997), also they eliminate virally infected cells by recognizing antigenic proteins bound to Major Histocompatibility Complex class I molecules at the surface of infected cells (Cheney et al., 2000).

Clinical Manifestations of Hepatitis C Infection:

(1) Acute Hepatitis C:

It may occur in all age groups but most cases occur in young adults with incubation period averages 7 weeks with a range of 2 to 30 weeks (Hoofnalge, 1998), patients who acquire the infection are usually asymptomatic or have a nonspecific clinical illness characterized by fatigue, malaise, anorexia and loss of weight and about 30% of symptomatic patients present with jaundice which is marked by prodromal period usually 3 or 4 days up to 2 weeks during which the
patient suffers headache, malaise, anorexia and mild fever, an ache develops in the right upper abdomen and pruritis may appear, darkening of urine and lightening of stool follows the prodromal period during which aminotransferases level may be increased tenfold with determination of HCV RNA by polymerase chain reaction but HCV antibody may not be measurable until several weeks or months after acute HCV infection (Cheney et al., 2000).

(2) Chronic Hepatitis C:

About 70-80% of patients with acute hepatitis C develop chronic hepatitis (El-Guindy, 1999) the patient may be completely asymptomatic and diagnosis is made only during blood donation or routine screening, fatigue is the common symptom and may be associated with non specific symptoms such as anorexia, nausea, loss of weight, pruritis and right sided upper abdominal discomfort or pain, mild hepatomegaly with or without splenomegaly, the course is very slow and determined by positive anti-HCV and HCV RNA and fluctuating aminotransferases levels over years (Sherlock and Dooley, 2002).

Extrahepatic manifestations of chronic HCV infection:

(1) Essential Mixed Cryoglobulinemia:

Cryoglobulins are serum proteins that precipitate at cold temperatures and result in deposition of immune complexes in medium sized blood vessels and it's characterized by triad of purpura, weakness and arthralgia and diagnosis is confirmed by presence of the specific immuno-globulin within the serum (Genereau et al., 1998) and lead to hepatic involvement, systemic vasculitis, glomerulonephritis and peripheral neuropathy and recently it appears that HCV is the primary etiologic agent in EMC and efficacy of treatment with interferon alpha for this condition (Casato et al., 1999).
2- Non Hodgkin's lymphoma:

Studies have shown increased evidence for a role for HCV in the development of non Hodgkin's lymphoma (NHL) and other lymphoproliferative disorders (Cheney et al., 2000) and that may be due to direct oncogenic effect of HCV on lymphocytes or by inducing monoclonal or polyclonal proliferation resulting in various lymphoproliferative disorders (Cheney et al., 2000).

3- Porphyria Cutanea Tarda (PCT):

The disease results from decreased enzymatic activity of uroporphyrinogen decarboxylase and it's characterized by increased skin fragility, easy bruisability, photosensitivity and development of vesicles. Studies have shown that HCV may be a triggering event for the development of PCT and other dermatological diseases like lichen planus, erythema nodosum, erythema multiforme, polyarteritis nodosum, vitiligo and Behcet's syndrome which can respond to treatment with interferon alpha (Cheney et al., 2000).

(4) Membranoproliferative Glomerulonephritis:

It's an immunologically mediated disorder characterized by deposition of circulating immune complexes within the subendothelium and mesangium with resultant proteinuria and progressive renal dysfunction (Cheney et al., 2000) and it has been suggested that 50% to 70% of patients with MPGN have concomitant hepatitis C and treatment of the underlying HCV can result in improvement of the renal disease (Ohta et al., 2000).

(5) Autoimmune Diseases:

HCV appeared to be linked to autoimmune liver disease because high levels of HCV antibodies were detected in patients with autoimmune
liver disease, also in other autoimmune disorders like autoimmune thrombocytopenia and autoimmune thyroiditis (Cheney et al., 2000).

Complications of Chronic HCV infection:

1) Post Hepatitis C Cirrhosis:
Cirrhosis is the final irreversible stage of chronic hepatitis C infection, about 20% to 30% of chronic hepatitis C patients will develop cirrhosis, portal hypertension and liver cell failure and it's more common in predisposed patients which are older than 40, male, long duration of infection, co-infection with other hepatotropic viruses, alcohol consumption and infection with genotype 1 (Sherlock and Dooley, 2002).

2) Hepatocellular carcinoma:
The frequency of HCC development among patients with cirrhosis ranges from 3% to 6% per year and majority of cases were associated with HCV infection (El Zayadi et al., 2001). Several factors have been implicated in development of HCC in patients with chronic HCV which are coinfection with HBV, infection with HCV genotype 1b, alcohol consumption, advanced age at infection and male gender (Cheney et al., 2000).

Diagnosis of Hepatitis C virus:

1) Serological assays:

(1) Screening tests for anti-HCV using ELISA:
Diagnosis of HCV infection is achieved by detecting the presence of HCV anti-bodies in serum by Enzyme Linked Immuno-Sorbent Assay (ELISA) which depend on using synthetic viral antigens to capture circulating HCV anti-bodies on wells of microtiter plates and presence of anti-HCV is revealed by anti-IgG antibodies labeled with enzyme which transform the substrate into coloured compound (Pawlotsky et al., 1998).
First generation ELISA was developed by Kuo and colleagues in 1989 but its sensitivity was only 70% to 80% then second generation ELISA was developed by Food and Drug Administration in 1992 with increased sensitivity of the test then third generation ELISA has been developed with increased specificity up to 99.3% to 100% but the major defect in this test is the false negative results especially with immunocompromised hosts who are unable to mount an adequate antibody response and in individuals early in the course of the disease before antibody develops (Cheney et al., 2000).

(2) Confirmatory Tests:

They include the recombinant immunoblot assay (RIBA) with three generations, the first generation contained two recombinant HCV antigens (C100-3,5) fused to human superoxide dismutase (Ebeling et al., 1990) then second generation utilized two additional recombinant antigens from the virus core (C22-3) and the NS3 region (C33c) (Vander Poel et al., 1992) then third generation used synthetic peptides for C100-3 and C22-3 to prevent the cross reactivity and RIBA is used only in individuals with a positive antibody test (Gretch, 1998) and dot blot assay and immunoblot assays (Chaudhary and Jacobsen, 1998).

3- HCV core antigen assays:

HCV core antigen is known to play an important role in viral replication cycle and it’s detected by microplate ELISA test (Shimotohno, 2000) and it can be detected 38 days before appearance of anti-HCV so the sensitivity of HCV core antigen assay is more than 90% (Dupressoir, 2000).

4- Serological determination of the HCV genotypes:

Serotyping techniques are based on the detection of antibodies directed to genotype specific HCV epitopes and they are done by ELISA
or immunoblotting tests and have sensitivity of 85% to 90% (Pawlotsky et al., 1998).

2- Molecular Biology Based Assay:

(1) Qualitative HCV RNA Detection assays:

Direct measurement of the HCV virus in the serum of infected individuals is the main test in the diagnosis of HCV and it's useful in case of seronegative patients with chronic hepatitis especially immunocompromised individuals and seropositive patients with a normal serum ALT (Lau et al., 1999), they are done by reverse transcription polymerase chain reaction (RTPCR) which involves separation of nucleic acid from the specimen, reverse transcribing the RNA to DNA and repeatedly amplifying the DNA by using specific primers until the amount of product reaches a level that be detected by autoradiography or calorimetric testing and by branched DNA amplification (Aoki et al., 1999). These tests are highly sensitive and specific but costly when compared to antibody testing and they are used to confirm the presence of the disease and to assess response to therapy and also used to diagnose HCV infection in new born of HCV mothers (Hodinka, 1999).

(2) Quantitative HCV RNA assays:

Viral load is the amount of circulating genomes per volume of plasma and measured by quantitative assays which are used for determination of the amount of virus in an individual who using therapy and observation of the decline rate of viral load in the first weeks of treatment (Zeuzem et al., 1999) and they are done by either target amplification methods which lack of standardization and have limited ability to measure samples with more than 1million copies/ml and by signal amplification which is highly accurate and standardized and with no limiting ability (Gretch, 1998).
(3) **Molecular Biology-based genotyping assays:**

Depends on examination of sequence polymorphism in variable regions by direct nucleic acid sequence analysis, restriction fragment length polymorphism analysis and recently by cleavage fragment length polymorphism and heteroduplex tracking assay techniques (*Marshall et al., 1999*).

**Prevention and Treatment of HCV infection:**

(1) **Primary (Pre-exposure) prevention:**

1- Effective screening of blood, tissue and organ donors and screening of individuals with a high prevalence of HCV infection [persons with chronic hemodialysis, children born to women infected with HCV and persons with chronic blood transfusion].

2- Prevention of nosocomial transmission by wearing gloves and using sterile instruments and needle exchange.

3- For households with an HCV positive member avoiding sharing razors, disposing of injection needles and safe sexual practice.

4- Educating healthcare and public safety workers about blood and body fluid precautions.

5- Trials are done for developing of a vaccine but this is difficult due to the many genotypes of HCV and high variability of envelope region and lack of efficient invitro culture system and trials are done by using E1, E2 and NS1 glycoproteins and by using DNA vaccines targeting the nucleocapsid protein (*MMWR Morb Mortal Wkly Rep., 2000*).

(2) **Post-exposure prevention:**

Unlike HBV and HAV there's no immune globulin preparation has been shown to be effective for post exposure prophylaxis but demonstration of serum neutralizing activity in early chronic infection

**Treatment of HCV:**

The goals of treatment are to eliminate the virus, prevent the progression to cirrhosis and prevent the development of hepatocellular carcinoma (Cheney et al., 2000) and the sustained response to treatment is defined as a normal ALT and a negative HCV RNA a year after stopping the treatment with histologically unproved disease activity, and it was found that favourable host factors include female sex, age less than 45 years, duration of infection less than 5 years, no coinfection with HBV, no alcoholism, ALT modestly increased and liver biopsy showing low activity score and favourable viral factors include low serum HCV RNA, genotype II or III and low viral diversity (Sherlock and Dooley, 2002).

**Treatment of acute attack:**

It consists of bed rest with bath room privileges until the patient is free of jaundice and convalescence is not allowed until the patient is symptomatically free and diet with low fat and high carbohydrate and avoid high protein intake with supplementation of vitamins, amino acids and lipotropic agents (Sherlock and Dooley, 2002).

**Treatment of chronic HCV:**

Treatment is recommended for patients with persistently (> 6 months) elevated amino-transferase levels, anti – HCV and HCV RNA positivity in serum and finding of fibrosis or necrosis in liver biopsy (European association for study of liver, 2000) and there are many regimens for the treatment including.
1- Regimen of interferon therapy:

Interferons are a class of naturally occurring compounds that have both antiviral and immunomodulatory effects and there are three types: Beta, gamma, and alpha which is the one used in treatment of HCV and the standard therapy consists of subcutaneous applications of 3 million units of IFN alpha three times per week for six months and the response to this regimen detected by clearance of HCV RNA and normalization of ALT levels and it was 30% and half of them relapse after stopping the treatment.

So trials were done to enhance this response by increasing the dose or duration by giving 3-5 million units daily or three times per week for 12 months up to 60 weeks and the response to treatment was increased to 50% and decreased cases of relapses (Cheney et al., 2000), but it’s associated with serious but not life threatening side effects which are symptomatic thyroid disease especially for those with positive thyroid microsomal antibody before IFN therapy, immune mediated dermatological diseases, cardiovascular diseases, peripheral neuropathy and hemolytic anemia (Cheney et al., 2000)

Regimen of interferon – ribavirin combination (dual therapy):

Ribavirin is a guanosine analogue with a broad spectrum of activity aganist RNA and DNA viruses including the flavivirus family and it temporarily lowers ALT but has little effect on HCV RNA values and it has the advantage of oral administration and minor side effects which are mild abdominal discomfort, insomnia, anorexia, vertigo, pruritis and hemolysis which are well tolerated and ribavirin like IFN has antiviral effect by inhibition of viral messenger RNA and inhibition of viral dependant polymerase and has also immunomodulatory effect (DiBisceglie, 1999) and it can be used alone or in combination with IFN-
alpha to enhance the anti-viral effect especially in those who failed to achieve a sustained response with IFN alone especially patients with genotype Ib and it’s given in a dose of 1000 –1200mg daily in two divided doses for six months for those with genotype II and III and for 12 months for those with genotype Ib and the response to this regimen was 40% and reduced number of relapsers (Cheney et al., 2000). Many studies were done to compare between the treatment with IFN alone, ribavirin alone and the combination of IFN and ribavirin and results showed that ribavirin alone has only transient effects while the combination therapy improved the rate of sustained complete response compared to IFN alone (Davis et al., 1999 and Kjaergard et al., 2001).

Regimen of triple therapy:

Amantadine is a synthetic and cyclic, primary amine which inhibits the in vitro and in vivo replication of type A and C influenza viruses, parainfluenza and rubella viruses and found that amantadine given in a dose of 100mg twice daily for 6 months is a safe and cheap drug that has therapeutic potential in HCV infection and so amantadine has been used in combination with IFN alpha and ribavirin as the triple therapy and side effects of amantadine include, hallucinations, insomnia, anorexia, confusion and irritability and so the development of rimantadine which is less toxic than amantadine (Munson et al., 1999). Many studies were done to evaluate the effect of combination of IFN- alpha and amantadine and ribavirin in patients not responding to dual therapy especially those with genotype I and results reported that triple therapy induced a positive virological response in 47% of these patients and high rate of sustained biochemical and virological responses (Brillanti et al., 2000) and (Menzek et al., 2000) so triple therapy is now widely used in a dose
of IFN alpha 3 million units three times per week with ribavirin 1000-1200mg daily and amantadine 200mg daily for six to twelve months.

**Regimen of ursodeoxycholic acid and hepatic iron removal:**

Ursodeoxycholic acid may improve liver function in patients with chronic hepatitis and it may have a specific effect on biliary component with reduction in transaminases and ductular metaplasia and can be added to IFN therapy in a dose of 10mg/kg/day for six to nine months with normalization of serum ALT but has no effect on the clearance of HCV RNA or on liver histology (Boucher et al., 1999). Phlebotomy to remove the hepatic iron combined with IFN may improve the response of serum ALT and HCV RNA and reduce the incidence of relapse (Caraceni et al., 1999).

**Regimen of pegylated interferon therapy:**

Pegylation is the process of attaching one or more chains of polyethylene glycol to another chemical entity and pegylated molecules have many different applications and used in foods, cosmetics and beverages, a large branched and mobile polyethylene glycol is attached to the interferon alpha-2a and provides a selectively protective barrier to interferon alpha 2a from rapid absorption, metabolism and elimination while maintaining the ability of the interferon alpha-2a to attack HCV (El Sayed et al., 2001).

