UNIVERSITY OF CRETE
SCHOOL OF MEDICINE

# EXPRESSION OF MATRIX METALLOPROTEINASES IN CHRONIC LIVER DISEASES

**Ph.D.**

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HERAKLION, 2004

**Acknowledgements**

I greatly thank Greek State Scholarship Foundation (IKY) for funding my studies and this research work.

This PhD thesis was held in the Liver Research Laboratory at the University of Crete School of Medicine under the supervision of Prof. Elias Kouroumalis.

For the realization of this work I greatly thank those who generously provided their help, in particular:

I thank my professor and adviser Prof. Elias Kouroumalis for the trust he gave me for carrying this research work, for his patience and goodwill to overcome any circumstances for the benefit of this work. I thank him for advising and directing me towards the successful fulfillment of my research work.

I thank Dr. Dimitris Arvanitis for his assistance and guidance in mastering the techniques used in this research.

I thank Associate Prof. Ioannis Diamantis, my adviser in the practical part of this scientific work for his ideas, leadership and suggestions for the benefit of this project and my scientific career.

I thank Thanasis Alegakis, my colleague and friend for his patient assistance in carrying out the statistical part of the work and for his helpful advice.

I greatly thank my English teachers Mrs.Vera Ladopoulou, Mrs. Natalie Ventura and Mrs. Kiki Divini for their efficient help in correcting this work and the moral support that they generously offered for the fulfillment of this work.

I thank Mrs. Hara Tomara, Mr. Dimitris Kounalakis and Mr. Vasilis Laios from the Physics Department for the free PC access without which this work would not be possible.

*Mojoj  porodici: Slavki i Radoslavu Ljumovic,*

*Nataliji,Milosu i Katarini*

*za pruzenu  ljubav, strpljenje i podrsku*

*(To my family: Slavka and Radoslav Ljumovic*

*Natalija,Milos and Katarina*

*for their love, patience and support)*

**CONTENTS**

## THESIS SUMMARY (ENGLISH VERSION)

### EXPRESSION OF MATRIX METALLOPROTEINASES IN CHRONIC LIVER DISEASES

Chronic liver injury occurs in response to a variety of causes, including viral hepatitis B and C. During liver fibrosis the pathological accumulation of extracellular matrix occurs as a consequence of the modifications that follow the synthesis and degradation of matrix proteins. The exact mechanism, by which specific matrix-degrading enzymes act during ECM remodelling, is not clear. Evidence of the involvement of MMPs, the most important family of ECM-degradative enzymes, in various liver diseases exists in a number of studies.

The majority of the findings regarding matrix metalloproteinase (MMP) expression in hepatitis B and C infected patients indicate the fluctuations of MMP's RNA, DNA or active enzyme levels in relation to the viral presence and chronic infections. However, better understanding is crucial for the management of these diseases since there are still many points to be answered. No study has attempted to study matrix metalloproteinase expression in non-alcoholic steatohepatitis (NASH).

To achieve more information about fibroproliferation in hepatitis B, C and NASH patients, the expression of MMPs in these diseases was studied checking the hypothesis that different MMPs might be involved in different liver diseases during the process of fibrogenesis.

For that purpose, we assessed the expression levels of MMPs by reverse transcription polymerase chain (RT-PCR) reaction and examined the presence of MMP-2, -9, -10, -11 total RNAs in the tissues from patients with viral and non viral hepatitis.73 patients were examined in this study: non-diseased controls (10), patients with chronic hepatitis B (14), chronic hepatitis C (33) and non-alcoholic steatohepatitis (16). All patients studied consumed no alcohol. Ten liver samples were taken at operation for cholecystectomy and used as healthy controls. Total RNA was isolated after immersion of the liver biopsies in liquid nitrogen and it was reversely transcribed by cDNA synthesis. Consecutive amplification of cDNA was performed by PCR and products were electrophorised through an 8% polyacrylamide gel and silver stained.

PCR products were semi-quantitatively analyzed by densitometric analysis using β-actin or β2-microglobulin as the house keeping gene. The RNA levels of each gene were expressed as a ratio of the intensity of the bands in diseased tissues versus the corresponding levels of the internal control. Non-parametric tests (Kruskal-Wallis and Mann-Whitney) were performed to examine differences on metalloproteinase expression amongst diseases and controls.

When we grouped together the expression values for metalloproteinases from HCV and HBV viral hepatitis patients and compared it with non-viral patients a statistically significant increase in levels of MMP-9 and MMP-10 in NASH ($P<0.05$) was obtained. In our studies in viral diseases a statistically significant increase in MMP-2 expression ($P<0.05$) was observed when compared to control values. We observed statistically significant increases in MMP-9 expression values in NASH when compared to controls ($P<0.05$).

We examined expression patterns of metalloproteinases in different histological stages of chronic hepatitis due to HBV or HCV viruses. In HBV cases, MMP-9 expression showed a statistically significant decrease ($P<0.05$) in the final stages of the viral infection.

When we investigated HCV related liver disease, a stronger presence of MMP-2 and MMP-9 in final disease stages was detected. MMP-10 and MMP-11 followed a decreasing pattern during disease development. Statistical significance was obtained only for the MMP-2 expression ($P<0.05$) at the final stages of HCV.

Few studies have assessed the expression of metalloproteinase network in chronic hepatitis. No study has attempted to study in detail their expression in non-alcoholic steatohepatitis. In the present results in non-alcoholic steatohepatitis we showed a significant increase in the expression of MMP-9 and MMP-10, when compared to viral hepatitis B and C. Previous reports on MMP-9 expression in the human liver have demonstrated an increase of MMP-9 gelatinolytic activity in hepatic tissues from patients with viral and alcoholic cirrhosis and an early increase in chronic viral

hepatitis C during all fibrotic stages including cirrhosis. We identified similar increase in the expression of MMP-9 when comparisons were made with normal livers.

In our viral hepatitis patients we identified MMP-10 expression with an increasing pattern in advanced fibrosis of HBV cases and a decreasing pattern in advanced HCV fibrosis when compared to the early fibrosis. The reason for this discrepancy is not clear. When HCV and HBV related chronic hepatitis were grouped together and compared to the controls, a significant increase in the MMP-2 expression was observed. Results from experimental animal and human studies agree with our findings.

In the present study, when the patients were analysed according to the viral aetiology and disease staging using the Ishak's system in the study of late stage HCV we obtained results which showed a different profile, with values of MMP-9 relatively increased. This increase did not reach statistical significance. Our findings in HCV agree with the findings of Lichtinghagen et al. [2003] who also observed an increase in MMP-9 mRNA expression in patients with advanced HCV fibrotic stages.

Gelatinases are the enzymes mostly responsible for collagen type IV degradation. We suggest that their increase might be a compensatory mechanism occurring as a result of the over accumulation of type IV collagen previously reported in fibrotic livers.

Our results indicate that apart from fibrotic stages, the viral aetiology should also be taken into account when MMP expression is examined. In addition to these findings our results on MMPs expression in NASH indicate that a different pattern emerges in this non-viral potentially fibrotic liver disease. This may be of importance when looking for diagnostic targets and therapy in the case of hepatic fibrosis. Further studies are warranted in order to elucidate the expression of the other MMPs and their role in hepatic diseases.

**THESIS SUMMARY (GREEK VERSION)**

Έκφραση των μεταλλοπρωτεασσών στις χρόνιες ηπατοπάθειες

Η χρόνια ηπατοπάθεια μπορεί να έχει πολλές αιτίες και κάποιες από αυτές είναι η ηπατίτιδα Β ή C. Στην ηπατική ίνωση η παθολογική αύξηση του εξωκυττάριου υποστρώματος οφείλεται στις αλλαγές που συμβαίνουν κατά την διάρκεια της σύνθεσης και αποικοδόμησης των πρωτεϊνών του υποστρώματος. Ο ακριβής μηχανισμός σύμφωνα με τον οποίο δρουν τα συγκεκριμένα αποικοδομητικά ένζυμα κατά την διάρκεια της ανάπλασης του εξωκυττάριου υποστρώματος δεν είναι γνωστός. Μελέτες που έχουν γίνει σε διάφορες ασθένειες του ήπατος επιβεβαιώνουν την εμπλοκή των MMPs, της πιο σημαντικής οικογένειας των αποικοδομητικών ενζύμων του εξωκυττάριου υποστρώματος.

Η πλειονότητα των ευρημάτων που αφορούν την έκφραση των MMPs σε ασθενείς με ηπατίτιδα Β και C δείχνει διακυμάνσεις στα επίπεδα DNA, RNA και ενεργού ενζύμου σε σχέση με την παρουσία ιού και χρόνιας λοίμωξης. Αυτό βοηθά στην καλύτερη κατανόηση και διαχείριση των ασθενειών αυτών. Δεν υπάρχει μελέτη που να εξετάζει την έκφραση των μεταλλοπρωτεασών στην στεατοηπατίτιδα μη αλκοολικής αιτιολογίας.

Για να πάρουμε περισσότερες πληροφορίες για την παθολογία της ηπατικής ίνωσης στις ηπατίτιδες Β, C και NASH μελετήσαμε την έκφραση των MMP στα νοσήματα αυτά.

Εξετάσαμε τα επίπεδα έκφρασης των MMP με RT-PCR σε επίπεδο RNAσε ιστούς ασθενών με ιογενή και μη ιογενή ηπατίτιδα. Οι περιπτώσεις 73 ασθενών ερευνήθηκαν στην παρούσα μελέτη. 10 από αυτούς ήταν οι υγιείς μάρτυρες, 10 ήταν ασθενείς με χρόνια ηπατίτιδα Β 33 έπασχαν από χρόνια ηπατίτιδα C και 16 υπέφεραν από μη αλκοολική στεατοηπατίτιδα. Κανένας από τους ασθενείς της μελέτης δεν κατανάλωνε αλκοόλ. Τα δέκα φυσιολογικά δείγματα ήπατος πάρθηκαν κατά τη διάρκεια αφαίρεσης χολής και χρησιμοποιήθηκαν σαν μάρτυρες. Το ολικό RNA απομονώθηκε μετά από εμβάπτιση των βιοψιών ήπατος σε υγρό άζωτο και χρησιμοποιήηκε ως μήτρα σε αντίδραση αντίστροφης μεταγραφής για την παρασκευή

cDNA. Εν συνεχεία το cDNA υπέστη αλυσιδωτή αντίδραση πολυμεράσης (PCR) και τα προϊόντα ηλεκροφορήθηκαν σε πήκτωμα πολυακρυλαμιδίου 8%.Μετά από χρώση νιτρικού αργύρου οι λαμβανόμενες ζώνες ημιποσοτικά με ψηφιακή απεικόνιση σε σχέση με τη β-ακτίνη/β-μικροσφαιρίνη.

Τα επίπεδα RNA του κάθε γονιδίου εκφράστηκαν ως ο λόγος της έντασης των ζωνών από τους ασθενείς ιστούς προς την ένταση των ζωνών των μαρτύρων. Μη παραμετρικές στατιστικές δοκιμασίες (Kruskal-Wallis, Mann-Whitney) χρησιμοποιήθηκαν για να εξεταστούν οι διαφορές στην έκφραση των MMPs ανάμεσα στις διάφορες ασθένειες και τους μάρτυρες.

Όταν ομαδοποιήθηκαν τα αποτελέσματα για τους ασθενείς με HCV HBV και συγκρίθηκαν με αυτά από ασθενείς με μη ιογενείς ηπατίτιδες, παρατηρήθηκε μια στατιστικά σημαντική αύξηση στην έκφραση των MMP-9, MMP-10 ($P<0.05$) σε περιπτώσεις NASH. Επίσης παρατηρήθηκε μια στατιστικά σημαντική αύξηση της MMP-2 ($P<0.05$) στις περιπτώσεις ιογενών ηπατιτίδων συγκρινόμενες με τους μάρτυρες ($P<0.05$).

Εξετάσαμε τα μοτίβα έκφρασης των MMP σε διάφορα ιστολογικά στάδια χρόνιας ιογενούς Β και C ηπατίτιδας. Στις Β περιπτώσεις παρατηρήθηκε μια σημαντική μείωση ($P<0.05$) στην έκφραση της MMP-9 στα τελικά στάδια της ιογενούς λοίμωξης.

Σε περιπτώσεις ιογενούς ηπατίτιδας C παρατηρήθηκε μια αύξηση των MMP-2 και MMP-9 στα τελικά στάδια της ασθένειας. Αντίθετα η έκφραση των MMP-10 και MMP-11 μειωνόταν κατά την ανάπτυξη της ασθένειας. Στατιστικά σημαντικά αποτελέσματα ($P<0.05$) πήραμε μόνο για την έκφραση της MMP-2 στα τελικά στάδια της ιογενούς ηπατίτιδας C.

Μόνο λίγες μελέτες έχουν αξιολογήσει την έκφραση του δικτύου των MMP σε περιπτώσεις χρόνιας ηπατίτιδας. Καμία μελέτη δεν έχει ασχοληθεί με την έκφραση τους σε περιπτώσεις με μη αλκοολική στεατοηπατίτιδα. Στην παρούσα μελέτη ανακαλύφθηκε μια στατιστικά σημαντική αύξηση στην έκφραση των MMP-9 και MMP-10 συγκρινόμενη με περιπτώσεις ιογενούς ηπατίτιδας Β και C. Παλιότερες

μελέτες που αφορούν την έκφραση της MMP-9 στο ανθρώπινο ήπαρ, δείχνουν μια αύξηση στην λυτική δραστηριότητα σε ηπατικούς ιστούς ασθενών με ιογενή και αλκοολική κίρρωση. Επίσης μια αρχική αύξηση σε περιπτώσεις χρόνιας ιογενούς ηπατίτιδας C, κατά τη διάρκεια όλων των ινωτικών σταδίων συμπεριλαμβανομένης και της κίρρωσης. Παρόμοια αύξηση στην έκφραση της MMP-9 εντοπίστηκε σε συγκρίσεις με φυσιολογικούς ιστούς και στην παρούσα μελέτη.

Επίσης, μελετώντας την έκφραση της MMP-10 σε ασθενείς με ιογενείς ηπατίτιδες, εντοπίσαμε μια αυξητική τάση σε προχωρημένα στάδια ίνωσης εξαιτίας της HBV και μια τάση μείωσης σε προχωρημένα στάδια HCV ίνωσης συγκρινόμενα με τα αρχικά στάδια ίνωσης. Ο λόγος για αυτήν την παρατήρηση δεν είναι γνωστός. Όταν και οι δυο ομάδες των B και C ομαδοποιήθηκαν και συγκρίθηκαν με τους μάρτυρες, παρατηρήθηκε μια στατιστικά σημαντική αύξηση στην έκφραση της MMP-2. Τα αποτελέσματα προηγούμενων μελετών με πειραματόζωα και ασθενείς συμφωνούν με τα δικά μας ευρήματα.

Στην παρούσα μελέτη όταν οι ασθενείς αναλύθηκαν σύμφωνα με το σύστημα του Ishak, σε περιπτώσεις προχωρημένης HCV πήραμε αποτελέσματα με διαφορετικές τάσεις. Συγκεκριμένα οι τιμές της MMP-9 έδειξαν αυξητικές τάσεις. Αυτές οι τάσεις δεν ήταν στατιστικά σημαντικές σε ασθενείς με χρόνια ιογενή ηπατίτιδα. Τα αποτελέσματα μας συμφωνούν με αυτά της ομάδας του Lichtinghagen [Clin Sci 2003].

Οι γελατινάσες είναι ένζυμα υπεύθυνα για την αποικοδόμηση του κολλαγόνου τύπου IV. Φαίνεται ότι η αύξηση τους συμβαίνει στα πλαίσια ενός αντισταθμιστικού μηχανισμού της υπερσυγκέντρωσης κολλαγόνου τύπου IV σε ηπατικούς ιστούς με ίνωση.

Τα αποτελέσματα μας δείχνουν ότι εκτός του σταδίου της ίνωσης του ήπατος, εκείνο που πρέπει να λαμβάνεται οπωσδήποτε υπ' όψιν κατά τη μελέτη των MMPs στο ήπαρ, είναι η αιτιολογία της νόσου και μάλιστα η συγκεκριμένη ιογενής αιτιολογία. Τα ευρήματα μας στη NASH δείχνουν ότι στις μη ιογενείς, ενέχονται πιθανόν διαφορετικές μεταλλοπρωτεϊνάσες στην διαδικασία της ίνωσης. Αυτό μπορεί να αποδειχτεί σημαντικό κατά την ανίχνευση διαγνωστικών και θεραπευτικών στόχων.

Είναι προφανές ότι απαιτούνται εκτενέστερες μελέτες για την έκφραση και άλλων μεταλλοπρωτεϊνασών και το ρόλο τους στις ηπατοπάθειες.

# CHAPTER 1

# Introduction

# SECTION 1

## 1. INTRODUCTION

### 1.1. MATRIX METALLOPROTEINASES

The metalloproteinases (MMPs) form a family of structurally related endopeptidases capable of degrading specific components of the extracellular matrix and basement membranes [Nagase and Woessner 1999]. Remodeling of the extracellular matrix is a crucial event in physiological processes, and matrix metalloproteinases are believed to participate in these events. MMPs play an important role in the processes of tissue morphogenesis [Cawston 1995], differentiation, wound healing [Ravanti and Kahari 2000], embryonic development, post-partum involution of the uterus, ovulation [Murdoch et al. 1992], bone remodeling, angiogenesis [John and Tuszynski 2001] and nerve growth [Price et al. 2001].

Abnormal production of metalloproteinases may contribute to a number of pathological conditions such as tumor invasion, metastasis [Stetler-Stevenson et al 1993], artherosclerosis [Price et al. 2001], liver fibrosis [Benyon et al. 1996], joint destruction in rheumatoid and osteoarthritis [Cawston and Billington 1996], periodontitis [Woessner 1991], arthritis, cardiovascular diseases and cancer progression [Velasco et al. 2000, Theret et al. 1997]. Active participation of matrixins in various physiological and pathological processes reinforces the interest in understanding their regulation and expression.