In 2001, Glue described a study of comparing standard interferon-alpha therapy of 3 million units three times weekly versus pegylated IFN alpha-2a given at a dose of 1-5 mg/kg weekly and results reported that all of the pegylated IFN had a greater drop in HCV RNA with less side effects than IFN alpha therapy, then he tried to detect the benefit from combined therapy of PEG interferon and ribavirn in a dose of 1.5 mg/kg
PEG IFN weekly and 1000-1200mg ribavirin daily and compare it with PEG interferon monotherapy and results showed higher virological response with the combined therapy, so PEG IFN will probably replace the conventional IFN because they are more effective and have to be injected only once weekly due to their prolonged life (Darius et al., 2001).

**Vaccine Development:**

Recently generation of HCV like particles (HCV-LPs) in insect cells by using recombinant baculovirus containing the complementary DNA of HCV structural proteins with similar morphological and biophysical properties as the putative virions and capable of inducing humoral response targeted against various regions of HCV structural proteins can be used as potential vaccine, also by DNA recombinant technology HCV E1 vaccine were developed and will be initiated in patients with chronic hepatitis C infection (Sherlock and Dooley, 2002).
HEPATO – CELLULAR CARCINOMA

Epidemiology of HCC:

It is the seventh most common tumor in males and the ninth in females and it is the direct cause of about 1 million deaths annually (Chen et al., 2000), there is a variable incidence of HCC between various countries and even between different groups living in the same area, high incidence areas include Taiwan, Sub-Saharan Africa and Eastern Asia where the reported incidence is 150/100,000 population per year (Chen et al., 2000), areas of intermediate incidence include Japan and the countries of Southern Europe, particularly Italy, Spain and the Middle East (DiBisceglie, 1998), in Egypt HCC incidence rate was estimated to be between 5 and 7 per 100,000 population per year, areas of low incidence include Northern Europe, the USA and South Africa where the rate may be as low as 5 per 100,000 population per year (Chen et al., 2000).

HCC in Egypt:

In Egypt there’s an apparent increase in the number of HCC patients attending the various oncology centers the incidence rates were estimated to be between 5 and 7 per 100,000 populations per year (El-Bolkeiny., 1998), hepatitis B and C are the major health problems in Egypt and both infections can lead to HCC in a 20-30 years period and prognosis may be worse in conjunction with shistosomiasis which is endemic in Egypt (Attia, 1999).

Factors associated with an increased risk of HCC in Egypt are age over 60 years old, farming, cigarette smoking, occupational exposure to chemicals such as pesticides and shistosomiasis and HBV with relative risk of 95% and found that shistosomiasis increase the severity of HBV
infection and elevate the risk of HCC over that associated with HBV infection alone (Badawi and Michael, 1999).

A study was done by National Liver Institute in the Delta region in 1999 on about 2359 hospitalized cases who performed liver biopsy, 388 cases (16.4%) proved to have HCC (Amer et al., 1999) and (El-Zayadi et al., 2001) found that 321/6850 cases who attended an outpatient liver center in Cairo had HCC and male to female ratio was 4:1 and the mean age was 50-60 years old and the National Cancer Institute in Cairo, 2001 ranked HCC as the 4\textsuperscript{th} among all types of cancer in Egypt.

**Aetiology and Risk factors:**

1- Chronic carriers of HBV and HCV.

2- Aflatoxin exposure, cigarette smoking, heavy alcohol consumption.

3- Low vegetable intake, inorganic arsenic ingestion, radio active thorium dioxide exposure.

4- The use of oral contraceptives and anabolic steroids.

5- Low serum retinol levels and high serum levels of testosterone (Chen et al., 2000) and (Rochen and Megerath, 2001).

**1) Relation to HBV:**

In 1996, the International Agency of Research on Cancer (IARC) concluded that chronic infection with hepatitis B virus is carcinogenic in humans (Engstorm et al., 1997). Molecular studies have shown that HBV DNA is integrated in the tumor tissues of HCC patients, and a direct interaction between HBVx protein and the product of the tumor suppressor gene P53 was found and this is important mechanism in hepatocarcinogenesis (Doria et al., 1998) and (Truant et al., 1999). Because of inactivation of the tumor suppressor protein P53 by HbxAg
results in genomic instability, a lower apoptotic rate and an extension in the life span of HBV infected cells so the HbxAg/ P53 complex formation represent one of important steps in the development of HCC (Bradley, 1999) and (Okuda et al, 1999). Also HbxAg enhances cell motility and induces a migratory phenotype in transformed cells by inducing the formation of pseudopodia protrusions (Enrique et al., 2001).

HCC risk has been associated with HbeAg carrier and high level of anti-Hbe antibodies in chronic hepatitis (Buendia et al., 1999) and about 80-90% of HCC cases contain multiple HbV DNA integration in their chromosomes especially chromosome 17 which the gene of P53 is found and this activates proto-oncogene expression leading to tumor development (Khakoo et al., 1999).

Relation to HCV:

Hepatitis C may be more important than hepatitis B in the aetiology of HCC as the incidence of HCC among anti-HCV positive patients is four times higher than among HBsAg carriers (Sherlock and Dooley, 2002) and increased risk of HCC with double HBV and HCV co-infection.

The (IARC) determined that chronic infection with HCV is carcinogenic in humans and although there’s no evidence that HCV is oncogenic as it doesn’t exert any effect on host cellular control it has been suggested that this agent promote carcinogenesis through the induction of chronic necro-inflammatory hepatic activity and liver cirrhosis and hepatocellular regenerative activity (Chen et al. 2000) and by using the polymerase chain reaction (PCR) technique, HCV RNA has been detected in serum and tumor tissue of anti-HCV patients with HCC (Colombo, 2000).
Relation to Cirrhosis:

The mechanism of the precancereous effect of cirrhosis is through regenerative hyperphasia which progress to liver cell dysplasia associated with cellular enlargement, nuclear pleomorphism and multinucleate cells (Abdel-Hamid, 1999). Macronodular or mixed macronodular and micronodular cirrhosis are more associated with HCC than micronodular cirrhosis and the risk of HCC increase with alcoholic cirrhosis or cirrhosis due to any cause associated with HCV or HBV (Buendia et al., 1999) HCC is a common cause of death in patients with hemochromatosis and alpha 1 antitrypsin deficiency and rare in patients with wilson’s disease and primary biliary cirrhosis (Sherlock and Dooley, 2002).

Relation to Aflatoxins and other toxins:

Aflatoxins are mycotoxins produced by the fungi Aspergillus flavus which may contaminate food stored in humid conditions or inadequate storage facilities, there are four major naturally occurring aflatoxins called aflatoxin B1, B2, C1 and C12 and AFB1 is the most toxic and animal studies suggest that AFB1 is a carcinogen but case studies failed to find an association between human HCC and aflatoxins exposure (Wild et al., 2000) but molecular studies have demonstrated that AFB1 has a specific effect and mutation on the third base of codon 249 in the P53 tumor suppressor gene by replacement of the guanine residues with thymidine residues which affect the DNA replication and cell growth and increase the susceptibility to the development of HCC (Kirk et al., 2001) and in liver cells aflatoxins is metabolized by microsomal oxidase and this metabolize of AFB1 is capable of binding to DNA and RNA and lead to carcinogenic effect.
Relation to alcohol:

Excessive alcohol ingestion may be considered a risk factor for the development of HCC and several mechanisms have been postulated to explain this role and these include activation of chemical carcinogens through the induction of the microsomal P450 dependent biotransformation system, suppression of the immune system, hepatocellular injury and reduction in the activity of the enzymes involved in the repair (Smith et al., 1999).

Relation to other causes:

1) In glycogen storage disease type I (Von Gerke’s disease) patients who survived beyond 10 years are prone to develop hepatocellular carcinoma and this may be due to a role of abnormal glycogen storage in initiating and promoting liver growth and malignancy through glucagon/insulin imbalance (Bianchi, 1999).

2) Prevalence of HCC in tyrosinemia patients over 2 years of age is high reaching 18% in an international survey and that may be due to accumulation of the intermediates of tyrosine metabolism which act as natural alkylating agents and cause genetic instability (Khakoo et al., 1999).

3) Oral contraceptives were reported to be associated with development of HCC, cholangiocarcinoma, also liver cancer has been reported within 2 months of androgen use and androgen receptors were detected in grade II HCC while estrogen receptors in grade I HCC and so some cases of HCC may be hormonal dependent and antihormonal therapy may be effective in treatment (Hassanein et al., 2000).
Histopathology of HCC:

The histopathology of HCC varies depending on combinations of the following features: structural pattern (trabecular, pseudoglandular, solid or scirrhous), degree of cell differentiation (well, moderately or poorly differentiated) and cytological features (clear cell type, pleomorphic cell type and spindle cell type) (Nakashima, 1999).

1- Structural pattern:

1- Trabecular (sinusoidal) type:

Tumor cells are arranged in cords of variable cell thickness separated by sinusoids lined by flat endothelial cells, fibrous connective tissue is absent but few collagen fibers may be detected in sinusoidal walls.

2- Pseudo glandular (Acinar) type:

Tumor cells form gland like structures, canaliculi with or without bile are common gland like spaces may derive from central degeneration and are filled with cellular debris, exudates and macrophages and the basic trabecular pattern often remains detectable.

3- Compact type:

Tumor cells grow in solid masses of cells, sinusoids are inconspicuous by compression.

4- Scirrhous type:

There is a significant fibrous stroma seperating cords of tumor cells which should be distinguished from cholangiocarcinoma and metastatic tumors.

2- Cytological features:

1- Clear cell type:

It’s characterized by tumor cells with a clear cytoplasm which is due to the presence of abundant glycogen and it has a favorable prognosis compared with that of other variants (Lai et al., 1998).
2- Pleomorphic cell type:
   It’s characterized by bizarre pleomorphic cells and it is common in poorly differentiated HCC (Nakashima, 1999).

3- Sarcomatoid (Spindle cell) type:
   This type of tumor is consisting of spindle shaped or pleomorphic anaplastic tumor cells which are found in part or most of the tumor with or without transitional feature between trabecular HCC and the sarcomatoid area and by immunohistochemically the sarcomatous tumor cells are positive for keratin, albumin, α fetoprotein and fibrinogen so sarcomatous appearance seen in HCC is due to morphological and phenotypic changes of the tumor cells (Nagamine et al., 1998).

Spread of HCC:
- Intra-hepatic metastases mainly by blood spread to form multiple metastases in one or both lobes.
- Extra-hepatic metastases by local extension to diaphragm and adjacent organs and lymphatic spread to portahepatic lymph nodes, mediastinal and cervical chains and may spread to peritoneum with hemorrhagic ascites and systemic spread to lung, bones and brain (Sherlock and Dooley, 2002).

Diagnosis of HCC:
1) Laboratory diagnosis of HCC:
   In patients with liver cirrhosis an unexplained increase in the serum alkaline phosphatase should suggest the possibility of HCC has developed and hypercholesterolemia in a patient with non cholestatic liver disease is a strong indication of HCC which is one of the common paraneoplastic phenomena and affects 30% of patients with HCC (Sherlock and Dooley, 2002). The CBC reveals leucocytic count
elevation with 80% polymorphnuclear cells, high platelet count, erythrocyte count is normal or mild anemia and erythrocytosis is seen in about 1% of cases which is due to increased erythropoietin production by the tumor and fibrinolytic activity is decreased and dysfibrinogenaemia may occur (Sherlock and Dooley, 2002).

Liver function tests are of little value in the diagnosis of HCC but one of the most important biochemical features of HCC is the difference between aspartate transaminase (AST) and alanine transaminase (ALT) which becomes greater with the progression of the disease (Kew et al., 2001).

2) Tumor markers in HCC:

1- Alpha – Fetoprotein (AFP):

AFP is a normal fetal serum protein which is synthetized by the perivascular parenchymal cells through the yolk sac and fetal intestine and it is present in high concentrations between 10.000 and 100.000 ng/ml at birth and gradually declines to < 20 ng/ml (adult level) before the first year of life (Kaneko et al., 2001), AFP level is midly increased in liver cirrhosis, acute and chronic hepatitis and in pregnant women during the second and third trimester especially if associated with fetal neural tube defect and elevated levels are found in about one third of patients with undifferentiated teratocarcinoma or embryonal cell carcinoma of ovary or testes (Entezami et al., 2000).

AFP is considered the most accurate tumor marker in the diagnosis of HCC and levels above 500 to 1000 ng/ml are diagnostic of HCC with 100% sensitivity and 70% specificity (Abd El Hamid, 1999), also rising titre is significant for HCC in at risk patients, and AFP usually correlates with tumor size but the tumor differentiation appears to be more important than tumor size in determining the level of AFP (Sato, 1999).
2) Carcino – Embryonic antigen (CEA):

CEA is a glycoprotein with a molecular weight of 200,000 and the upper limit of normal value ranging between 5 and 20 ng/ml and mild elevation occur in certain inflammatory diseases such as colitis and raised levels are seen with some benign tumors but grossly high level is suggesting of malignancy and mostly it is diagnostic of colonic cancer and also primary and secondary liver tumors (Okuda et al., 1999).

3) Des-Gamma Carboxy-prothrombin: DCP

DCP is released into blood in case of inhibition of vitamin K-dependent gamma carboxylation of the liver which is produced by HCC due to failure of the tumor to express the prothrombin carboxylase gene and values > 100 ng/ml are highly suggestive of HCC and although the specificity of DCP is similar or higher than that of AFP for HCC it is not sensitive as AFP as abnormal elevations have been reported in patients with chronic hepatitis and liver cirrhosis (DiBisceglie, 1998).

3) Imaging technique for diagnosis of HCC:

1- Ultrasonography:

Ultrasound is known to be highly sensitive in the detection of nodular lesions in the liver as well as highly cost – effective and easy to perform so considered as the first choice for screening high risk patient populations like cirrhotic patients and patients infected by hepatitis B or C virus (Takano et al., 1999).

Ultrasound angiography is one of the most sensitive tools for detecting small hypervascular HCC tumors and it is performed by injecting microbubbles of CO₂ through a catheter placed into the hepatic artery after conventional hepatic angiography and the typical angiographic finding of HCC is abundant tumor vessels with hypervascular stain and the angiographically undetected HCC can be
diagnosed by ultrasound angiography and the typical vascular patterns of HCC are homogenous or mosaic hypervascular pattern with sensitivity and specificity about 90% and 89% (Kudo, 2001).