#### A. CLASSIFICATION, STRUCTURE AND FUNCTION

At present, nineteen different human MMPs have been identified, cloned and characterized at the amino acid sequence level [Uria and Lopez-Otin 2000]. Based on cellular localisation mammalian metalloproteinases can be classified into two categories: soluble (secreted)-type and membrane-bound [Seiki 1999, Pei et al. 2000]. All members of the matrixins family are synthesized as pre-proenzymes. In most cases soluble-type MMPs are secreted as inactive pro-enzymes, while membrane-anchored MMPs are activated intracellularly and mostly expressed on the cell surface as active enzymes [Nagase and Woessner 1999].

According to the substrate specificity, structural and functional characteristics, soluble MMPs can be further classified into at least five different subfamilies: the collagenases, gelatinases, stromelysins, matrilysins, MT-MMPs, and other MMPs (Table 1) [Nabeshima et al. 2002]. Collagenases have the most specific substrate profile and they degrade the native forms of fibrillar collagens type I [Arthur 1990]. Gelatinases degrade type IV collagen, the major component of basement membranes, type V collagen, elastin and denatured collagen (gelatin) [Morgunova et al. 1999]. Stromelysins have broad substrate specificity and degrade proteoglycans, glycoproteins and have some activity on type IV collagen and elastin [Knittel et al. 1999]. Stromelysins are involved in the activation of procollagenase and progelatinase B.

The membrane-type metalloproteinases (MT-MMPs) include type I transmembrane MMPs, the glycosylphosphatidylinoditol (GPI)-anchored MMPs and type II transmembrane MMPs (Table 1) [Pei et al. 2000]. MT-MMPs contain a transmembrane domain and localize proteolysis to the cell surface through a ternary complex containing an activated MT-MMP, progelatinase-A and tissue inhibitor of matrix metalloproteinases 2 (TIMP-2) [Basbaum and Werb 1996]. MT-MMPs cleave fibronectin, tenascin, nidogen, aggrecan and perlecan [D'Ortho et al. 1997].

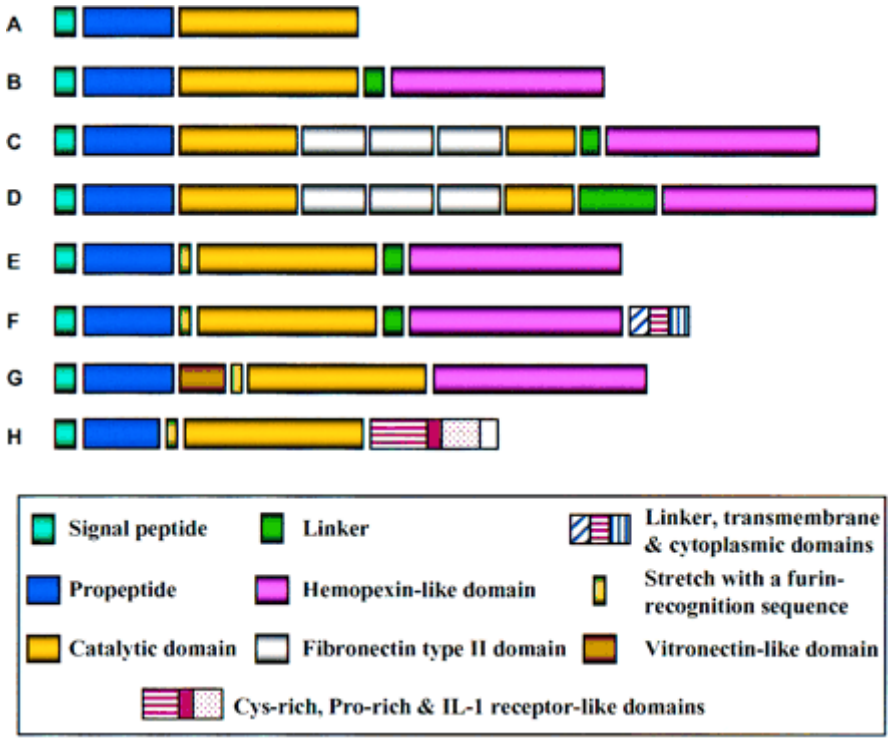


**Figure 1. Domain arrangements of vertebrate matrix metalloproteinases.**

The structure of most of the MMP family members is organized into four well-defined domains including a signal peptide to direct secretion from the cell, a propeptide with a conserved cysteine residue involved in maintaining enzyme latency; a catalytic domain with Zn-binding site; and a hemopexin-like domain at the COOH-terminal region [Nagase and Woessner 1999] (Figure 1).

**Table 1. The matrix metalloproteinase (MMP) gene family**

| Protein | MMP | MW (kDa) | Domain structure |
|---|---|---|---|
| Soluble-type MMP | | | |
| Collagenase | | | |
| Collagenase 1 | MMP-1 | 52/41 | C-I, II, III, X |
| Collagenase 2 | MMP-8 | 85/64 | C-I, II, III |
| Collagenase 3 | MMP-13 | 65/55 | C-I, II, IV, X, XIV; FN, aggrecan, tenascin |
| Stromelysin | | | |
| Stromelysin 1 | MMP-3 | 57/45, 28 | PG, FN, C-III, IV, VII, IX; gelatin, LN |
| Stromelysin 2 | MMP-10 | 56/47, 24 | C-III, IV, V; gelatin, PG, FN |
| Gelatinase | | | |
| Gelatinase A | MMP-2 | 72/67 | Gelatin, C-IV, V, VII, XI; FN, LN, elastin |
| Gelatinase B | MMP-9 | 92/67 | Gelatin, C-III, IV, V; ?2(I), elastin |
| Matrilysin | | | |
| Matrilysin 1 | MMP-7 | 28/19 | Gelatin, C-IV, FN, PG, LN |
| Matrilysin 2 | MMP-26 | 29/19 | Gelatin, C-IV, FN, fibrinogen |
| Others | | | |
| Stromelysin 3 | MMP-11 | 58/28 | Gelatin, PG, laminin, FN |
| Epilysin | MMP-28 | 56/45 | Casein |
| No trivial name | MMP-19 | 57 | Gelatin, aggrecan, COMP, LN, nidogen, tenascin C-IV, FN |
| Metalloelastase | MMP-12 | 54/45, 22 | Elastin |
| Enamelysin | MMP-20 | 54/43 | Amelogenin, aggrecan, COMP, FN, C-IV, LN |
| Membrane-anchored MMP | | | |
| Type I transmembrane-type | | | |
| MT1-MMP | MMP-14 | 66/60 | C-I, II, III; gelatin, PG, FN |
| MT2-MMP | MMP-15 | 68/62 | FN, aggrecan, nidogen, tenascin, perlecan, LN |
| MT3-MMP | MMP-16 | 64/55 | C-III, FN, gelatin |
| MT5-MMP | MMP-24 | 73/64 | PG |
| GPI-type | | | |
| MT4-MMP | MMP-17 | 71/67 | Fibrin, fibrinogen |
| MT6-MMP | MMP-25 | 62/58 | Gelatin |
| Type II transmembrane-type | | | |
| CA-MMP | MMP-23 | ~66 | Gelatin |

C-I, II, III, IV, V, VII, IX, X or XIV, type I, II, III, IV, V, VII, IX, X or XIV collagen, respectively; CA, cysteine-array; COMP, cartilage oligomeric matrix protein; FN, fibronectin; GPI, glycosylphosphatidyl-inositol anchor signal; LN, laminin; PG, proteoglycan [Nabeshima 2002].

## B. REGULATION, ACTIVATION AND INHIBITION

The activity of metalloproteinases is tightly coordinated by complex mechanisms at several levels including transcriptional regulation, activation of latent zymogen, and interaction with endogenous inhibitors [Price et al. 2001]. The later mechanisms are regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) that bind to the catalytic site of the active enzyme as well as to the carboxyl domain of the proenzyme, thereby preventing their activation [Cawston 1995, Birkendal-Hansen 1995].

Regulation of metalloproteinases and their expression is strongly controlled by a variety of biological and external agents including cytokines, polypeptide hormones, chemical agents like phorbol esters, physical stress, steroid hormones and oncogene products [Geisler et al. 1997]. Transcription of many MMP genes can be induced by IL-1, TNF-α, TGF-α, EGF, FGF, PDGF [Birkendal-Hansen 1995], changes in cell-cell and cell-extracellular matrix interactions [Stamenkovic 2000] or repressed by TGF-β, IL-4, retinoid acid, glucocorticoids [Nagase and Woessner 1999]. Many of these co-regulate expression of several metalloproteinases while others regulate expression of individual members of this gene family.

Activation consists of a two-step reaction. An initial step involves the N-terminal domain, known as the pro domain, which is present in all MMPs and is responsible for the latency of the zymogen [Van Wart and Birkendal-Hansen 1990]. The cysteine residue present in the pro domain coordinates the active site zinc allowing proteolytical cleavage at the propetide region. The disruption of the cysteine-zinc interaction (the 'cysteine switch') leads to disconnection of the N-terminal polypeptide [Springman et al. 1990, Van Wart and Birkendal-Hansen 1990]. Removal of the propeptide by proteolysis results in zymogen activation (latent form). The following autoproteolytic reaction generates stable, permanently active enzymes [Woessner 1991, Massova et al. 1998, Nabeshima et al. 2002] (Figure 2).

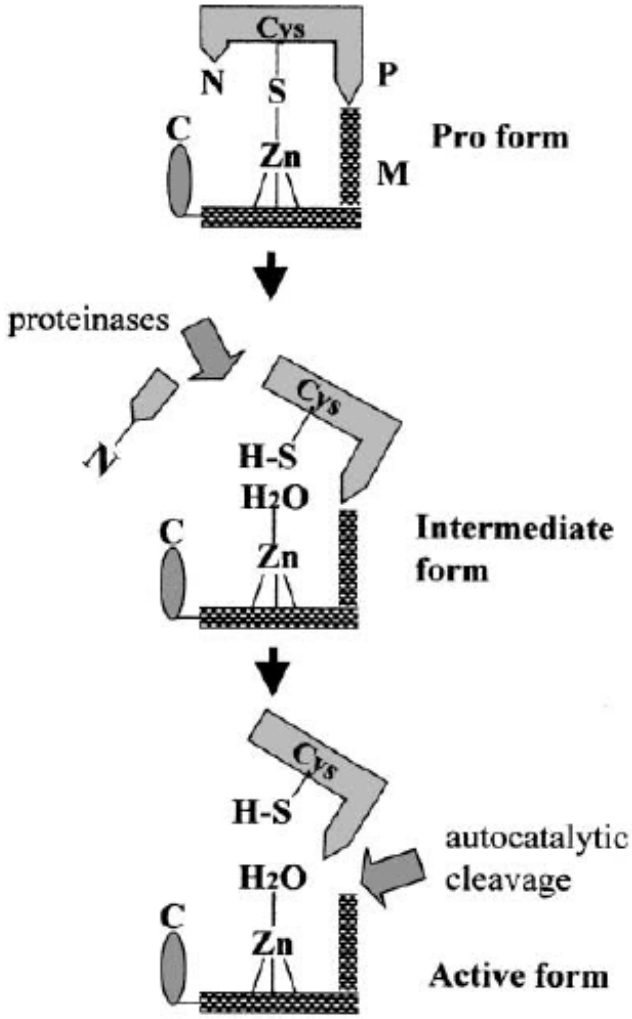


**Figure 2. Activation of proMMP**. Activation process consists of a two-step reaction: an initial cleavage at the propeptide bait region leading to removal of the N-terminal polypeptide, and the second step, an autoproteolytic reaction that generates the stable active enzyme. C, C-terminal; M, catalytic domain; MMP, matrix metalloproteinase; N, N-terminal; P, prodomain [Nabeshima et al. 2002].

Matrixins are secreted as latent proenzymes, which must be cleaved in the extracellular space before they exhibit degradative activity. The best characterised mechanism of metalloproteinase activation involves urokinase- or tissue-plasminogen activator (uPA or tPA) which converts plasminogen to plasmin. This serine proteinase then cleaves and activates prostromelysin while procolagenase is partially activated. In turn, the active form of stromelysin fully activates interstitial collagenase [He et al. 1989]. Activation of proMMPs by this mechanism, including collagenases, stromelysins and matrilysins, can occur distant from cells in ECM or at the cell surface through the uPA plasminogen cascade [Carmeliet et al. 1997]. Progelatinase

A has a unique mechanism of activation, which involves binding to a cell membrane-associated protein (MT-MMP) which induces self-cleavage to active gelatinase A through formation of trimolecular complex between progelatinase A, MT1-MMP and TIMP-2 [Fernandez-Catalan et al. 1998, Cao et al. 1995] (Figure 3).

Regulation of the activation and inhibition is achieved by the formation of stoichiometric, inactive 1:1 complexes of MMPs with their specific inhibitors TIMPs [Van Wart and Birkendal-Hansen 1990, Jimenez et al. 2000]. TIMPs exist in equilibrium with MMPs, and their interaction is largely responsible for modification of the extracellular environment [Jimenez et al. 2000]. Specific inhibitors of metalloproteinases are small proteins of 21-28 kDa [Cawston 1995, Stamenkovic 2000]. Their action involves binding to the catalytic site of an active matrix enzyme [Birkendal-Hansen 1995, Basbaum and Werb 1996, Cawston 1995, Murphy et al. 1981].

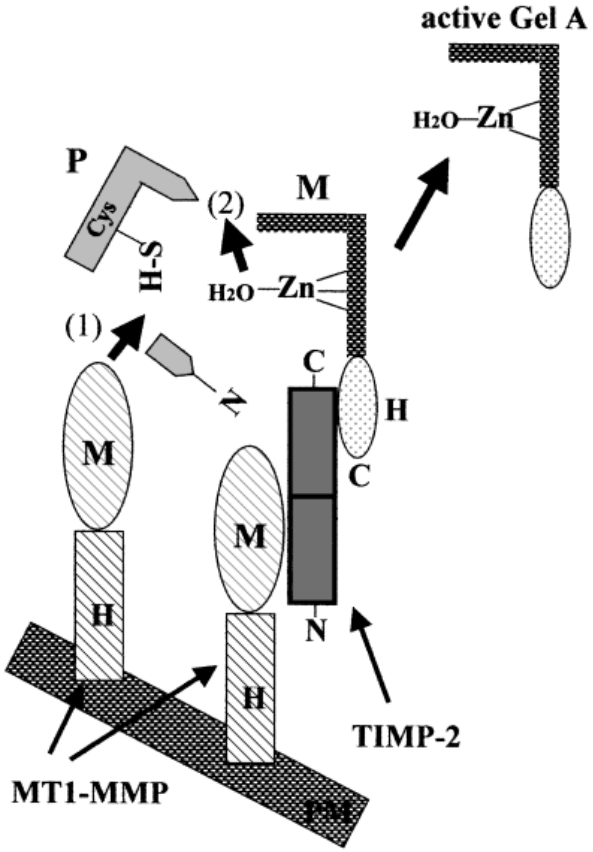


**Figure 3. Schematic presentation of proGelatinase A activation by MT1-MMP.** Free MT1-MMP activates proGel A trapped in a tri-molecular complex of MT1-MMP/TIMP-2/proGel A. C, C-terminal; H, hemopexin-like domain; M, catalytic domain; MMP, matrix metalloproteinase; N, N-terminal; P, prodomain; PM, plasma membrane [Nabeshima et al. 2002].

In addition to direct binding to the catalytic site, TIMPs also bind to the carboxyl-terminus of certain prometalloproteinases to inhibit their activation. All metalloproteinases except matrilysin have the C-terminal domain, which contains an amino acid sequence similar to hemopexin and a plasma heme binding protein, which interacts with the TIMPs [Massova et al. 1998]. Prevention of the MMP activation through TIMPs binding plays a highly important role in the activation of progelatinase A where MMP-2 is secreted from the cells as a proenzyme-inhibitor complex with the TIMP-2 [Howard and Banda 1991, Fridman et al. 1992].

Action of metalloproteinases is a complex process. It involves a variety of mechanisms occurring during degradation of the basement membrane and extracellular matrix remodelling. A vast amount of work verifies the significance of MMPs in physiology and pathology of tissue formation, repair and breakdown showing that disordered regulation of matrixins may contribute to number of diseases including progressive liver fibrosis [Arthur 1995, Woessner 1999]. Despite the detailed studies on substrate specificities, the exact role of different metalloproteinases in liver fibrosis is not known. Studies based on the expression of gelatinases A and B, and stromelysins -2 and -3 in viral and non-viral liver diseases should help us to understand better the mechanisms characterising liver fibrosis.

### 1.1.1. GELATINASE A

Gelatinase A has type IV collagenolytic activity and is involved in basement membrane remodeling [Theret et al 1997]. MMP-2 gene differs from the other members of matrixin family in three internal repeats, which resemble the collagen-binding domains of fibronectin justifying its enzymatic activity for this substrate [Huntala et al. 1990]. Gelatinase A substrate specificity for gelatin (denaturated interstitial collagens) [Nagase 1997], types IV, V, VII, X collagens, elastin [Theret et al. 1999], laminin, fibronectin [Murphy et al. 1991, Collier et al 1988] and a variety of other matrix and non-matrix proteins [Price et al. 2001] is associated with extracellular matrix remodeling in wound healing, development, inflammation, fibrosis, angiogenesis, and tumor invasion [Corcoran et al. 1996, Yu et al. 1996].