2) Computed Tomography (CT):

Conventional CT has allowed successful detection of large HCC (more than 2 cm in diameter) and the differentiation of HCC from other space occupying lesions, conventional CT is being replaced by spiral CT where a continuous spiral exposure is made, can be completed during a single breath hold scanning of the entire liver and so it permits the detectability of small hypervascular tumors (2cm or smaller) (Catalano et al., 2000).

The dual phase helical CT is used to differentiate dysplastic hepatic nodules and HCC on the basis of separation of images of the hepatic arterial phase and the portal venous phase of hepatic enhancement as HCC is supplied by hepatic artery and dysplastic nodules by portal vein and this technique sensitivity is 93% with hepatic nodules less than 3cm in diameter (Catalano et al., 2000), sensitivity of CT can be improved by enhancement with intravenous contrast medium, so HCC of 1cm and more in diameter can be detected by CT enhancement (Sherlock and Dooley, 2002).

Lipiodol angiography followed by CT appears to be one of the most sensitive tools to detect small HCCs with a sensitivity of about 93% to 97% and it is performed by intra-hepatic arterial injection of lipiodol and CT scan of the liver is performed 10 to 14 days later where the lipiodol has cleared from the non-neoplastic liver parenchyma but is retained by HCC due to lack of kupffer cells and so HCC as small as 2-3 mm can be detected (Spitz et al., 1999).
3) Magnetic Resonance Imaging (MRI):

It depends on detection of energy refused from hydrogen protons after forcible alignment in a strong magnetic field and it is safe but expensive and of two types T1 and T2 weighted images, T1 weighted images of HCC have a hypo-intense peripheral ring and are isodense while T2 weighted images show good tumor – liver contrast and can detect vascular invasion and satellite nodules (Sherlock and Dooley, 2002). Other techniques are intra-venous iodine – containing contrast (Gadolinium) or magnesium contrast agents (Ros et al., 2000). Magnetic resonance angiography with reticuloendothelial and hepatobiliary agents which leads to specific blood distribution within the liver and so gives good informations about anatomic relation of the tumor and major vessels (Spitz et al., 1999).

4) Radio-Isotope Scanning:

Technique depends on 99m TC labelled colloids of human albumin are taken up by reticulo – endothelial cells so used to detect hepatic tumors but could not differentiate cystic form solid ones and lesions 4cm in diameter are demonstrated but sensitivity falls below this size (Sherlock and Dooley, 2002) 99m TC – dimercaptosuccinic acid (DMSA) has been used to image HCC as it is accumulated in HCC more than the adjacent liver tissue and it’s sensitivity was found to be 89% (Wang et al., 2000).

5) Histological diagnosis “Liver Biopsy”:

Liver biopsy is often required to confirm the diagnosis of HCC, biopsy of the non – tumorous portion of the liver is done to evaluate the severity of the underlying liver disease (DiBiseglie, 1998) the biopsy should be done under imaging control (ultrasound or CT) and the reported complications include vasovagal reactions, injury to important structures, infection and haemorrhage and the risk of tumor seedling
along a percutaneous needle tract or systemic and local spread of malignancy by ultrasound guided biopsy and this is very rare \citep{Spitz1999}, recently laparoscopy or laparotomy with wedge biopsy may be the best approach when the neoplastic status of a mass is unclear and histologic diagnosis is essential \citep{Laurence2000}.

**Staging of HCC:**

1) Old staging of HCC is that developed by Okuda which has 4 criteria:
   - tumor size > 50%, presence of ascites, Albumin < 3g/dL and Bilirubin > 3 mg/dL
   - Stage I: none of the above and median survival is 1-5 months.
   - Stage II: 1 or 2 of the above and median survival is 3 months.
   - Stage III: 3 or 4 of the above and median survival is 0.9 months.

2) More recent staging is the Cancer of The Liver Italian Program (CLIP) scoring system which has 4 criteria: Child Pugh classification, Tumor morphology, AFP levels, portal vein thrombosis.

**Score 0:** Child classification A, uninodular and extension < 50%, AFP < 400 ng/dL and absent portal vein thrombosis with median survival 42.5 months.

**Score I:** Child classification B, uninodular and extension < 50%, AFP < 400 ng/dL and absent portal vein thrombosis with median survival 32 months.

**Score II:** Child classification B, multinodular and extension < 50%, AFP < 400 ng/dL and absent portal vein thrombosis with median survival 16.5 months.

**Score III:** Child classification B, multinodular and extension < 50%, AFP> 400 ng/dL and absent portal vein thrombosis with median survival 4.5 months.
Score IV: Child classification B, multinodular and extension < 50%, AFP > 400 ng/dL and present portal vein thrombosis with median survival 2.5 months.

Stage V: Child classification C, multinodular and extension < 50%, AFP > 400 ng/dL and present portal vein thrombosis with median survival 1 month.

Stage VI: Child classification C, multinodular and extension > 50%, AFP > 400 ng/dL and present portal vein thrombosis with median survival less than 1 month.

3) The TMN staging:

a) Primary tumor: Tx primary tumor can not be assessed.

T0: No evidence of tumor.

T1: Solitary tumor ≤ 2cm without vascular invasion.

T2: Solitary tumor ≤ 2cm with vascular invasion or multiple tumors < 2cm limited to one lobe with or without vascular invasion or solitary tumor >2cm without vascular invasion

T3: Solitary tumor > 2cm with vascular invasion or multiple tumors < 2cm limited to one lobe with vascular invasion or multiple tumors any > 2cm limited to one lobe with or without vascular invasion.

T4: Multiple tumors in more than one lobe or tumor(s) involving a major branch of portal or hepatic vein(s) or invasion of adjacent organs other than gall bladder or perforation of visceral peritoneum.

b) Lymphnodes: Nx regional lymphnodes can not be assessed:

N0 No regional lymphnodes metastases

N1 regional lymphnodes metastases

c) Distant metastasis: Mx distant metastases can not be assessed

Mo No distant metastases

M1 distant metastases are present
Stage I $T_1$ No Mo
Stage II $T_2$ No Mo
Stage III A $T_3$ No MO
Stage III B $T_{1-3} N_1$ Mo
Stage IV A $T_4$ any N Mo
Stage IV B any T any N M

**Treatment of HCC:**

HCC is difficult to be treated because of it is deeply seated and background liver parenchyma is cirrhotic and cirrhotic liver can not tolerate major surgery, hepatocytes are radioresistant, cirrhotic liver can not tolerate high doses of chemotherapy and cirrhosis is precancerous so some of the cirrhotic nodules may turn malignant even after primary lesions are removed (Okuda et al., 1999).

1) **Surgical therapy:**

1- **Hepatic Resection:**

Surgery is the only treatment with a definitive chance of cure and it depends largely on the clinical condition of the patient to be done to patients with child classification A, TNM stages I or II or okuda stage I, unifocal mass $\leq 5$cm in diameter and encapsulated and no evidence of portal or hepatic vein invasion (El-Assal et al., 1999) and the normal liver can tolerate removal up to 45% of parenchyma and will return to normal size with several months (Okuda et al., 1999) and the operative mortality for liver resections in non cirrhotics is below 3% and 23% in cirrhotics (Brancatisano et al., 1999).

2- **Liver Transplantation:**

Orthotopic liver transplantation (OLT) is the attractive option for patients with primary HCC and cirrhosis because it offers the possibility of tumor excision and replacement of dysfunctional liver parenchyma and
avoid the morbidity of post operative liver failure, eliminate the incidence of tumor recurrence in the remaining liver disease and prevent the progression of portal hypertension (*Usatoff and Habib, 2000*) and the results of OLT for advanced HCC are poor and so OLT is best indicated for small HCC in liver with advanced cirrhosis as recurrence is rare and a long survival is expected and for small HCC and fibrolamellar HCC in a non cirrhotic liver there are two options OLT and hepatic resection (*Okuda et al., 1999*). Most centers restrict transplantation to patients with less than 3HCC nodules with tumors less than 3cm in diameter and with no vascular invasion and these centers reported 5 years survival rate of OLT ranging from 20% to 80% and mortality rate is due to recurrence of tumor in the transplanted liver (*Usatoff and Habib, 2000*).

3- **Cryosurgery and heat coagulation:**

Cryosurgery has been used as an alternative to resection for HCC and to treat residual nodules in the remaining liver after resection of the main tumor but has specific complications such, bile leakage, haemorrhage, liver abscess and renal failure and it is done by injection of liquid nitrogen through a vacuum insulated metal probe into the tumor guided by ultrasound and it can be done in combination with resection with 3 years survival rate about 20% (*Spitz et al., 1999*). Heat coagulation is done by the application of 60-watt microwave generated heat at 2450 MHz of frequency for 60 to 120 seconds through a probe inserted into the tumor and it is used for lesions up to 5cm in diameter with 5 years survival rate about 60% but it’s not used for multifocal or metastatic tumors (*Lin et al., 2000*).

2) **Adjuvant therapy:**

1- **Systemic chemotherapy:**

It is used for patients who are not eligible for surgery and who cannot receive other local treatment (*Okuda et al., 1999*).
specific chemotherapeutic drug for HCC but combination of agents like 5-fluorouracil with leucovorin and 5-FU with INF alpha and other agents include doxorubicin, cisplatin, mitoxantrone, isophosphamide, epirubicin and used alone or in combinations have been used with response rate ranging from 10% to 30% (Fuloria et al., 2000) and development of liposomal doxorubicin preparation which is retained by HCC cells has very promosing effect and recent studies showed that in patients with viral hepatitis, the rate of HCC development was lower with the treatment of INF alpha so combined therapy of intra-arterial cisplatin and gemeitabine infusion and systemic IFN – alpha had shown promosing results as palliative therapy (Chung et al., 2000).

2) Radiotherapy:

Generally, hepatocytes are radioresistant and the external beam radiation has a limited use in treatment of HCC as it requires about 50Gy to destroy the HCC cells and this is associated with radiation induced hepatitis and hepatic failure so the recommended dose should be 30-35 Gy over 3-4 weeks with survival period about 6 months, and with the development of three dimensional beam focusing methods it minimizesd beam scatter and delivered the required dose more specifically to the liver tumor with 3 years survival rate of 40% (Sptiz et al., 1999). Proton beam therapy is a newer method in which large dose of radiation can be focused on the lesion with minimal skin exposure and minimal exposure to rest of liver parenchyma with 50% reduction in tumor size and it is used in patients with child A cirrhosis and tumors smaller than 8cm in diameter or with child B cirrhosis and tumors smaller than 5 cm in diameter and it is not dealing with multifocal or metastatic lesions (Okuda et al., 1999).
3) Percutaneous Ablation therapy:

1- Transarterial ablation therapy (Targeted chemotherapy, arterial embolism and chemoembolism):

Selective intra – hepatic arterial infusion of single and multiple chemotherapeutic agents has adverse effect less than systemic chemotherapy on HCC as targeted chemotherapy allows the delivery of high concentrations of cytotoxic drugs directly to the tumor with minimal side effects (Spitz et al., 1999) it is appropriate only in patients with HCC confined to the liver and drugs used include doxorubicin, 5- fluorouracil, mitoxantrone, epirubicin and cisplatin used alone or in combinations and complications include biliary sclerosis and liver abscess (Chung et al., 2000). AS HCC depends on arterial blood supply except the capsule cancer cells die when arterial blood flow is interrupted so (Yamada et al., 1988) developed a way of transcatheter arterial embolization for treatment of HCC by injection of 1-2 mm gelfoam particles into the artery that was feeding the tumor and after embolization, embolized HCC undergo necrosis and this technique became widely used (Okuda et al., 1999).

Transarterial chemoembolization (TACE) consist of the intra arterial infusion of an anticancer agent in the feeding artery of the tumor followed by embolization (Bronowicki et al, 2001) and it is done by using lipiodol which remains in the neoplastic tissue for an extended time mixed with anti-cancer agent injected intra arterially and this mixing lowers the plasma concentration and increases the intratumoral concentration of the anti-cancer agent followed by injection of embolizing material like gelatin sponge, starch, polyvinyl alcohol and mainly Gelfoam particles and it is carried out in patients with unresectable HCC and any type of HCC whether large or multiple and for
recurrent lesions after surgery and it causes more than 50% reduction of tumor size and of serum AFP level (Okuda et al., 1999).

2- Local ablation therapy:

a) Percutaneous ethanol injection (PEI):

This technique is done by placement of needle in the tumor and through this needle 8-10 ml of 95% ethanol is injected and guided by ultrasound and is repeated once or twice a week until the original echopattern is completely replaced by different one (Hyperechoic or Heterogenous) and may be associated with local pain or low grade fever in about 50% of patients and this lasts for only 3 days and it was done in Chiba University in Japan by (Surgiuta et al., 1998) and PEI acts by diffusing within the neoplastic cells which cause immediate dehydration of cytoplasm and coagulation necrosis followed by fibrous reaction with minimal injury to the surrounding liver (Okuda et al., 1999).

PEI is highly effective for tumors of 3 cm in diameter with 80% cure rate and for tumors 5 cm in diameter with 50% cure rate but not used for multilocal or metastatic tumors (Spitz et al., 1999).

b) Percutaneous Hot saline injection (PSI):

PSI causes target destruction through heat induced coagulation necrosis rather than protein denaturation, studies done an HCC patients treated with PSI reported no major complications except for burning pain of moderate severity and transient fever and CT done showed decrease in lesion size after about 6 months and liver biopsy showed necrosis without residual viable tumor and follow up showed no local recurrences after 2-36months (Honda et al., 1999).

4) Other Modalities of therapy:

1- Hormonal therapy:

As estrogen receptors are present in 33% of HCC’s, studies were done to detect the effect of using anti – estrogen drug like tamoxifen in
the treatment of HCC with a dose of 40mg daily but results were discouragement as they concluded that tamoxifen has no efficacy in the treatment of patients with advanced HCC but other studies used octreotide which is somatostatin analogue in a small number of inoperable HCC and results were encouragement with decrease in the AFP levels and improved survival period in these cases ranging from 6 to 12 months (Reubi et al., 2000).

2- Immunotherapy:

Immunotherapy by using lymphokine activated killer cells in advanced HCC was done by (Okuno et al., 1990), by intra-arterial administration of $2.4 \times 10^9$ LAK cells through the hepatic artery and the serum AFP levels were decreased, also interferon alpha given systemically results in reduction of proliferation and produces a response rate of 31% in advanced HCC (Lai et al., 1999).

3- Gene therapy:

The Basic approach of gene therapy is to replace non – functional anti – oncogenes to protect normal tissue or to increase the level of expression of anti – oncogenes to target the malignant cells themselves and an example of that is the insertion of the tumor suppressor gene p53, introduction of this gene into patients with HCC has been shown to produce a reduction of tumor volume and a significant fall in serum AFP levels (Habib et al., 2000).
MATRIX METALLOPROTEINASES (MMPS)

Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell matrix composition and they are zinc dependent endopeptidases known for their ability to cleave one or several extra-cellular matrix (ECM) constituents as well as non matrix proteins, they comprise a large family of proteases that share common structural and functional elements and products of different genes (Woessner et al., 2000). MMPs have an important role in normal and pathological processes including embryogenesis, wound healing, inflammation, arthritis, cardio-vascular diseases, pulmonary diseases and cancer and inhibition of MMPs have been shown to prevent progression of these diseases (Mandal and Chakraborti, 2003).

Most cells synthesize and immediately secrete MMPs into the ECM, however, inflammatory cells store MMPs such as neutrophil collagenase and gelatinase B so the tissue distribution of these proteinases varies widely, some are constitutively synthesized such as 72kDa gelatinase by many cells while others are synthesized mainly upon stimulation such as collagenases (Chakraborti et al., 2002), MMPs are mobilized by cells in at least three different ways: 1) by initiating transcription of growth factor responsive MMP genes such as collagenase, 92kDa gelatinase and putative metalloproteinases in macrophages and endothelial cells, 2) by constitutive expression of MMPs that are largely unresponsive to growth factors and cytokines such as 72kDa gelatinase in most cells and 3) by the triggered release of prepackaged MMP from granule storage sites such as 92kDa gelatinase in polymorphnuclear leucocytes (Chakraborti et al., 2002)
The first MMP activity discovered was a Collagenase in the tail of tadpole undergoing metamorphosis and to date, 24 different vertebrate MMPs have been identified of which 23 are found in humans and they are classified into six groups which are Collagenases, Gelatinases, Stromelysins, membrane type MMPs and other MMPs on the basis of substrate specificity, sequence similarity and domain organization as seen in this table:-
Three- Dimensional (3D) Structures of MMPs:

X-ray crystallography and nuclear magnetic resonance (NMR) have determined the 3D structures of a number of MMPs such as MMP-2, MMP-3 and MMP-9 which consist of basic distinctive and well conserved domains which are: a prodomain, a catalytic domain, a hinge region and a hemopexin domain.

1- The prodomain: it consists of three α-helices and connecting loops, the first loop between helix 1 and 2 is a protease sensitive “bait region” with an extended peptide region after helix 3 and this region contains the conserved cysteine switch which forms a fourth ligand (water molecule) with the active site zinc of the catalytic domain keeping the zymogen inactive (proMMP) and removal of the propeptide by proteolysis results in zymogen activation.

2- The catalytic domain: it consists of a 5 stranded β pleated sheet, three α-helices and connective loops and it contains one catalytic zinc involved in the catalytic process of MMPS, one structural zinc and three calcium ions essential for the proteolytic activity of MMPS and there are three histidines coordinate the active site zinc and the glutamic acid adjacent to the first histidine is essential for catalysis (Bode et al., 2000). Three repeats of fibronectin type II domains found in MMP-2 and MMP-9 are inserted between the fifth β strand and the catalytic site helix (Briknarova et al., 2001).

3. The hemopexin domain: It consists of four bladed β propeller fold with a single stabilizing disulfide bond between blades I and IV and its sequence is similar to the plasma protein hemopexin and it plays a functional role in
substrate binding and specificity and in interactions with the tissue inhibitors of MMPs (TIMPS). The hemopexin domain of MMP-9 binds the C-terminal domain of TIMP-1 and hemopexin domain of proMMP-2 binds the C-terminal domain of TIMP-2, the hemopexin domain is important for substrate specificity.

**Substrate Specificity of MMPs:**

Substrate specificities of MMPs have been studied either by identifying the cleavage sites of protein substrate or by a series of synthetic peptide substrates. In general MMPs cleave a peptide bond before a residue with a hydrophobic side chain and the hydrophobic residues fit into the S1 specificity pocket whose size and shape differ among MMPs (*Nagase et al.*, 2003).

**Regulation of MMPs:**

The biosynthesis and extracellular activity of MMPs are regulated so as to exert control over degradation of ECM proteins which is important in regulating physiological processes such as morphogenesis, organogenesis, embryogenesis, tissue regeneration and wound healing, disordered regulation of MMPs could contribute to pathological processes such as neoplasia, metastasis, connective tissue destruction, cardiovascular diseases, arthritis, pulmonary diseases and progressive fibrosis.

1) **Regulation of gene expression:**

Expression of most MMPs is normally low in tissues and is induced when remodeling of ECM is required, MMP gene expression is primarily regulated at the transcriptional level and this is done by growth factor/cytokine-dependent and independent mechanisms among the members of the MMP gene family, two pairs of enzymes (MMP-1 and MMP-8, MMP-2 and MMP-9) have been identified with almost identical
substrate specificity but with different transcriptional regulation as one member of each pair (MMP-1 and MMP-9) responds to growth factors and cytokines while the other one (MMP-2 and MMP-8) doesn’t respond to growth factors and cytokines (*Matrisian, 1999*), stimulation of growth factor and cytokine responsive MMP genes (MMP-1, MMP-9, MMP-3 and MMP-11) in many cases results in 20-50 fold changes in mRNA and protein levels and this is induced by interleukin –1β (IL-1β), tumor necrosis factor-α (TNF-α) tumor growth factor-α (TGF-α), nerve growth factor (NGF) and tumor growth factor-β (TGF-β) (*Salo et al., 1999*).

The signaling pathways that lead to induction of expression of MMPs are still under study and recent studies have suggested the protein kinase C to act as an important messenger for the transcriptional regulation of growth factor responsive MMP genes, also the role of 3’-5’ cyclic adenosine monophosphate (cAMP) in which a rise of the cytoplasmic cAMP in some systems leads to stimulation of MMP expression and in others to repression, also the signaling pathways by mitogen activated protein kinases (MAPKs) and serine/threonine kinases that mediate signals from cell membrane receptors triggered by growth factors, cytokines, hormones and cell-cell and cell-matrix interactions (*Robinson et al., 2001*)

**Activation of pro MMPs:**

Degradation of extracellular matrix is a tightly controlled process under normal circumstances, insufficient degradation would prevent normal cell migration while excessive degradation would result in loss of cell attachment to the ECM as well as pathologic destruction of connective tissue, so physiological activation of MMPs is an important point, plasmin and urokinase type plasminogen activator (UPA) and
tissue type plasminogen activator (tPA) were implicated as the important physiological activators of MMPs (Mandal and Chakraborti, 2003).

The presence of uPA bound to a cell surface receptor provides a mechanism for the cell to activate an array of proteases in close proximity to the cell surface with the potential to restrict this activation to only a portion of the cell surface, interaction between the metalloproteinases exists and can further enhance activity as has been suggested for stromelysin activation of interstitial collagenase and MMP-9, like the plasmin/plasminogen activator system, MMP-2 may be controlled by a cell surface associated activator receptor and this system would allow the cells to activate MMP-2 close to or actually bound to the cell membrane, in fact MMP-2 was found to be activated by membrane type MMPs (MT-MMPs), MT1- MMP is the most predominant and the most clearly regulated by cytokines and growth factors and both MT2- MMP and MT3 – MMP share the ability to initiate the activation of proMMP2 with MT1-MMP, in contrast MT4-MMP has negligible proMMP-2 processing activity (Mandal and Chakraborti, 2003).

**Stepwise Activation Mechanism:**

MMPs can be activated by proteinases or invitro by chemical agents such as thiol-modifying agents (4-aminophenylmercuric acetate, HgCL2 and N-ethylmaleimide), oxidized glutathione, chaotropic agents and reactive oxygens, low PH an heat can also lead to activation and these agents work through the disturbance of the cysteine-zinc interaction of the cysteine switch (Nagase et al., 2003).

Proteolytic activation of MMPs is stepwise in many cases, the initial proteolytic attack occurs at an exposed loop region between the first and the second helices of the propeptide, once a part of the propeptide
is removed, this probably destabilizes the rest of the propeptide including the cysteine switch-zinc interaction which allows the inter-molecular processing by partially activated MMP intermediates or other active MMPs, thus the final step in the activation is conducted by an MMP (Gu et al., 2002).

Activation of proMMPs by plasmin is a relevant pathway in vivo, plasmin is generated from plasminogen by tPA bound to fibrin and uPA bound to specific cell surface receptor, both plasminogen and uPA are membrane associated so creating localized proMMP activation and ECM turnover, plasmin has been reported to activate proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10 and proMMP-13.

Activated MMPs can participate in processing other MMPs, the step wise activation system may have evolved to accommodate finer regulatory mechanisms to control destructive enzymes, TIMPs may interfere with activation by interacting with the intermediate MMP before it is fully activated (Suzuki et al., 2000).

**Intracellular activation:**

Most proMMPs are secreted from cells and activated extracellularly, first demonstrated that proMMP-11 is activated intracellularly by furin, proMMP-11 has a furin recognition sequence at the C-terminal end of the propeptide, several other MMPs including the six MT-MMPs, MMP-23 and MMP-28 have a similar basic motif in the propeptide and because these proteins are secreted as active enzymes, their gene expression and inhibition by endogenous inhibitors would be critical for the regulation of activity (Nagase et al., 2003).
Inhibition of active MMPs:

MMPs are inhibited by two types of proteinases inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) [TIMP-1, TIMP-2, TIMP-3 and TIMP-4] and Inhibitors of metalloproteinases specifically inhibit this class of enzymes, all active forms of MMPs are inhibited by TIMP-1 and TIMP-2. TIMP-1 is a 28.5 KDa glycoprotein that is synthesized by most connective tissue cells and macrophages while TIMP-2 is a 23 KDa unglycosylated protein and is found at lower concentrations than TIMP-1 in tissue (Woessner et al., 2000).

TIMP-1 and TIMP-2 rapidly inhibit active MMP-2, these inhibitors interact with the active site plus a site in the carboxyl terminal hemopexin-like region, the C-terminal region of the TIMPs interacts with the C-terminal region of the enzyme and the C-terminal hemopexin like region contributes stability to complexes of TIMP-1 with other MMPs, in addition to the inhibition of active MMPs, TIMP-1 can regulate the activation of MMP-9 while TIMP-2 is an effective regulator of MMP-2. In addition to MMP inhibiting activities, TIMPs have other biological functions such as TIMP-1 and TIMP-2 have erythroid protentiating activity, cell growth promoting activities, degradation of joint cartilage and mammary gland involution, TIMP-2 participates in metanephric mesenchymal growth and in the morphogenesis of ureteric bud, also over expression of TIMP-1 and TIMP-2 reduces tumor growth, TIMP-3 has proapoptotic activity possibly through the stabilization of TNF-α cell receptor and TNF-related apoptosis while TIMP-1 and TIMP-2 have antiapoptotic activity (Ahonen et al., 2003).

Two other classes of MMPIs have also been identified, these are the smaller inhibitors of metalloproteinase (IMPs) and the Large inhibitor
of metalloproteinase (LIMP), IMPs are smaller than TIMPs and the molecular mases of IMP-1,2 and 3 are 26, 21 and 18 K Da and are found in many cells while LIMP is a complex composed of TIMP-2 and proMMP-2 and this complex inhibits collagenase, gelatinase A and stromelysin, the ability of the TIMP-2/proMMP-2 complex to inhibit these enzymes indicates that the inhibitory site is exposed in the TIMP-2 molecule (Mandal and Chakraborti, 2003).

α2-Macroglobulin:

α2- Macroglobulin is an inhibitor of all MMPs known to date, it inactivates susceptible proteinases by entrapment following cleavage of the bait region.

Synthetic Inhibitors:

Nearly all of the synthetic inhibitors analyzed so far in MMP complexes contain a chelating group (such as a hydroxamic acid, a carboxylate or a thiol group) for zinc ion ligation, chelating agents that interacts with or remove zinc ion at the active site such as 1,10 phenanthroline and EDTA are potent inhibitors of MMP but are of limited therapeutic potential. Tetracyclines and certain synthetic analogues without antibiotic activity have been shown to inhibit PMN-CL and the mechanism is unknown but probably by chelating calcium ion that is required for activation of MMPs and they are less effective against FIB-CL but the reason is unknown (Bode et al., 2000).

Next generation Inhibitors:

Glycomed has completed the initial trials for the peptide hydroxamate inhibitor galardin (GM-6001) a couple of years ago and the drug showed a statistically significant benefit as a topical treatment of corneal ulcers, the MMP inhibitor with the most advanced clinical development at this time is British Biotech’s Marimastat (BB-2536) a
broad spectrum peptide inhibitor effective for the advanced stage refractory cancers, recently more selective compounds have been developed such as compounds from Bayer (BAY12-9566) and Roche (Ro 32-3555) effective for osteoarthritis and rheumatoid arthritis and Agouron (AG-3340) effective for advanced lung and prostate cancers (Moy et al., 2002).

MMPIs extracted from different natural products are also being increasingly explored as anti-tumor agents for example, green tea components such as catechins that inhibit both MMP production and activity, Neovastal which is derived from extracts of shark cartilage has potent MMP inhibitory and anti-angiogenic properties and is under trial for treatment of advanced non small cell lung cancer, metastatic renal cell carcinoma and multiple myeloma (Garbisa et al., 2001) and (Falardeau et al., 2001).

**Biological functions of MMPS:**

A major function of MMPs is thought to be the removal of ECM in tissue resorption, however the ECM is not a simply matrix but it also acts as a reservoir of biologically active molecules such as growth factors and some ECM components can express biological functions on proteolysis so degradation of ECM components by MMPs can alter cellular behavior and phenotypes (Nagase et al., 2003).

**Role of MMPS in many physiological processes**

**1-Wound Repair**

It’s a physiological event in which tissue injury results in a repair process which leads to restoration of structure and function of the tissue, proteolytic degradation of ECM is required in many stages of wound repair, it was found that following tissue injury, MMP-1 is expressed by basal keratinocytes at the migration front of the epidermis in several types
of cutaneous wounds including incision wounds and blistering skin diseases and this expression is most abundant at the edge of the wound and it diminishes away from the edge and it’s essential for keratinocyte migration and essential for re-epithelialization during wound healing and it also expressed by stromal dermal fibroblasts in both acute and chronic dermal wounds and important in remodeling of granulation tissue (Ravanti et al., 2000).