Gelatinase A is expressed by most connective tissue cells including endothelial cells, osteoblasts, fibroblasts and myoblasts [Price et al. 2001]. In the liver, MMP-2 is produced by lipocytes (hepatic stellate cells, Ito cells) [Arthur et al. 1989]. Hepatic stellate cells (HSC) play an important role in ECM breakdown and liver fibrosis due to their localization within the space of Disse [Minato et al. 1983 and Yokoi et al. 1988]. MMP-2 is secreted as an inactive proenzyme and the activation of proMMP-2 is mediated at the cell surface [Butler et al. 1999]. Recent studies demonstrate increased gelatinolytic activity on the HSC surface as a product of metalloproteinase MMP secretion from stellate cells. This kind of change of ECM might disturb communication between HSC and ECM within the space of Disse [Theret et al. 1999, Preaux et al. 1999].

Activation of proMMP-2 can not be maintained by the action of serine proteases such as plasmin, neutrophil elastase and trypsin. For its activation the presence of the tri-molecular complex of MT1-MMP, TIMP-2 and carboxyl end domain of proMMP-2 at the cell surface is required [Strongin et al. 1995, Takahara et al. 1997, Seiki 1999]. MT1-MMP serves as a cell surface receptor for MMP-2 when combined with TIMP-2 [Sato et al. 1994]. TIMP-2 associates with MT1-MMP at equimolar concentrations, forming a binary complex which binds progelatinase A and leads to the MMP-2 activation [Theret et al. 1998, Zucker et al. 1998, Preaux et al. 1999]. Excess of TIMP-2 inhibits progelatinase A activation and blocks the activity of gelatinase A [Kinoshita et al. 1998, Butler et al. 1999]. While activating proMMP-2, MT-MMP may possibly serve to concentrate activity of the enzyme to the cell surface promoting proteolysis of the extracellular space and disrupting cell-matrix interactions [Benyon et al. 1999]. The disruption of this interaction can intensify the HSC activation leading to the formation of irreversible fibrosis in patients with chronic liver injury [Gilles et al. 1998].

Numerous studies have shown the implication of gelatinase A in fibroproliferation during the HSC transformation to myofibroblasts. In human liver fibrosis as well as in $CCl_4$ induced liver fibrosis, increased levels of MMP-2 mRNA were detected in intermediate stages of the disease [Takahara et al. 1995]. In the normal liver, the expression of MMP-2 was determined as a part of the ECM remodeling process, although in small and hardly traceable amounts [Geisler et al. 1997]. In chronic viral

hepatitis, increased transcription levels of MMP-2 were reported in the studies of hepatic tissues taken from patients with chronic viral hepatitis and cirrhosis [Takahara et al. 1997, Lichtinghagen et al. 2003], primary biliary cirrhosis (PBC), primary sclerosing cholangitis and biliary arthesia [Benyon et al. 1996]. Despite numerous findings emphasizing the profibrogenic functions of gelatinase A, mechanisms leading to MMP-2 activation in fibrotic liver have not been clearly defined. Further research is required to outline the role of MMP-2 in the cascade of events leading to the degradation of fibrotic matrix in the liver.

### 1.1.2. GELATINASE B

Gelatinase B (MMP-9, 92 kDa collagenase type IV, 92-kD gelatinase) is capable of degrading basement membrane collagen IV playing an important role in the early steps of tissue remodeling in chronic liver diseases and in the development of organ fibrosis [Lichtinghagen et al 2000]. 92-kDa type IV collagenase substrate specificity includes denaturated collagens (gelatins), type V, VII, X, XIV collagens, elastin, plasminogen, fibronectin and TGF-β [Stamenkovic 2000, Arthur 1995, Masure et al. 1991, Bernhard et al. 1994, Nagase and Woessner 1991]. Gelatinases are not capable of degrading triple helix of native type I and III collagens like interstitial collagens are. They can degrade the α2 chain of type I and the α1 chain of type III collagen, which are major components of fibrotic liver [Ashida et al 1996]. Gelatinases differ from other metalloproteinase family members in three repeats of fibronectine-type II domains inserted in the catalytic domain which allow its interaction with collagens and gelatins providing different developmental regulation and biological function [Allan et al. 1995, Steffensen et al. 1995, Huhtala et al. 1991].

Gelatinase B is synthesised by both resident and invasive cells playing a role in matrix turnover through the basement membrane breakdown [Winwood et al. 1995]. MMP-9 synthesis and secretion plays an important role in physiological and pathological processes like fibrosis, hepatocellular carcinoma, inflammatory response, wound healing [Sakamoto et al. 2000, Arii et al. 1996], primary liver tumors [Terada et al. 1996], invasive and metastatic tumors [Okada et al. 2001, Hayasaka et al. 1996, Nagakawa et al. 2002, Turner et al. 2000].

MMP-9 is released as a proenzyme predominantly from neutrophils and macrophages [Arthur 1995, Devarajan et al. 1992]. It can also be secreted by normal human skin fibroblasts, transformed lung fibroblasts, [Wilhelm et al. 1989] and polymorphonuclear leukocytes [Masure et al. 1991].

Gelatinase B participates in the myofibroblastic transformation of Ito cells with the mechanism similar to MMP-2 activation [Winwood et al. 1995]. Unlike MMP-2, which is mostly secreted by Ito cells (HSC), MMP-9 is produced by Kupffer cells and its production increases after activation [Winwood et al. 1995, Knittel et al. 1999]. Its activity is stimulated by factors known to promote Kupffer cell activation such as phorbol ester and endotoxin in both rat and human cells [Arthur 1995]. Knowing that Kupffer cells play a significant role in host defense by increasing in number and state of activation during liver injury and fibrosis, it is believed that Kupffer cells may serve as an indicator of gelatinase B secretion during liver pathogenesis [Arthur et al. 1986, Geerts et al. 1988].

Gelatinase B is produced as an inactive proenzyme (progelatinase B) and it is activated by stromelysin and plasmin through urokinase type plasminogen activator. Its activity is controlled via inhibition by specific inhibitor TIMP-1, which binds in a non-covalent manner to the enzyme forming 1:1 stoichiometric complex [Chung et al. 2002].

A vast amount of studies have examined the participation of MMP-9 in ECM remodeling in the liver. Involvement of MMP-9 in fibroproliferation through the TGF-β activation mechanism in tumor cell cultures revealed the ability of this enzyme to adhere to the membrane receptors (like CD44) forming molecular complex that possibly attaches to TGF-β1, -2 and –3 to activate them proteolytically [Yu and Stamenkovic 2000]. Studies investigating the role of gelatinase B in the normal liver confirmed its presence in traceable amounts [Geisler et al. 1997, Lichtinghagen et al. 1995]. Experimental study in patients with chronic hepatitis detected increased plasma levels of MMP-9 [Hayasaka et al. 1996] and transiently elevated MMP-9 mRNA expression [Lichtinghagen et al. 2003]. In subjects from the HCV and HBV positive patients, significant changes in MMP-9 activities were observed as a result of

viral influence on type IV collagenase activity in serum. Analysis evaluating the activity of MMP-9 in groups of hepatitis B and C carriers reported low activity values compared to the healthy controls [Kuo et al. 2000]. Due to the diverse behavior of MMP-9 in various liver diseases, studies of its expression raise interest and promise additional information on fibroproliferative processes.

### 1.1.3. STROMELYSIN 2

Human stromelysin 2 is metalloproteinase capable of degrading a variety of non-collagen connective tissue components including proteoglycan, fibronectin, laminin, gelatin, elastin, casein and type IV, V, IX, X collagen. It does not have the affinity for the interstitial type I collagen. The ability of degrading ECM components places it in the assembly of important modulators of ECM composition in liver injury [Muller et al. 1988, Bodey et al. 2000]. It is a secreted metalloproteinase synthesised in a preproenzyme form predominantly expressed by epithelial cells, and it can also be found in carcinomas [Stamenkovic 2000]. Even though stromelysin 2 (MMP-10) shares 82% sequence homology with stromelysin 1 (MMP-3), their expression appears to be differently regulated by growth factors, cytokines and oncogenes [Bord et al. 1998].

Primary structure analysis shows that human stromelysin 2 has high homology with the analogue of rat transin, which is an oncogene transformation-induced protease [Matrisian et al. 1986]. MMP-10 RNAs shows the ability to hybridize to a rat stromelysin cDNA in human tumors [Muller et al. 1988]. Studies made on normal and tumorigenic cells reveal the ability of human skin fibroblasts to constitutively secrete stromelysin in vitro [Wilhelm et al. 1987].

Stromelysin 2 is expressed mostly by HSC in rat liver. It is also detectable in lower amounts in Kupffer cells, myofibroblasts and hepatocytes. The expression studies reported reduced MMP-10 levels during the activation of HSC primary culture [Knittel et al. 1999]. A survey involving MMP-10 expression in HCC patients by indirect alkaline phosphatase conjugated immunocytochemical technique revealed strong expression in the ECM adjacent to blood vessels [Bodey et al. 2000]. In a research study carried out on the normal human liver by western blot analysis, MMP-

10 was found present in a proenzyme form, while activated enzyme was not detectable [Lichtinghagen et al. 1998]. In the normal human liver stromelysin 2 was hardly detectable when analysed by antibodies against various metalloproteinases. In HCC it was strongly positive [Lichtinghagen et al. 1995]. Common findings of increased levels of stromelysin 2 during various pathological liver disorders underline its importance during the course of fibrolysis.