II- Cellular fibrinolytic activity:

Interactions between MMP and the fibrinolytic system may affect cellular fibrinolysis as MMP-3 specifically hydrolyzes human α2-antiplasmin (α2-AP) and inactivation of α2–AP by MMP-3 may lead to flavouring the local plasmin mediated proteolysis, also MMP-3 specifically hydrolyzes and inactivates human plasminogen activator inhibitor –1(PAI-1) and this may lead to decreasing the anti-proteolytic activity of PAI-1 and impairing the potential inhibitory effect of vitronectin bound PAI-1 on cell adhesion and migration so these molecular interactions of MMP-3 with enzymes, substrates and inhibitors of the fibrinolytic system may play a major role in the regulation of cellular fibrinolysis (Lijnen, 2002).

III) Human endometrium:

Menstruation is an event of tissue destruction that results from partial breakdown of the functional layer of endometrium at the onset of reproductive cycle in women and it consists of number of inflammatory responses such as leucocyte infiltration, proliferation and activation and it was found that interaction between leucocyte and the epithelial cells of the endometrium releases and activates MMPs as evidenced by the expression of MMPs such as matrilysin in the epithelium and glandular epithelial cells and MMP-1 and MMP-9 in the luminal region of tissue.
and MMP-2 and MMP-11 in the stromal component of tissue and this suggests the role of MMPs in the breakdown of endometrial tissue during menstruation, it was also found that the mRNA of all MMPs is reduced and even absent during the secretory phase of the cycle when progesterone levels are elevated suggesting an inhibitory effect of progesterone on the expression of MMPs which may be important for maintaining endometrial environment for embryo implantation and development (Salamonsen et al., 2000).

Epithelial Remodeling:

A common role of the mucosal epithelium is to function as an active barrier against the external environment and secretion of antimicrobial peptides by epithelial cells which are known as α and β defensins which kills bacteria by membrane disruption, paneth cells are specialized epithelial cells that secrete defensins and other anti-microbial molecules and it was found that matrilysin is responsible for the activation of prodensins in the secretion pathway and it is most expressed in tissues with a heavy bacterial load such as in lungs with cystic fibrosis, so matrilysin plays an important role in the innate immunity of the mucosal epithelium (Parks and Shapiro, 2001).

Also it has been demonstrated a functional role for matrilysin in airway re-epithelialization by repairing of injured tracheas as evidenced by expression of matrilysin in airway epithelial cells but the mechanism is not well known and may be due to loosen cell-matrix and cell-cell contacts and matrilysin is not the only MMP involved in repairing of airway epithelial wounds but MMP-9 is also expressed upon injury and (Legrand et al., 2000) have demonstrated that the activity of gelatinase-B is required for the migration of isolated airway epithelial cells over matrix substratum (Mandal and Chakraborti, 2003)
## Aging:-

Aging is the major risk factor for the development of vascular diseases such as atherosclerosis and it was found that intimal growth factor during aging in the absence of experimental injury resembles the neointimal formation in response to injury and it contains markedly higher levels of MMP-2, fibronectin and tumor growth factor β (TGF-β) and platelet derived growth factor (PDGF), recent studies have demonstrated that chemotactic invasion of reconstituted basement requires MMP-2 activity in smooth muscle cells (SMCs), also the expression and activity of MMP-14, MMP-2 and MMP-9 increase during mechanical injury to arteries, MMP-2 has been shown to be present within atherosclerotic lesions as the proteolytic activity weaken the fibrous cap resulting in its rupture, both MMP-2 and MMP-9 exhibit elastase activity and a significant amount of MMP-2 activity has been found to be localized to the intima and elastic lamella and accumulates in the area surrounding SMCs located just beneath the broken internal elastic lamina indicating that MMP-2 may have a role in fragmentation of the elastic laminae with aging (Li et al., 2000). SMCs are potentially a source of age associated increase in MMP-2 in the aortic wall as early passage SMCs from aged aorta secrete more MMP-2 than those from young aorta and this is triggered by stimulation with cytokines including interleukin-1, TNF-α and TNF-β (Jenkins et al., 2000).

### Pathological consequences:-

1) **Angiogenesis:-**

It's a process of neovascularization which can occur throughout one’s adult span life, during angiogenesis capillary endothelial cells (EC)of parent venule are stimulated by an angiogenic stimulus such as fibroblast growth factor (FGF), vascular endothelial cell growth factor (VEGF) and vascular permeability factor (VPF). The perivascular...
ECM is composed of type I collagen, two specific MMPs (MMP-1 and MMP-8) which are capable of degrading type I collagen, in a recent study it has been demonstrated that MMP-1 activity appears to be required for angiogenesis. MMPs facilitate EC release through the degradation of the venules basement membrane and proteolytic activity is required for the migration of EC into the perivascular stroma and these events are followed by extension and formation of a new vessel lumen, also it was suggested that the remodeling of the capillary endothelium which is required during angiogenesis can be induced by thrombin most probably by inducing the activation of MMP-2 (Mandal and Chakraborti, 2003).

Tumor cell invasion and angiogenesis share a number of functional similarities, initiation of cellular invasion in both processes requires attachment to a basement membrane followed by creation of a proteolytic defect in the basement membrane and migration through this defect thus tumor invasion and metastasis formation are closely linked to tumor induced neo-angiogenesis. Evidence for the role of MMPs and TIMPs in angiogenesis come from a number of studies as nanomolar concentration of TIMP-2 has been shown to block the angiogenesis produced by cytokines which are produced by vascularized tumors, TIMP-1 has been shown to inhibit EC invasion of human amniotic membranes invitro (Mandal and Chakraborti, 2003).

2)Lung Diseases:-

Production of MMP-2 by airway smooth muscle suggested that it contributes to extra-cellular matrix turnover and airway remodeling in inflammatory diseases such as asthma as airway smooth muscle has important secretory functions and participates in pro and anti-inflammatory responses, also MMP-2 may be produced by other airway cells including fibroblasts and macrophages (Jhonsen et al., 2000). The
involvement of MMPs in tissue remodeling have been reported in a variety of diseases including pulmonary fibrosis, it was found that MMP-9 activity is significantly increased in the acute respiratory distress syndrome (ARDS) and this high level could lead to matrix degradation that occurs in this syndrome and it was suggested that both neutrophils and macrophages are potent sources of MMP-9 which not only degrades matrix but on contrary could also prevent fibroproliferation and allow the clearance of the abnormal interstitial matrix to facilitate reconstitution of the normal lung and it's now believed that cytokines and MMPs interact closely to produce ARDS as IL-1 and TNF-α enhance matrix degradation by stimulating protease production (Ricou et al., 1999).

Several MMPs are produced in human emphysematous lung and it was found that overexpression of interleukins result in the production of several MMPs which lead to structural damage associated with emphysema and COPD by degradation of selective ECM components especially elastin, metalloelastase is not the only proteinase responsible for the disease but also Collagenase-1,2 and 3 contribute to loss of the airspace and an increase in collagen deposition leading to airway obstruction (Zheng et al., 2000).

3) Rheumatoid Arthritis:-

In rheumatoid arthritis (RA) and osteoarthritis the progressive cartilage and bone destruction is considered to be driven by an excess of MMP enzymes, in (RA) MMPs such as MMP-1,2,9 and 13 contribute to joint destruction in at least two ways, first they can directly degrade the cartilage and bone, second they are important during angiogenesis which is a prominent feature of RA as during angiogenesis ECs degrade two barriers which are microvascular basement membrane and the interstitium (Close DR, 2001).
Several studies have shown an increase in collagenase activity in rheumatoid synovial tissues and cells, collagenase-1 and stromelysin-1 have been identified near the areas of destruction at the cartilage pannus junction and in the synovial lining cells and in the chondrocytes, also Collagenase-TIMP complexes were found in the rheumatoid synovial fluids, it was found that higher levels of MMP-3 mRNA and protein were detected in the human synovium and lining cells from patients with RA than those with osteoarthritis (Jackson et al., 2001), recently a new therapeutic approach is to use MMP inhibitors to prevent the slow joint destruction in both RA and osteoarthritis (Gordan et al., 2002).

4) Cardio-vascular Diseases:

The MMPs system has been implicated in the pathogenesis of atherosclerosis and aneurysm formation, human atherosclerosis appears to be an important example of the potentially harmful effect of MMP-9 production as immunostaining has shown that in both stable and unstable angina MMP-9 production in arteries is increased whereas in normal arteries MMM-9 is not expressed and that MMP-9 production by macrophages in human aortic aneurism has recently been observed and this may contribute to plaque rupture and formation of aortic aneurism, collagenases and stromelysins have also been identified in atherosclerotic coronary arteries (Ross, 1999).

The factors regulating the production of MMPs in atherosclerosis are TNF-α and IL-1β which increase the production of MMP-9 by human macrophages and SMCs and these factors are released by the inflammatory cells of the arterial wall, also thrombin has been shown to proteolytically activate proMMP-2 at sites of vascular injury and promote plaque instability and rupture by increasing local matrix degrading activity of MMPs, also pericellular activation of proMMP-2 can be
achieved by MT-MMPs expressed by vascular ECs and SMCs in response to cytokines and oxidized lipoproteins (Rajavashisth et al., 2000).

MMPs are expressed at very low levels in normal myocardium such as MMP-13 and MT1-MMP but they are upregulated in congestive heart failure (CHF) and relationship has been demonstrated between myocardial MMP expression and the left ventricular remodeling process which is accompanied by changes in the structure and composition of the myocardial ECM and this is done by TIMP/MMP imbalance that leads to MMP activation, studies have noted that an increase in the release of TNF-α can contribute to LV myocardial remodeling in CHF and these effects of TNF-α are mediated through TNF-α receptors that are present in all the heart and they lead to induction of specific MMPs such as MMP-9 and MMP-13 which degrade ECM components (Bradham et al., 2002).

Cardiac fibrosis:

MMPs no only play a role in degrading matrix components but also modulate collagen synthesis, an increase in the level of MMPs is accompanied with increased fibrosis as seen in failing heart, MMPs may participate in the fibrosis and remodeling process through direct digestion of matrix components and regulation of the formation of matrikines, imbalance of extracellular matrix turnover (synthesis and degradation) is the cause and not the effect of structural disease in the heart, a balance between ECM, MMP and TIMPs concentrations are required for normal structure remodeling (Li et al., 2000).

Inflammatory myopathy:

MMPs have been suggested to play a role in inflammatory myopathies, MMP-1 and MMP-9 levels were elevated in polymyositis and dermatomyositis and to a lesser extent in inclusion body myositis,
whereas the level of expression of TIMP remained unchanged in comparison with controls, these observations indicate a pathogenic role for specific MMPs in the genesis of inflammatory myopathies (Kieseier et al., 2001).

Role of matrix metalloproteinases in tumor invasion and metastasis:

MMPs mediate ECM and basement membrane degradation during the early stages of tumor genesis contributing to the formation of a microenvironment that promote tumor growth, MMPs are also active in the latter stages of cancer development in that they promote metastasis as well as other aspects of tumor growth, it has been shown that MMP activity is required for growth of metastasized tumor cells and that MMPs may play an essential role in angiogenesis and tumor cell intravasation, both of which are required for tumor cell growth and metastasis (Mandal et and Chakraborti, 2003).

MMPs promote the initiation and sustained growth of both primary tumors and metastatic foci by activating growth factor, by activating growth factors binding protein, or by releasing mitogenic molecules from matrix proteins that are sequestered in the peritumor ECM, thus MMP activated growth factors directly induce tumor cell proliferation or indirectly regulate the behaviour of fibroblasts or endothelial cells that support tumor growth, MMPs also have cell adhesion molecules, tumor derived MMPs assist in overcoming the host anti-tumor defense system by destroying chemokine gradients that are laid down in the peritumor stroma which attract immune cells, also MMPs promote tumor angiogenesis by activating angiogenic factors such as VEGF, TGF β and BFGF (Mandal and Chakraborti, 2003).
a- Gelatinases:

Studies have shown that gelatinases capable of destroying type IV collagen are involved in degradation of basal membranes which is required for tumor invasion and metastasis, both MMP-2 and MMP-9 were shown to be two of the key enzymes in invasion and metastasis of various tumors, high expression level of mRNA for MMP-2 and MMP-9 were found during the progress of mammary gland carcinoma, also immunochemical studies revealed the presence of MMP-2 on the invasive boundaries of rectal carcinoma and MMP-2 mRNA in the stromal cells and MMP-2 was found to bind to vitronectin receptors on the surface of invasive cells and this influence the ability of tumor cells for growth and differentiation (Tokumaru et al., 2000).

b- Stromelysins:

Stromelysin-1 expression is usually absent in steady state tissue and it is induced by cytokines and growth factors and it was found that its activity is increased in tissues of stomach carcinoma, also high activity of stromelysin-3 is observed invasive mammary gland carcinomas, squamous cell carcinoma and rectal tumors and its expression in stroma fibroblasts is controlled by factors produced by the tumor cells (Okada et al., 1999).

c- Matrilysins:

MMP-7 plays an important role in tumor invasion by its ability to destroy type IV collagen and laminin of basal membranes and in tumor migration by its ability to destroy the fibrillar form of fibronectin which weaken the attachment of tumor cells to the connective tissue stroma, the expression of MMP-7 mRNA is increased in carcinomas of prostate, lungs, stomach, rectum and mammary gland (Ozaki et al., 2000).
d- Collagenase-3:

Collagenase-3 can destroy fibrillar collagens, fibronectin, tenascin C and fibrillin and display gelatinolytic activity when involved in the degradation of collagen fragments so expression of MMP-13 plays an important role in the development of many malignant tumors such as mammary gland carcinoma, chondrosarcoma and carcinomas of head and neck as MMP-13 was found in epithelial tumor cells, stroma cells and MMP-13 mRNA in cells located on the invasive boundary of the tumor (Answorth et al., 2000).

3- Membrane Bound MMPs:

MB-MMPs play a dual role in the degradation and remodeling of the matrix, first they are proteolytic enzymes which act on fibronectin, laminin, proteoglycan and collagens, second they activate MMP-2 and collagenase-3 on the cell surface, MB-1 MMB is located on the surface of various tumor cells and seems to influence their invasion, studies have shown a direct correlation between the expression of MB1- MMP in stomach tumor cells and the activation of MMP-2 which leads to tumor invasion and metastases also expression of other MMPs such as MB2 – MMP and MB4 – MMP were observed in the cells of mammary gland carcinoma (Velasco et al., 2000).

Increased production of proMMP-2 and the rate of its activation in human brain malignant tumors such as astrocytoma and glioblastoma directly correlate with expression of MB1 – MMP and MB2 – MMP and recently found metalloproteinases such as MB5 – MMP and MB6- MMP which is also expressed in intestinal carcinoma, cooperation of tumor cells with the adjacent stroma cells promotes the invasiveness and facilitates metastasis and this is influenced by expression of MB1 – MMP as in case of mammary gland and lung tumors and the mRNA of MB1-
MMP is expressed in fibroblasts which are in contact with the malignant cells (Nakada et al., 2000).