### 1.1.4. STROMELYSIN 3

Stromelysin 3 (ST3, MMP-11) is secreted matrix metalloproteinase that is expressed in tissues undergoing the active remodelling associated with embryonic development, wound healing and tumour invasion [Basset et al. 1990, Matrisian 1990, Lefebvre et al. 1992, Wolf et al. 1993, Lefebvre et al. 1995]. Even though it belongs to matrixins, MMP-11 differs from other family members in its distinct activation properties, substrate specificity and interactions with metalloproteinase inhibitors [Levy et al. 1992, DeClerck et al. 1989, Stetler-Stevenson et al. 1989]. Like other members of the metalloproteinase family, stromelysin 3 is synthesized as an inactive precursor that has to be processed to its mature form for the enzymatic activation [Matrisian 1992]. As an active enzyme formed in the process of intracellular proteolysis, it possesses a unique feature among all matrixins, to be directly secreted into the extracellular space. Its intracellular activation is regulated by furin type convertases after the proteolytic removal of the propeptide domain [Pei and Weis 1995, Masson et al. 1998].

Metalloproteinases are known to degrade at least one component of the ECM prior to the secretion of their latent form which requires proteolysis for the activation. However, mature forms of MMP-11 appear to be unable to degrade any major component of ECM [Pei et al. 1994, Noel et al. 1995]. Studies show that MMP-11 acts as powerful endopeptidase with restricted substrate specificity distinct from all other matrix metalloproteinase [Pei et al. 1994]. This distinction may be attributed to the localisation of the stromelysin-3 gene. While the majority of matrixins are assigned to chromosome 11, MMP-11 is localised on chromosome 22 as in situ hybridisation analysis reveals [Basset et al. 1990]. The ST3 gene was originally identified on the basis of its specific expression in stromal cells surrounding invasive breast carcinomas [Levy et al. 1992, Asch et al. 1999].

The expression of stromelysin 3 is related to mesenchymal cells and predominantly fibroblasts located in the vicinity of epithelial cells [Lefebvre et al. 1992, Anglard et al. 1995, Stamenkovic 2000]. Its expression was also observed in fibroblastic cells of most types of human carcinomas including colon, skin, head, breast and neck [Rouyer et al. 1994]. ST3 transcripts were detected in fibroblasts in the immediate surrounding of degenerative ducts, suggesting the association of the gene expression with the basement membrane dissolution [Lefebvre et al. 1992].

Application of monospecific polyclonal antibodies and high sensitivity immunocytochemical procedures in studies made on the human liver revealed localization of MMP-11 at the surface of bile canaliculi. Some enzymatic presence was detected in portal fibrocytic cells, while hepatocytes did not show any immunoreactivity [Geisler et al. 1997]. In the normal human liver, fibroblast analysis detected negligible expression of MMP-11 [Giambernardi et al. 1998, Lichtinghagen et al. 1995]. During the course of chronic hepatitis C mRNA expression data identified transiently elevated expression of MMP-11 [Lichtinghagen et al. 2003], while studies in cirrhotic patients identified increased mRNA expression levels [Lichtinghagen et al. 1998]. Similar findings in a number of liver diseases address research on the importance of stromelysin 3 as to an important indicator of liver pathogenesis.

## SECTION 2

### 1.2. LIVER FIBROSIS AND REMODELING

Liver fibrosis is a wound healing or scarring process occurring in response to ongoing chronic liver injury. It is a programmed, dynamic and reversible response to injury [Olaso and Friedman 1998]. Hepatic fibrosis involves multiple cellular and molecular events that lead to deposition of excess matrix proteins in the extracellular space. It involves a cascade of events which, when combined with ineffective regeneration and repair, increase the distortion of the normal liver architecture resulting in cirrhosis [Arthur 2000].

Cirrhosis was considered to be an irreversible end stage consequence of fibrosis with the outcome of liver failure [Friedman 2003]. Recent advances in characterization of resident liver cells show that cirrhosis can be reversible. The origin of hepatic stellate cells and their fate after liver injury resolution are still connected to number of controversies. The exact stage at which fibrosis or cirrhosis becomes truly irreversible is not known [Safadi and Friedman 2002].

Chronic liver injury occurs in response to a variety of causes, including viral hepatitis (hepatitis B and C), toxic damage (e.g. alcohol abuse), drugs, metabolic diseases due to overload of iron or copper, autoimmune attack of hepatocytes, billiary cirrhosis or congenital abnormalities [Friedman 2000]. Hepatic scar formation involves inflammation, recruitment and local proliferation of myofibroblast-like cells and matrix remodelling regardless of the type of initial injury. Remodeling of the liver matrix involves an overall increase in the extracellular matrix components (Figure 1). It is accompanied by a shift from the normal low density basement membrane-like matrix containing non-fibril-forming collagens (e.g. types IV and VI) to one rich in fibril-forming collagens (types I and III) [Olaso and Friedman 1998].

### 1.2.1. PATHOGENESIS

Fibrosis is a dynamic pathophysiological process that includes phases of progression and regression [Friedman 2003]. It is characterized by quantitative and qualitative changes in the composition of the hepatic extracellular matrix (ECM) that affect matrix secretion and degradation rate [Arthur 2000]. Accumulation of fibrilar collagens in fibrosis occurs when collagen deposition exceeds its degradation. Experiments on animal models of induced liver injury show that removal of hepatotoxin within a short period of time brings histological normality and liver recovery [Iredale et al. 1998]. Excessive accumulation of extracellular matrix components occurs in part due to increased synthesis and deposition of various collagens (type I collagen in particular) and noncollagenous glycoproteins by hepatic mesenchymal cells, such as stellate cells (Ito cells, lipocytes) and hepatic myofibroblasts. Various studies indicate that important factor contributing to the pathological accumulation of hepatic extracellular matrix is insufficient ECM degradation [Milani et al. 1994].

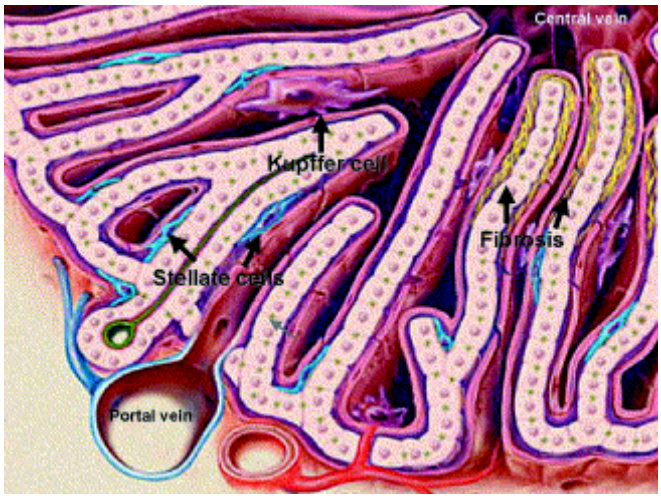


**Figure 1. Sinusoidal architecture and the localisation of hepatic stellate cells and Kupffer cells [Friedman 2003].** Cords of hepatocytes surrounded by a fenestrated endothelial lining are shown in the normal liver. Accumulation of extracellular matrix due to the HSC activation is presented.

The normal liver includes the epithelial layer mostly formed by hepatocytes, the endothelial lining distinguished by fenestrae (pores), tissue macrophages (Kupffer cells), and the perivascular mesenchymal stellate cells. Hepatic stellate cells (HSCs) are embedded in a basement membrane-like matrix rich in type IV collagen in the normal liver. Hepatocytes are separated from the sinusoidal endothelium by a subendothelial space of Disse, which contains a low-density matrix similar to the basement membrane type (Figure 1).

Such law density ECM is necessary to maintain the function of surrounding cells through the signalling [Friedman 2003]. Subendothelial matrix contains a network of ECM molecules that provide cellular support and allow transport of solutes and growth factors [Friedman 2003]. It is suggested that during fibrogenesis, alterations in subendothelial matrix composition promote activation of HSC. Experiments done with cell cultures strongly support this assumption showing rapid losts of the quiescent HSC phenotype when the cells are cultivated on type I collagen or on plastic, whereas the cultivation on a basement membrane-like matrix maintains the phenotype [Preaux et al. 1999].

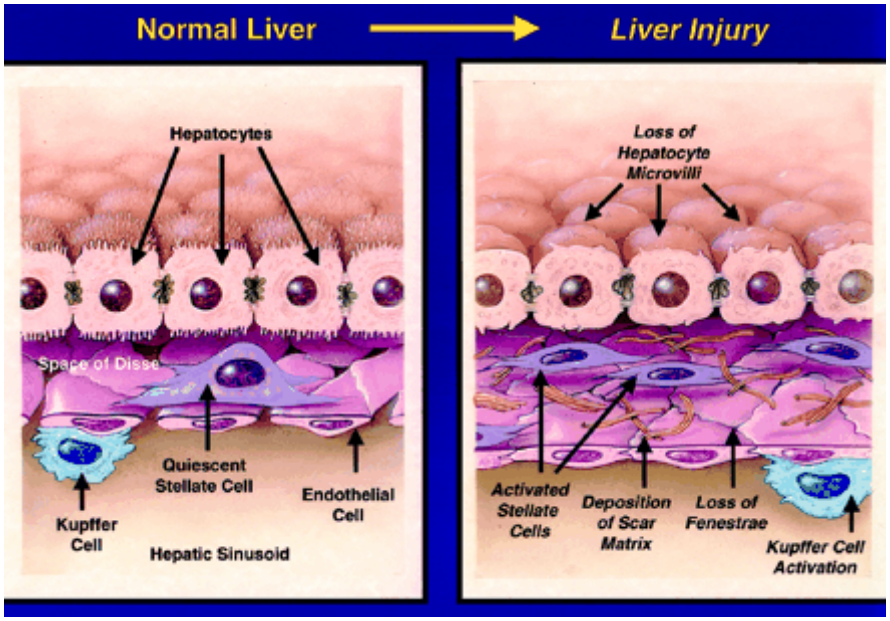


**Figure 2. Subendothelial changes during stellate cell activation accompanying liver injury [Friedman 2000].** Liver injury affects the composition of subendothelial matrix by modifying it into the collagen rich matrix simultaneously initiating alterations of hepatocellular function.

Development of liver fibrosis disrupts the normal hepatic architecture affecting the subendothelial space of Disse and through the cell-matrix interactions possibly modifies hepatocyte and sinusoidal cell function. Synthesis and degradation of matrix components occur at varying rates altering ECM degradation and ECM protein synthesis. Key mediators of these events are believed to be MMPs, which are the main degrading enzymes of extracellular matrix proteins [Murawaki et al. 1999].

### 1.2.2. ACTIVATION OF HSC

The central event in hepatic fibrosis is the activation of hepatic stellate cells (HSC). Upon their activation series of changes commence including degradation of the normal ECM of the liver, deposition of scar molecules, vascular and organ contraction, release of cytokines, and synthesis of matrix proteins [Safadi and Friedman 2002]. As a part of the fibrosing process, formation of stellate cell subpopulation occurs with particular cytoskeletal and phenotypic profiles [Friedman 2003].

Stellate cell activation is a well-programmed response to various stimuli derived from injured hepatocytes, endothelial and Kupffer cells. It is a process which leads to the accumulation of scar-like fibril-rich matrix. Modification of stellate cells contributes to the loss of hepatocyte microvilli and endothelial fenestrae, which results in deterioration of hepatic function (Figure 2). As a part of the process, Kupffer cells also undergo activation contributing to paracrine activation of stellate cells [Friedman 2000]. Activation of hepatic stellate cells involves their transdifferentiation from quiescent, vitamin A-storing cells into proliferative, fibrogenic, and contractile myofibroblast-like phenotype characterized by the appearance of smooth muscle α-actin [Eng and Friedman 2001]. Phenotypic changes involve series of events including proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, retinoid loss and white blood cells chemoattraction (Figure 3) [Friedman 2000].

The conversion of hepatic stellate cells is a complex process promoted by a variety of cytokines or soluble factors released by Kupffer cells, inflammatory cells, and injured hepatocytes, as well as by alterations in the composition of the perisinusoidal matrix surrounding stellate cells [Preaux et al. 1999]. Changes in cellular function of hepatic cells involve stages of events like initiation, perpetuation and resolution (Figure 3). Initiation of HSC activation encloses progressive changes in gene expression and phenotype that trigger the cells responsive to cytokines and other local stimuli. Consequently, initiation is associated with transcriptional events and induction of immediate early genes. Due to disruptive effects of liver injury on the homeostasis of neighbouring cells, paracrine stimulation occurs causing an early change in ECM composition [Friedman 2000].

Perpetuation is a dynamic process, which results from autocrine and paracrine stimulation, as well as from accelerated ECM remodeling. It incorporates cellular events that amplify the activated phenotype through enhanced cytokine expression and responsiveness. Resolution of liver injury is the uncertain stage of fibrosis possibly including reversion of HSC to a quiescent phenotype and /or clearance by apoptosis [Friedman 2000]. Resolution is a step towards the reversibility of fibrosis related to a number of questions ascribed to the fate of stellate cells. It is supposed that during the resolution of liver fibrosis a number of stellate cells becomes

diminished by apoptosis, or by reversion to a more quiescent phenotype [Friedman 2003].

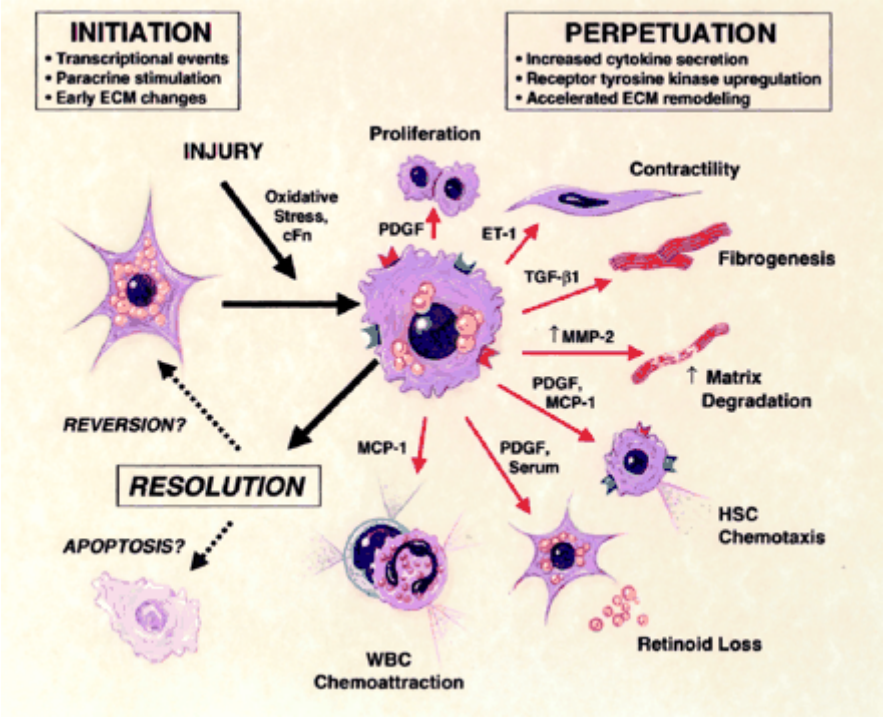


**Figure 3. Stellate cell activation and/or resolution pathways during liver injury (phenotypic features) [Friedman 2000].**

During fibrosis development, activation of HSC and their dedifferentiation into myofibroblast-like cells trigger the proteins and enzymes secreted by these cells to the effect that more collagens and more metalloproteinase inhibitors have been produced [Lichtinghagen et al. 1998, Murawaki et al. 1999]. Since matrix metalloproteinases are key enzymes responsible for the extracellular matrix degradation and remodeling in the liver, the deregulation of their activities may be of special importance for the development of liver fibrosis. As liver fibrosis progresses, expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) increases. Experimental studies on rats involving hepatic injury showed increased expression of gelatinase A (MMP-2), membrane type MT1-MMP, TIMP-1 and TIMP-2 during the animal exposure to $CCl_4$ [Benyon and Arthur 2001]. Alterations of the extracellular matrix disturb the balance of collagen turnover in the fibrotic liver affecting collagen production and breakdown. Various studies reported that collagen breakdown is

largely attributable to the effect of MMPs. Gelatinases degrade basal membrane collagen and are involved in the early steps of tissue remodeling during fibrogenesis [Lichtinghagen et al. 2001]. Experiments show increased collagen content contributing up to 30% of total liver weight in the liver in the course of the disease. Insufficient activation or excess inhibition of MMPs in response to mild but continuous tissue injury may represent an important factor for the development of liver cirrhosis [Geisler et al.1997].

### 1.2.3. MMPs IN FIBROSIS

Obscure knowledge exists regarding changes in MMPs production that may contribute to the tilting of balance between matrix production and degradation during fibroproliferation [Lichtinghagen et al. 1998]. There is an absence of conclusive data demonstrating matrix degradation during or after liver injury in fibrosis progression. Extensive data show the accumulation of matrix proteins like type IV collagen, laminin and proteoglycans in the fibrotic liver. This paradox can be viewed as an outcome of matrix degradation in a pericellular environment affecting HSC function, followed by subsequent matrix deposition. Another possible explanation for this occurrence could lie in matrix composition formed during the pathological modification of the liver and its disability to keep cell-matrix interactions with HSC in the same manner as the normal liver. Additional clarification might be related to the modification in the MMP function in response to the substrate composition changes during fibrogenesis [Arthur 2000].

Increased matrix degradation is also associated with the regression of liver fibrosis. Various experiments have shown that liver fibrosis may resolve spontaneously after successful treatment of chronic liver disease, as in the case of hepatitis C virus clearance in animal models [Iredale et al. 1998]. In experimentally initiated liver injury in rats with $CCl_4$ despite the significant development of liver fibrosis, complete resolution of normal liver histology was obtained after the hepatotoxin removal [Arthur 2000].