**Role of Matrix Metalloproteinase inhibitors in tumor invasion and metastasis:**

Disorders in the balance between the activities of MMP and TIMP resulting in excess degradation of the matrix which leads to tumor invasion and metastasis, the expression level of mRNA for TIMP-1 was shown to be higher in stroma cells of mammary gland carcinoma and malignant ovary tumors than in adjacent normal tissues, also high level of TIMP-1 expression is specific for progressing stages in development of some lymphomas. A correlation was observed between an increase in TIMP-2 expression and the progress of mammary gland carcinoma, colorectal carcinoma and blood cancer (Takahashi et al., 1999).

On the other hand the increased expression of TIMP-1 and TIMP-2 suppressed the invasion of melanoma cells and the tumor growth so studies were done for development of gene therapy by application of MMP inhibitors for suppression of tumor invasion and metastasis for example transfection of the TIMP-1 gene into tumor cells such as melanoma, astrocytoma and stomach cancer decreased the ability of these cells for invasion and metastasis, also transfection of the TIMP-2 gene into hepatocytes decreased the growth rate of metastasing foci in the liver, also the transfection of TIMP-3 gene into the cells of leukomyosarcoma resulted in changes in the cell morphology and growth rate which could be considered as suppression of the invasive phenotype, so the balance between MMPs and TIMPs regulate the tumor invasion and metastasis (Yoshiji et al., 2000).

**Matrix Metalloproteinase-2**
1- Definition and sources:

MMP-2 is a matrix degrading enzyme of gelatinase group called gelatinase A which degrade denatured collagen or gelatins and also digest native type IV collagen which is an important component of the normal basement membrane.

- MMP-2 contain an extra 175 amino acids stretch with three collagen-binding fibronectin type II that precede the zinc binding region of the catalytic domain, and this type II defines the substrate specificity (Arthur, 2000).

- MMP-2 is secreted by fibroblast, tumor cell lines such as transformed human bronchial epithelial cells, MMP-9 which is closely related [92-kDa type IV collagenase/gelatinase) called gelatinase B is secreted by keratinocytes, polymorphnuclear leucocytes, tumor cell lines such as transformed human lung fibroblasts and fibrosarcoma cells and mononuclear phagocytes, these gelatinases are considered to serve not only as type IV collagenase but also as proteases cleaving other molecules such as type V and VII collagens, elastin and fibronectin also they degrade the denatured type I collagen (Arthur, 2000)

Cell Surface Activation of ProMMP-2:

ProMMP-2 is not activated by general proteinases but it's activation takes place on the cell surface and is mediated by five MT-MMPs which are MT-MMP-1,2,3,5 and MT-MMP-6, the MT-1MMP mediated activation of proMMP-2 requires the assistance of TIMP-2 as proMMP-2 forms a tight complex with TIMP-2 through their C-terminal domains permitting the N-terminal inhibitory
domain of TIMP-2 to bind to MT-1MMP on the cell surface and the cell surface bound proMMP-2 is then activated by an MT-1MMP that is free of TIMP-2, alternatively, MT-1MMP inhibited by TIMP-2 can act as a receptor of proMMP-2, this MT-1MMP-TIMP-2-proMMP-2 complex is then presented to an adjacent free MT-1MMP for activation (*Wang et al.*, 2000)

ProMMP-2 activation by MT-2MMP is direct and independent of TIMP-2, the extra-cellular activity of MMP-2 is regulated by interaction of activation systems and specific inhibitors and the activation systems are the uPA and tPA while inhibitors are TIMP-2 and TIMP-3 (*Morrison et al.*, 2001)

**Mechanisms responsible for increased MMP-2 in cirrhotic livers:**

Increased TGF-B1 in fibrotic livers stimulates MMP-2 synthesis and it upregulates MMP-2 expression of the transcriptional and post-transcriptional levels (*Bedoss and Paradis, 1999*).

1- In advanced liver fibrosis overexpression of MMP-2 maybe induced by increased amount of type IV collagen and activity of MMP-2 is increased in parallel with increased collagen accumulation in the liver and this leads to compensatory mechanism against further deposition of type IV collagen (*Murawaki et al.*, 1999).

2- The progression of liver fibrosis may lead to increase in collagen synthesis or to decrease in collagen degradation or both and this is suggested due to decrease the collagenase activity and so decrease the collagen degradation which occurs in progressive liver fibrosis and this could be due to decreased pro-collagenase biosynthesis, decreased
procollagenase activation or specific inhibition of activated collagenase (*Arthur, 2000*).

Synthesis of ECM components could modulate MMP-2 processing as the collagen 1 synthetized at high levels by cultured hepatic stellate cells promotes MMP-2 activation also the hepatocytes which don't secrete the gelatinase activity induce the activation of MMP-2 by hepatic stellate cells through a plasma-membrane dependent mechanism and the role of MT-1MMP in activation of MMP-2 (*Sato et al., 1999*).

- Recent studies showed that circulating levels of pro MMP-2, TIMP-1 and TIMP-2 increase with advancing liver disease while pro MMP-9 levels decrease with advancing liver disease but only MMP-2 / TIMP-1 ration which reflects an increasing dysbalance toward the TIMP-1 concentration correlates to both inflammatory activity and extent of fibrosis (*Lichtinghagen et al., 2000*).

- There is a long standing assumption that degradation of fibrillar matrix in liver is mediated by MMP-1 in humans and MMP-13 in rats, in contrast to MMP-1 and MMP-13, MMP-2 and MTI-MMP are both expressed in stimulated HSCs and expression of both is increased in liver fibrosis, during regression of liver fibrosis expression of both MMP-2 and MT-1 MMP gradually returns to base line control values in a similar manner to that of TIMP-1 and TIMP-2 (*Mandal and Chakraborti, 2003*).

**Methods of detection of MMP-2 :**

1- **Enzyme Immuno Assay (EIA) :**

   Serum MMP-2 is measured by one step sandwich EIA using a combination of 2 monoclonal antibodies against proMMP-2 purified from condition medium of human skin fibroblasts and against a synthetic peptide corresponding to the N-terminal domain of human proMMP-2 (*Fujimoto et al., 1999*).
The antibodies specifically react with proMMP-2 and show negligible cross-reaction with proMMP-1, proMMP-3, proMMP-9, TIMP-1 and TIMP-2, the EIA is done to recognize both the free form of pro MMP-2 and its complex form with TIMP-2 but not active MMP-2 or active MMP-2 complexed with TIMP-1 or TIMP-2 and so human pro MMP-2 protein is used as the standard protein (Murawaki et al., 1999).

2- Gelatin zymography:

Zymographic analysis using Na dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatin, serum aliquots (10ml) were electrophoresed in a 10% polyacrylamide gel embeded with 1mg/ml of gelatin with 25mMtris Hcl at PH 8.3 after electrophoresis the gel was washed 2.5% riton x-100 in 50 mMtris - Hcl at PH 7.5 for 1h to remove the SDS then the gel was incubated for 18 h at 37°c in 50 mMtris Hcl at PH 7.5 followed by staining for 10min with 10% coomassie Brilliant blue G250 and destaining in 10% methanol /5% acetic acid, pro MMP-2 and prestained SDS molecular weight standard mixture were used as marker proteins (Murawaki et al., 1999).

3- Gel filtration of serum on Sephadex G-100 column:

The serum 1ml was applied to column (1.6×100cm) of Sephadex G-100 equilibrated with 67mM sodium phosphate buffer at PH 7 at flow rate of 15ml/h, the column was eluted with this buffer at 4°c and 100drop fractions were collected, each fraction is then analyzed directly using MMP-2 assay kit, Protein peaks were monitored by absorbance at 280nm and the column was calibrated with blue dextran 2000, bovine serum, albumin, ovalbumin, chymotrypsinogen A and ribonuclease A (Murawaki et al., 1999).

4- Immunohistochemistry:
Performed by indirect immunoperoxidase method using monoclonal antibodies against MMP-2 which also recognizes proMMP-2, cryosections are incubated with the antibodies and the reaction is visualized by diaminobenzidine (Takahara et al., 1999).

5-MMP-2 quantitative test assay (Amersham Biosciences):

The assay is based on a two site ELISA "sandwich" format, standards and samples are incubated in microtiter wells precoated with anti-MMP-2 antibody, any MMP-2 present will be bound to the wells, other components of the sample being removed by washing and aspiration, the amount of peroxidase bound to each well is determined by the addition of TMB ready to use substance, the reaction is stopped by addition of an acid solution and the resultant color read at 450nm in a microplate spectrophotometer, the concentration of MMP-2 in a sample is determined by interpolation from a standard curve.
PATIENTS AND METHODS

1) Patients:
This study was conducted on 50 patients, they were 32 males (64%) and 18 females (36%), their ages ranged between 30 years and 80 years with a mean age of 54.2 years, all cases were selected from the Internal Medicine Department of Benha University Hospital. Another 25 healthy individuals of matched age and sex served as a control group.

Subjects were divided into 3 groups:

Group I: Included 25 hepatic patients with positive hepatitis – C – virus, they were 14 males (56%) and 11 females (44%), their age ranged between 32 years and 75 years with a mean age of 53.12 years.

Group II: Included 25 hepatic patients with hepato-cellular carcinoma and positive hepatitis – C – virus, they were 18 males (72%) and 7 females (28%), their ages ranged between 30 years and 80 years with a mean age of 55.64 years.

Group III: (control group) included 25 apparently healthy individuals, they were 8 males (32%) and 17 females (68%), their ages ranged between 23 years and 80 years with a mean age of 50.04 years.

All cases were subjected to:
Full history taking of present illness.
Thorough clinical examination.
Abdominal ultrasonography.
Routine laboratory investigations.
CBC.
Urine and stool analysis.
Serum creatinine.
Liver function tests including:
Total protein.
Serum Albumin.
Bilirubin (total – direct).
Alanine transaminase (ALT).
Aspartate transaminase (AST).
Alkaline phosphatase (ALP).
Prothrombin time (PT).
e- Hepatitis C virus marker.
Determined by Enzyme Linked Immunosorbant Assay (ELISA) and Polymerase Chain Reaction (PCR) for hepatitis – C – virus.
f- Specific laboratory investigations including.
Alpha feto-protein (αFP).
Matrix Metalloproteinase-2 (MMP-2).

2) Methods:
Matrix Metalloproteinase 2 (MMP-2):
All cases and controls were subjected to assessment of serum MMP-2 by Quantikine MMP-2 immunoassay which is a 4.5 hour solid phase ELISA.

Collection of samples:
Blood samples were collected by vein puncture in a serum separator tube and were allowed to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000xg and the centrifuged sera were separated and kept frozen at –20°C and they are stable for 3 months at – 20°C.
Principle of the assay:
This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for MMP-2 has been precoated onto a microplate, standards and samples were pipetted into the wells and MMP-2 was bound by the immobilized antibody, after washing away unbound substances, and enzyme linked polyclonal antibody specific for MMP-2 was added to the wells, following a wash to remove unbound antibody enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of total MMP-2 bound in the initial step and the color development was stopped and the intensity of the color was measured.

Reagents:
1) Total MMP-2 Microplate (Part 892253). 96 well polystyrene microplate (12 strips of wells) coated with a polyclonal antibody against human MMP-2.
2) Total MMP-2 Conjugate (Part 892254)-21mL of polyclonal antibody against human MMP-2 conjugated to horseradish peroxidase with preservative.
3) Total MMP-2 Standard (Part 892255)-100ng of recombinant human pro MMP-2 in a buffered protein base with preservative, lyophilized.
4) Assay Diluent RD1-74 (Part 895809) 11mL of a buffered protein base with preservative.
5) Calibrator Diluent RD5-32 (Part 895810)-21 mL of a buffered protein base with preservative.
6) Wash Buffer Concentrate (Part 895003)-21mL of a 25 fold concentrated solution of buffered surfactant with preservative.
7) Color Reagent A (Part 895000)-12.5mL of stabilized hydrogen peroxide.
8) Color Reagent B (Part 895001)-12.5mL of stabilized chromogen (tetramethyl benzidine).
9) Stop Solution (Part 895032) 6mL of 2N sulfuric acid.
10) Plate Covers-4 adhesive strips.

**Other supplies required:**
- Microplate reader capable of measuring absorbance at 450nm, with the correction wavelength set at 540nm or 570nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multichannel pipette, squirt bottle, manifold dispenser or automated microplate washer.
- Horizontal orbital microplate shaker (0.12 orbit) capable of maintaining a speed of 500 ± 50 rpm.
- 500mL graduated cylinder.
- Human Total MMP-2 Controls.
- 12mm 75mm polypropylene test tubes.

**Reagent preparation:**
First all reagents were bought to room temperature before use then:

1) **Wash Buffer:**
If crystals have formed in the concentrate, they were warmed to room temperature and mixed gently until the crystals have completely dissolved then 20ml of Wash Buffer Concentrate were diluted into deionized or distilled water to prepare 500ml of Wash Buffer.

2) **Substrate Solution:**
Patients and Methods

Color Reagents A and B were mixed together in equal volumes within 15 minutes of use and were protected from light and 200ml of the resultant mixture was required per well.

3) **Total MMP-2 Standard:**

The MMP-2 standard was reconstituted with 1ml of deionized or distilled water and this reconstitution produced a stock solution of 100ng/mL and then the standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

4- **Use polypropylene tubes:**

200mL of Calibrator Diluent RD5-32 were pipetted into each tube and the stock solution was used to produce a dilution series, then each tube was mixed thoroughly before the next transfer and the 50ng/mL standard serve as the high standard and the Calibrator Diluent RD5-32 serve as the zero standard (0ng/mL)

**Assay procedure:**

All reagents and samples were brought to room temperature before use and it was recommended that all samples, standards and controls be assayed in duplicate.

1) All reagents, working standards and samples were prepared.
2) Excess microplate strips were removed from the plate frame and returned to the foil pouch containing the desiccant pack.
3) 100 mL of Assay Diluent RD1-74 were added to each well.
4) 50mL of standard, control or sample were added to each well and then covered with the adhesive strip provided and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12 orbit) set at 500 ± 50 rpm and a plate layout was provided to record the standards and assayed samples.
5) Each well was aspirated and washed and this process was repeated three times for a total of four washer and each well was washed by Wash buffer (400mL) using a squirt bottle, multi channel pipette, manifold dispenser or auto washer and complete removal of liquid was done at each step which is essential to good performance and after the last wash, any remaining Wash Buffer was removed by aspirating or decanting and the plate was inverted and blotted against clean paper towels.

6) 200mL of MMP-2 Conjugate were added to each well and covered with a new adhesive strip and incubated for 2 hours at room temperature on the shaker.

7) The aspiration/ wash was repeated as in step (5).

8) 200mL of Substrate Solution were added to each well and incubated for 30 minutes at room temperature on the benchtop and protected from light.

9) 50mL of Stop Solution were added to each well and if color change does not appear uniform, gently taping of the plate to ensure thorough mixing.

10) The optical density of each well were determined within 30 minutes and by using a microplate reader set to 450nm and if wave length correction is available we set to 540nm or 570nm and if wave length correction is not available, we subtract readings at 540nm or 570nm from the readings at 450nm and this subtraction will be correct for optical imperfections in the plate and readings made directly at 450nm without correction may be higher and less accurate (Murphy, 1998; Fernandez-Patron et al., 1999; Nagase, 1999; McQuibban et al., 2000; Fang et al., 2000 and Nguyen et al., 2002).

Statistical analysis:
Patients and Methods

Data were tabulated and statistically analysed to evaluate the difference between the groups under study as regards the various parameters. Results are expressed as mean ± SD and the statistical significance of differences between groups was assessed by an analysis of variance (ANVOA) and t-test. The correlation were evaluated by person’s test. Results are significant if P < 0.05, highly significant if P < 0.01 or P< 0.001 and non significant if P > 0.05.
### Results

Table (1): Distribution of Total bilirubin of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>Total Bilirubin X ± SD</th>
<th>Test of significance</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 25)</td>
<td>0.97 ± 0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>4.17 ± 3.2</td>
<td>t2 = 4.91</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>5.73 ± 6.3</td>
<td>t3 = 3.79</td>
<td>t1 = 1.11</td>
</tr>
</tbody>
</table>

F = 8.89 P < 0.001

This table shows the mean ± standard deviation of Total bilirubin between group I and group II and the control.

And t1 which shows the relation of GIVs GII is of non significance.

And t2 which shows the relation of GIVs control is of highly significance.

And t3 which shows the relation of GIIVs control is of highly significance.
Table (2): Distribution of Direct bilirubin of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>X ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 25)</td>
<td>0.28 ± 0.12</td>
<td>t2 = 4.97 p &lt; 0.001</td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>2.73 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>3.39 ± 3.88</td>
<td>t3 = 4.01 t1 = 0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt; 0.001 &gt; 0.05</td>
</tr>
</tbody>
</table>

F = 9.54 P < 0.001

This table shows the mean ± standard deviation of Direct bilirubin between group I and group II and the control.

And t1 which shows the relation of GI IVs GII is of non significance.

And t2 which shows the relation of GI IVs control is of highly significance.

And t3 which shows the relation of GII IVs control is of highly significance.
### Table (3): Distribution of AST of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>AST X ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 25)</td>
<td>25.7 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>94.2 ± 77.6</td>
<td>t2 = 4.4 &lt; 0.001</td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>126.2 ± 123.4</td>
<td>t3 = 4.07 t1 = 1.09</td>
</tr>
</tbody>
</table>

F = 9.28  \[ P < 0.001 \]

This table shows the mean ± standard deviation of AST between group I and group II and the control. And t1 which shows the relation of GIVs GII is of non significance. And t2 which shows the relation of GIVs control is of highly significance. And t3 which shows the relation of GIIVs control is of highly significance.
Table (4): Distribution of ALT of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>ALT</th>
<th>X ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=25)</td>
<td>23.96 ± 9.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI (n=25)</td>
<td>68.92 ± 74.93</td>
<td>t2 = 2.98</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GII (n=25)</td>
<td>84.76 ± 76.3</td>
<td>t3 = 3.96</td>
<td>t1 = 0.74 &lt; 0.001</td>
</tr>
</tbody>
</table>

F = 6.48        P < 0.001

This table shows the mean ± standard deviation of ALT between group I and group II and the control. And t1 which shows the relation of GIVs GII is of non significance. And t2 which shows the relation of GIVs control is of highly significance. And t3 which shows the relation of GIIIVs control is of highly significance.
Table (5): Distribution of ALP of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>ALP ( \bar{X} ) ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>t</td>
</tr>
<tr>
<td>Control (n = 25)</td>
<td>74.5 ± 19.3</td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>231.6 ± 67.7</td>
<td>t2 = 11.15</td>
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<tr>
<td>GII (n = 25)</td>
<td>281.1 ± 117.6</td>
<td>t3 = 8.67</td>
</tr>
</tbody>
</table>

F = 46.44        P < 0.001

This table shows the mean ± standard deviation of ALP between group I and group II and the control
And t1 which shows the relation of GIVs GII is of significance.
And t2 which shows the relation of GIVs control is of highly significance.
And t3 which shows the relation of GII vs control is of highly significance.
Results

Table (6): Distribution of PT of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>PT</th>
<th>X ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>t</td>
</tr>
<tr>
<td>Control (n = 25)</td>
<td>15.44 ± 10.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>18.98 ± 2.02</td>
<td>t2 = 1.68</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>18.4 ± 2.7</td>
<td>t3 = 1.38</td>
<td>t1 = 0.86</td>
</tr>
</tbody>
</table>

F = 2.27  P > 0.05

This table shows the mean ± standard deviation of prothrombin time between group I and group II and the control. And t1 which shows the relation of GIVs GII is of non significance. And t2 which shows the relation of GIVs control is of significance. And t3 which shows the relation of GIIV control is of non significance.
Table (7): Distribution of ALB of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>ALB</th>
<th>X ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 25)</td>
<td>4.53 ± 1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>3.04 ± 0.77</td>
<td>t2 = 3.62</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>2.9 ± 0.72</td>
<td>t3 = 4.01</td>
<td>t1 = 0.66 &lt; 0.001</td>
</tr>
</tbody>
</table>

F = 12.89  P < 0.001

This table shows the mean ± standard deviation of Albumin between group I and group II and the control. And t1 which shows the relation of GIVs GII is of non significance. And t2 which shows the relation of GIVs control is of highly significance. And t3 which shows the relation of GIIVs control is of highly significance.
Results

Table (8): Distribution of AFP of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>X^- ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>t</td>
</tr>
<tr>
<td>Control (n = 25)</td>
<td>1.54 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>26.21 ± 72.53</td>
<td>t2 = 1.71</td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>951.1 ± 900.1</td>
<td>t3 = 5.27</td>
</tr>
</tbody>
</table>

F = 26.94        P < 0.001

This table shows the mean ± standard deviation of alpha-feto-protein between group I and group II and the control.
And t1 which shows the relation of GIVs GII is of highly significance.
And t2 which shows the relation of GIVs control is of significance.
And t3 which shows the relation of GIIVs control is of highly significance.
# Table (9): Distribution of MMP2 of different study groups

<table>
<thead>
<tr>
<th>St. groups</th>
<th>X' ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 25)</td>
<td>254.99 ± 83.9</td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>523.4 ± 207.4</td>
<td>t2 = 5.99</td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>553.9 ± 112.1</td>
<td>t3 = 10.7</td>
</tr>
</tbody>
</table>

F = 32.42  
P < 0.001

This table shows the mean ± standard deviation of Matrix Metallaproteinase-2 between group I and group II and the control. And t1 which shows the relation of GIVs GII is of non-significance. And t2 which shows the relation of GIVs control is of highly significance. And t3 which shows the relation of GIIVs control is of highly significance.
### Table (10): Distribution of creatinine of different study groups

<table>
<thead>
<tr>
<th>Creatinine St. groups</th>
<th>X ± SD</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 25)</td>
<td>0.8 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>GI (n = 25)</td>
<td>1.15 ± 0.5</td>
<td>t2 = 3.08 &lt; 0.01</td>
</tr>
<tr>
<td>GII (n = 25)</td>
<td>1.06 ± 0.5</td>
<td>t3 = 2.43 t1 = 0.63 &lt; 0.01 &gt; 0.05</td>
</tr>
</tbody>
</table>

F = 4.3 P < 0.01

This table shows the mean ± standard deviation of creatinine between group I and group II and the control. And t1 which shows the relation of GIVs GII is of non significance. And t2 which shows the relation of GIVs control is of highly significance. And t3 which shows the relation of GIIVs control is of highly significance.
Table (11) Correlation coefficient ($r$) between AFP and different variables among group I

<table>
<thead>
<tr>
<th>Variables</th>
<th>FP</th>
<th>($r$)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.36441</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>0.1337</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0.0886</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>0.06498</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>0.38002</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>0.39876</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>0.37404</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>0.09626</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>ALB</td>
<td>0.0542</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

This table shows correlation coefficient between alpha-feto protein and different variables among group I and it shows significance with creatinine, AST, ALT, ALP.
Table (12) Correlation coefficient (r) between AFP and different variables among group II

<table>
<thead>
<tr>
<th>Variables</th>
<th>FP</th>
<th>(r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.35502</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>0.1344</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0.08994</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>0.0466</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>0.53288</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>0.46822</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>0.3731</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>0.08044</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
<tr>
<td>ALB</td>
<td>0.03513</td>
<td>&gt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

This table shows correlation coefficient between alpha-feto protein and different variables among group II and it shows highly significance with ALT, AST and significance in creatinine, ALP.
Table (13) correlation coefficient ($r$) between AFP and different variables among control

<table>
<thead>
<tr>
<th>Variables</th>
<th>(r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.13146</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.01075</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0.015211</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>0.09962</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>AST</td>
<td>0.29776</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ALT</td>
<td>0.23094</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ALP</td>
<td>0.20524</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>PT</td>
<td>0.16635</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ALB</td>
<td>0.1003</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

This table shows correlation coefficient between alpha-feto protein and different variables among control and it shows non significance.
Table (14) correlation coefficient (r) between MMP2 and different variables among group I

<table>
<thead>
<tr>
<th>Variables</th>
<th>(r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.3587</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.2662</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0.3372</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>0.3831</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>AST</td>
<td>0.3355</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ALT</td>
<td>0.33979</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ALP</td>
<td>0.3386</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>PT</td>
<td>0.3517</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ALB</td>
<td>-0.3399</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>αFP</td>
<td>0.0919</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

This table shows correlation coefficient between Matrix Metalloproteinase-2 and different variables among group I and it shows significance (positive with creatinine, AST, ALT, ALP, PT, Bilirubin “Total- Direct”) and negative with Albumin and non significance with AFP and Total protein.
Table (15) correlation coefficient (r) between MMP2 and different variables among group II

<table>
<thead>
<tr>
<th>Variables</th>
<th>(r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.33387</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.1218</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0.3532</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>0.3991</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>AST</td>
<td>0.37868</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ALT</td>
<td>0.3654</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ALP</td>
<td>0.35544</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>PT</td>
<td>0.37261</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ALB</td>
<td>-0.3408</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>αFP</td>
<td>0.21236</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

This table shows correlation coefficient between Matrix Metalloproteinase-2 and different variables among group II and it shows significance (positive with ALT, AST, creatinine, Bilirubin (Total-Direct), ALP, PT and negative with Albumin and non significance with AFP and Total protein.
Table (16) correlation coefficient (r) between MMP2 and different variables among control

<table>
<thead>
<tr>
<th>Variables</th>
<th>(r )</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>0.07403</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.08395</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>0.07987</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>0.04137</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>AST</td>
<td>0.02437</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ALT</td>
<td>0.22665</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ALP</td>
<td>0.11708</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>PT</td>
<td>0.04784</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ALB</td>
<td>0.05267</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>α FP</td>
<td>0.20257</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

This table shows correlation coefficient between Matrix Metalloproteinase-2 and different variables among control and it shows non-significance.
Table (17): Distribution of MMP2 between different classes among group I

<table>
<thead>
<tr>
<th>St. group</th>
<th>MMP2 X ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 5)</td>
<td>464.6 ± 205.8</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>B (n = 7)</td>
<td>496.8 ± 208.6</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>C (n = 13)</td>
<td>568.02 ± 202.4</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

F = 0.57 \quad P > 0.005

This table shows distribution of MMP2 between different classes according to Child-Pugh classification and it shows higher levels in class C than class B than class A among group I.
Table (18): Distribution of MMP2 between different classes among group II

<table>
<thead>
<tr>
<th>St. group</th>
<th>MMP2</th>
<th>X ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 8)</td>
<td>460.9</td>
<td>± 270.6</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>B (n = 5)</td>
<td>572.05</td>
<td>± 144.93</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>C (n = 12)</td>
<td>600.1</td>
<td>± 335.1</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

F = 0.58    P > 0.05

This table shows distribution of MMP2 between different classes according to Child-Pugh classification and it shows higher levels in class C than class B than class A among group II.
Results

Chart (1) Means of Total bilirubin of different study groups

Chart (2) Means of Direct bilirubin of different study groups
Results

Chart (3) Means of AST of different study groups

Chart (4) Means of ALT of different study groups
Results

Chart (5) Means of ALP of different study groups

Chart (6) Means of ALB of different study groups
Results

Chart (7) Means of PT of different study groups

Chart (8) Means of alpha FP of different study groups
Results

Chart (9) Means of MMP2 of different study groups

Chart (10): Correlation coefficient (r) between MMP2 and different variables among group I
Results

Chart (11): Correlation coefficient (r) between MMP2 and different variables among group II

Chart (12): Correlation coefficient (r) between AFP and different variables among group I
Chart (13): Correlation coefficient (r) between AFP and different variables among group II

Chart (14): distribution of MMR2 between different classes among group I
Chart (15): distribution of MMR2 between different classes among group II
CONCLUSION

From the present study we can conclude that:

1- MMP-2 is secreted by hepatic stellate cells (HSCs) as proMMP-2 which is activated by membrane type-1 MMP (MT1-MMP) on the cell surface of both normal and tumor cells and in contrast to other proMMPs, proMMP-2 cannot be activated by serine proteinases such as plasmin or by catalytic quantities of other MMPs and inhibited by TIMP-2 by forming a complex with it and the binding of TIMP-2 to the carboxyterminus of pro MMP-2 prevents the activation and stabilizes pro MMP-2, so MMP-2 theoretically may exist in serum as a free pro MMP-2, a pro MMP-2 complexed with TIMP-2, a free active MMP-2 and an active MMP-2 complexed with TIMP-2 but the majority are pro MMP-2.

2- Serum levels of MMP-2 were highly significantly elevated in patients with chronic hepatitis C (group I) and in patients with hepatocellular carcinoma (group II) than the controls with no significant statistical difference between both patient groups.

3- Serum levels of alpha-feto protein were highly significantly elevated in patients with hepatocellular carcinoma (group II) than in patients with chronic hepatitis-C (group I) than the controls.

4- Serum levels of MMP-2 correlated significantly with the severity of liver disease as it showed a significant elevation in child C class than child B class than child A class, according to Child-Pugh classification.

5- Serum MMP-2 is considered to be derived from the cirrhotic liver in patients with chronic viral liver disease and correlated with the degree and staging of fibrosis, so the measurement of serum MMP-
2 is the best test for diagnosing liver cirrhosis in patients with chronic viral liver disease.

6- Serum MMP-2 levels had a strong correlation with serum markers of liver function (positive for Total bilirubin, Direct bilirubin, Aspartate transaminase, Alanine transaminase, Alkaline phosphatase and Prothrombin time and negative for albumin) in both patient groups (group I and II) while serum αFP levels had a strong correlation with some of serum markers of liver function (AST, ALT and ALP) in both patient groups.

7- No correlation could be detected between MMP-2 and alpha-feto protein, tumor size or tumor differentiation in both patient groups, so high serum levels of MMP-2 in patients with HCC may result from the non tumorous part of the liver rather than from the carcinoma.

8- The MMP-2 levels in an individual patient cannot be used as a marker for liver function because of a wide overlap in levels between the different child-Pugh classes but could be used as a substitute for liver biopsy to assess the extent of liver fibrosis and fibrogenesis especially when liver biopsy is contraindicated.

9- Due to similar levels in patients with HCC (group II) and patients with chronic hepatitis-C (group I) and due to absence of significant statistical difference in serum MMP-2 levels between both patient groups and due to absence of correlation between MMP-2 serum levels and α FP serum levels in both patient groups, serum levels of MMP-2 cannot be used as a diagnostic marker for HCC in the context of chronic liver diseases.
RECOMMENDATION

From the results of the present study measurement of serum MMP-2 is the best test for diagnosing liver cirrhosis in patients with chronic viral liver diseases and also can be used as a substitute for liver biopsy to assess the extent of liver fibrosis and fibrogenesis especially when liver biopsy is contraindicated, In further study it is recommended to study a larger number of patients with variable etiologies of chronic hepatitis-C and larger number of patients with hepatocellular carcinoma to clarify the role of MMP-2 in these patients especially with HCC and the role of the imbalance of MMP-2/ TIMP-2 in HCC.
SUMMARY

Conventional biochemical and serological tests are of little value in the diagnosis of the liver cirrhosis and the degree of fibrosis and the activity of fibrogenesis and the presence of hepatocellular carcinoma so percutaneous liver biopsy is used to assess the extent of liver fibrosis and fibrogenesis and the presence of hepato-cellular carcinoma but a liver biopsy is sometimes of questionable value because of the heterogenous distribution of pathological changes in the liver, so non invasive biochemical markers for assessing liver cirrhosis in chronic hepatitis and for detecting malignancy in hepato-cellular carcinoma are being actively sought to help evaluating the histologic damage and monitor the progression of fibrosis

Matrix metalloproteinases are key enzymes in the regulation of the cell matrix composition and in the metabolism of collagen and in the physiopathology of fibrosis by their ability to cleave and degrade one or several ECM constituents as well as non-matrix proteins and their activity is regulated by interaction with a group of TIMPs (tissue inhibitor of metallo-proteinases).

Gelatinases (type IV collagenases) may be especially important for the development of organ fibrosis because they degrade type IV (basement membrane) collagen and thus are involved in the early steps of tissue remodeling that characterizes chronic viral liver diseases, also may be especially important in invasion and metastasis of malignant tumors because they destroy the surrounding extra-cellular matrix (Arthur, 2000)
There are two types of gelatinases which are MMP-2 and MMP-9 and studies have shown that serum levels of MMP-2 are increased in chronic liver disease so the aim of this study is to assess the clinical significance of MMP-2 as a diagnostic marker in both chronic hepatitis-C and hepatocellular carcinoma and if there’s any difference between both conditions and to evaluate if there’s any correlation between MMP-2 and liver functions (namely, Bilirubin, Albumin and Prothrombin time) and also with the severity of liver disease, this study was conducted on 50 patients, they were 32 males and 18 females their ages ranged between 30 years and 80 years and all cases were selected from the Internal Medicine Department of Benha University Hospital in addition to 25 healthy individuals of matched age and sex served as a control group.

**The studied cases included the following groups:**

Group I: Included 25 hepatic patients with positive HCV comprised 14 males and 11 females with their mean age (53.12 ± 10.5 years) ± SD.

Group II: Included 25 hepatic patients with positive HCV and with hepatocellular carcinoma comprised 18 males and 7 females with their mean age (55.64 ± 10.5 years) ± SD.

Group III (control group): Included 25 apparently healthy individuals comprised 8 males and 17 females with their mean age (50.04 ± 10.5 years) ± SD.

**All patients and controls were subjected to:**

1- Full history taking of present illness.
2- Thorough clinical examination.
3- Abdominal ultrasonography to study the size and pattern of liver and spleen and presence or absence of ascites.
4- Routine laboratory investigations:
Summary

a) Complete blood picture.
b) Urine and stool analysis.
c) Serum creatinine.
d) Liver function tests: (Total protein, Serum Albumin, Bilirubin (Total and Direct), ALT, AST, Alkaline phosphatase and prothrombin time).
e) Serological test:
Hepatitis-C virus marker determined by Enzyme Linked immunosorbant Assay (ELISA) and Polymerase Chain Reaction (PCR).
f) Specific laboratory investigations included:
- Alpha fetoprotein (α FP).
- Matrix metalloproteinase-2 (MMP-2).

The present study revealed that:

1- Serum levels of MMP-2 were highly significantly elevated in patients with chronic hepatitis-C (group I) and in patients with HCC (group II) than the controls with no significant statistical difference between both patient groups.

2- Serum levels of MMP-2 correlated significantly with the severity of liver disease as it showed a significant elevation in child C class than child B class than child A class.

3- Serum MMP-2 is considered to be derived from the cirrhotic liver in patients with chronic viral liver disease and correlated with the degree and staging of fibrosis, so the measurement of serum MMP-2 is the best test for diagnosing liver cirrhosis in patients with chronic viral liver disease.

4- Serum MMP-2 levels had a strong correlation with serum markers of liver function (positive for Bilirubin, AST, ALT, ALP and PT
Summary

and negative for albumin) in both patient groups (group I and group II).

5- The MMP-2 levels in an individual patient cannot be used as a marker for liver function because of a wide overlap in levels between the different child-Pugh classes but could be used as a substitute for liver biopsy to assess the extent of liver fibrosis and fibrogenesis especially when liver biopsy is contraindicated.

6- High serum levels of MMP-2 in patients with HCC may result form the non tumorous part of the liver rather than from the carcinoma and no relation between MMP2 and tumor size or tumor differentiation so it cannot be used as a diagnostic marker for HCC in the context of chronic liver diseases.
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المختصر العربي

إن الاختبارات الكيميائية والمصلية التقليدية ذات أهمية قليلة في تشخيص التليف الكبدى ودرجة التليف وكذلك النشاط المؤدي لتكوين الألياف وجود سرطان الكبد من عدمه لذا فإن أخذ عينة من الكبد وتحليلها بالطريقة ضروري في تحديد مدى التليف الكبدى وكذلك نشاطه ولتشخيص سرطان الكبد ولكن أخذ عينة من الكبد يكون أيضاً مشكلاً فيه وذلك نظراً للتوزيع الغير متساوي للتحولات الباثولوجية في الكبد مما يؤدي إلى قلة كفاءة العينة الباثولوجية في تحديد المرض بدقة وبناءً على ذلك كان من الضروري البحث عن دلالات كيميائية أخرى غير نافعة لتقديم درجة التليف الكبدى في مرض الالتهابات الكبدية المزمنة وكذلك اكتشاف حدوث أورام سرطانية في الكبد ومن ثم المساعدة في تقييم أفضل للمضух ومتابعة تقدم التليف الكبدى.

النيتروتييناز هي الإتيزيمات الرئيسية في تنظيم مكونات النسيج الكبدى خصوصاً وكذلك في تنظيم عمليات الأيض لمادة الكولاجن والطبوعة الباثولوجية للتليف وذلك بقدرها على شق وإحلال مكونات النسيج الكبدى خلوى بالإضافة إلى الروتينيات الأخرى بين الخلايا وهذه المقدرة يتم تنظيمها بالتفاعل مع الإتيزيمات المثبتة للميتالبروتيناز.

تحتوي عائلة الميتالبروتيناز على العديد من الإتيزيمات من أهمهم إنزيم الجيلاتيناز الذي له أهمية في حدوث تليفات الأعضاء حيث أنه يمثل القدرة على إحلال النوع الرابع لمادة الكولاجن وبالتالي يكون له أهمية في الخطوات الأولى المسؤولة عن التغيرات الحادة في بناء الأنسجة التي هي مميزة لالتهابات الكبد الفيروسية المزمنة وكذلك في الإجهاض والانتشار الخاص بالأورام السرطانية وذلك بقدرته على تدمير النسيج الكبدى خلوى.

هناك نوعان أساسيان لإتيزيم الجيلاتيناز وهما (النيتروتييناز-2 والميتالبروتيناز-4) وقد أظهرت الدراسات أن مستوى الميتالبروتيناز-2 في الدم يزداد في حالات أمراض الكبد المزمنة ولذلك تهدف هذه الدراسة إلى تقييم الدالالة الإكلينيكية لإتيزيم الميتالبروتيناز-2 كبنية تشخيصي في كلاً من حالات التليف الكبدى المزمن وسرطان الكبد وذلك تهدف هذه الدراسة إلى تحديد مدى وجود علاقة بين مستوى إنزيم الميتالبروتيناز-2 في الدم ومدى تأثر وظائف خلايا الكبد (خاصة نسبة الصفرا والألبومين بالدم وزمن البروثرمدين) وكذلك مع درجة شدة المرض.
ركزت هذه الدراسة على خمسين مريضاً ما بين اثنين وثلاثين رجلاً وثمانية عشرة أثناً تراوح العمر بين سن الثلاثين وسن الخامسة عشرة، وقد تم اختيارهم جميعاً من قسم الباطنة العامة في المستشفى الجامعي بالإضافة إلى خمسة وعشرين ظلناء كانت أعمارهم ونسنهم مماثلة في المرضى ممثلة في دراسة. الهدف من هذه الدراسة هو مقارنتهم بمجموعة المرضى محل الدراسة.

حالات الدراسة تضمنت هذه المجموعات:

المجموعة الأولى: اشتملت على خمسة وعشرين مريضاً بالالتهاب الكبدى الفيروسى-ج يشكلون أربعة عشر رجلاً واحد عشرة أثناً ووسط أعمارهم (12.5 ± 1.0 سنة).
المجموعة الثانية: اشتملت على خمسة وعشرين مريضاً بالالتهاب الكبدى الفيروسى-ج وأيضاً بسرطان الكبد يشكلون ثمانية عشر رجلاً وسبعة إناث ووسط أعمارهم (45.5 ± 6.0 سنة).
المجموعة الثالثة: اشتملت على خمسة وعشرين أصحاء يشكلون ثمانية رجلاً وسبعة عشر أثناً ووسط أعمارهم (45.0 ± 6.0 سنة).

كل المرضى والأصحاء خضعوا للاختبارات التالية:

1- إدراك تاريخ المرض بالكامل.
2- فحص إكلينيكي كامل.
3- موجات فوق الصوتية على منطقة البطن لدراسة حجم وشكل الكبد والبطال.
4- تحاليل معملية روتينية.
   أ- صورة دم كاملة.
   ب- تحليل بول وبراز.
   ج- نسبة الكرياتينين بالدم.
   د- وظائف الكبد كاملاً (بروتين كلي ونسبة الألبومين ونسبة الصفراء (كلي – مباشر) والألاتين وراتازينى واسبنتات وراتازينى والألكالين فوسفاتاز وزمن البروثرمين).
الملخص العربي

- تحاليل مصلية: دلالات الالتهاب الكبدي الفيروسي-J وذلك باستخدام الـ ELISA وال PCR

- تحاليل معملية خاصة اشتملت على :
  - الألفا فيتوبروتين.
  - الميتاللوبروتيناز-2.

وبعد التحليل الإحصائي لنتائج الفحص الطبي والتحليل الطبي اتضح الآتي:

1- أن مستوى إنزيم الميتاللوبروتيناز-2 في الدم لدى المرضى المصابين بالالتهاب الكبدى الفيروسي-J العالى (المجموعة الأولى) ولدى المرضى المصابين بسرطان الكبد (المجموعة الثانية) كان مرتفعاً بدرجة مميزة إحصائياً مقارنة بالأشخاص الأصحاء مع عدم وجود فروقات إحصائية بين كلاً من مجموعتي المرض.

2- يناسب مستوى إنزيم الميتاللوبروتيناز-2 في الدم لمنشأ طردياً مع شدة المرض كما هو مبين بالانقاص الملحوظ لله في مرضي التصنيف-J أكثر من مرضي التصنيف-B أكثر من مرضي التصنيف-A.

3- يزيد مستوى إنزيم الميتاللوبروتيناز-2 في الدم كلما زادت درجة التليف الكبدى ولذلك يمكن اعتباره كأفضل اختبار لتشخيص حدوث التليف الكبدى في مرضى الالتهابات الكبدية الفيروسية المزمنة.

4- يناسب مستوى إنزيم الميتاللوبروتيناز-2 في الدم لمنشأ طردياً مع مدى ودرجة وظائف الكبد المختلفة (إيجابياً مع نسبة الصرفاء في الدم وانزيمات الكبد وسلبية مع نسبة الألبومين في الدم) في كلاً من مجموعتي المرضى (المجموعة الأولى والمجموعة الثانية).

5- يمكن أن نستنتج من هذه الدراسة أن مستوى إنزيم الميتاللوبروتيناز-2 في الدم يمكن استخدامه كبديل لأخذ عينة باتولوجية من الكبد لتقييم مدى التليف الكبدى خاصة عندما يكون أخذ عينة من الكبد ممنوعاً ولكن لا يمكن استخدامه كبديل لمدى تأثر وظائف الكبد في المريض الفردى.
- يمكن أن نستنتج أيضاً من هذه الدراسة أن مستوى إنزيم الميتايلوبروتيناز-2 في الدم لا يمكن استخدامه كمَّثيل تشخيصي لسرطان الكبد حيث أنه لا توجد أي علاقة بينه وبين حجم الورم أو الأشكال المختلفة للورم كما أنه يمكن أن يكون ناتج من الجزء الخالى من الورم في الكبد.
الدلالات الإكلينيكية للميتالوبروتيناز-2
في مصل مرضى التهاب الكبد المزمن سي
ومرضى سرطان الكبد

دراسة
توطنة للحصول على درجة الماجستير
في أمراض الباطنة العامة

مقدمة من
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