Recent studies have shown that the recovery stage of the fibrotic process involves a decrease in the number of activated stellate cells and retrieval of tissue integrity. An important question regarding the fibrosing process refers to the mechanism of

restoration [Arthur 2000]. A possible solution lies in reversion of hepatic stellate cells to their quiescent state or in their apoptosis. Referred to as programmed cell death, apoptosis is viewed as a potential way of clearing stellate cells during the resolution of the liver injury, in order to control the overall cell number within a liver tissue [Iredale 2001, Eng and Friedman 2000]. Additional studies along with elucidation of cellular and molecular events following natural resolution of the liver architecture should give more light to this outcome [Arthur 2000].

The life of stellate cells and their fate during development of hepatic fibrosis remain unclear. Further research is required to explicate various mechanisms of HSC activation and key genes initiating these processes. The importance of vitamin A loss and its connection to the stellate cell activation during fibrogenesis need further clarification and analysis along with the fate of HSC during the resolution of liver injury [Li and Friedman 1999]. The answers to these questions along with the advances in gene therapy and drug technology should advance the development of new approaches to the understanding of liver fibrosis and ECM remodelling aiming at an effective antifibrotic therapy [Iredale 2001].

**SECTION 3**

## 1.3. HEPATITIS B

### 1.3.1. PREVALENCE AND TRANSMISSION

Hepatitis B is one of the major diseases of mankind and is a serious global health problem [Alter 2003]. Chronic hepatitis B virus (HBV) infection affects approximately 400 million people worldwide [Esteban 2002]. One third of the world's population, or about 2 billion people, have serologic evidence of past or present HBV infection [Kane 1995], of which 15%-40% develop complications like cirrhosis, hepatocellular carcinoma and liver failure [Esteban 2002]. Each year over a million people die from HBV-related chronic liver disease, including cirrhosis and hepatocellular carcinoma [Alter 2003].

Transmission of hepatitis B occurs horizontally through blood products and sexual transmission, and vertically from mother to infant in the perinatal period [Worman 1999]. As a worldwide health problem, HBV is specifically endemic in developing

areas. In these regions it is more prevalent in groups of immigrants from endemic areas, men homosexuals, injecting drug users, and persons with multiple sexual partners (Table 1). HBV is transmitted by perinatal, percutaneous, and sexual exposure, as well as by close person-to-person contact presumably by open cuts and sores, especially among children in hyperendemic areas [Alter 2003, Lok and McMahon 2001]. In endemic areas it is transmitted perinatally, whereas in developed countries infection is usually transmitted by blood transfusion, intravenous drug abuse, or sexual contact [Esteban 2002].

**Table 1. Prevalence of HBV serologic markers in population groups [Lok and McMahon 2001].**

| Population | Prevalence of HBV Serologic markers (%) HBsAg | All markers |
|---|---|---|
| Persons born in endemic areas | 13 | 70-85 |
| Men homosexuals | 6 | 35-80 |
| Injecting drug users | 7 | 60-80 |
| Dialysis patients | 3-10 | 20-80 |
| HIV-infected patients | 8-11 | 89-90 |
| Pregnant women (USA) | 0.4-1.5 | |
| Family/household and sexual contacts | 3-6 | 30-60 |

Hepatitis B has a complex natural history influenced by various factors including age at infection, viral factors (HBV genotype, viral mutations, level of HBV replication), host factors (gender, age, immune status), and exogenous factors such as coinfection with other hepatotropic viruses or alcohol. Difficulties in defining the natural history of chronic hepatitis B include the indolent course of the disease, the lack of symptoms during early stages, and the heterogeneity of the disease [Fattovich 2003].

### 1.3.2. INFECTION AND PATHOGENESIS

HBV belongs to the family of hepadnaviruses. It is an extremely resistant strain capable of withstanding extreme conditions. The HBV genome consists of circular, partially double-stranded DNA, which contains approximately 3,200 base pairs that encodes 4 overlapping open reading frames. One open reading frame encodes the precore polypeptide, which is modified into a soluble protein, the hepatitis B e antigen

(HBeAg) and the nucleocapsid protein, hepatitis B core antigen [Lok and McMahon 2001]. The presence of stable, covalently closed circular HBV DNA (ccc DNA) in the hepatocyte nucleus, and the asymmetric replication of the HBV genome, by reverse transcription of an RNA intermediate, make the virus resistant to antiviral agents [Fattovich 2003].

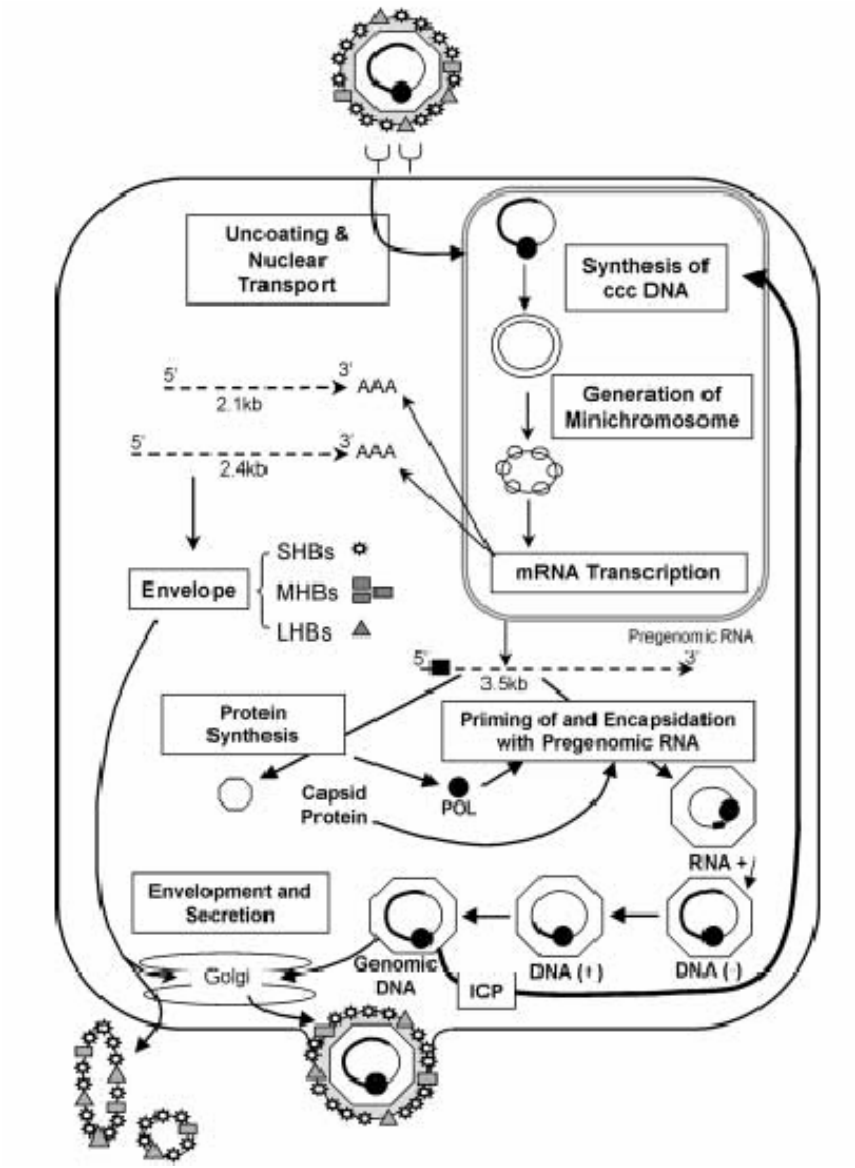


**Figure 1. Hepatitis B virus reproduction cycle [Locarnini et al. 2003].**

This unique feature of the virus allows genome replication through an RNA intermediate using its own reverse transcriptase. In this fashion, the hepatitis B virus has evolved a unique life cycle resulting in the production of enormous viral loads during active replication without directly killing the infected cell [Locarnini et al. 2003].

The HBV replication cycle in the liver begins with the attachment of the virion to the hepatocyte (Figure 1). Synthesis of the positive strand HBV DNA occurs inside the hepatocyte nucleus and the conversion of the viral genome into a covalently closed circular DNA (ccc DNA) follows. The cccDNA serves as a template for the pregenomic RNA, which is reversely transcribed into the minus strand HBV DNA. The exact mechanism by which ccc DNA is eliminated is unclear, though several studies suggest that cell death and/or cell division is necessary [Zhu et al. 2002, Lok and McMahon 2001].

HBV infection is a dynamic process with replicative and nonreplicative phases based on virus-host interaction (Figure 2). The first stage is immune tolerance, which lasts approximately 2-4 weeks in healthy adults and involves the incubation period. Presence of hepatitis B e antigen (HBeAg), increased HBV DNA serum levels and normal alanine aminotransferase (ALT) are the main characteristics of this phase. In the second stage, an inflammatory reaction with a cytopathic effect occurs. HBeAg can be identified in the serum, decrease of HBV DNA in serum is detectable along with the increased liver pathogenesis.

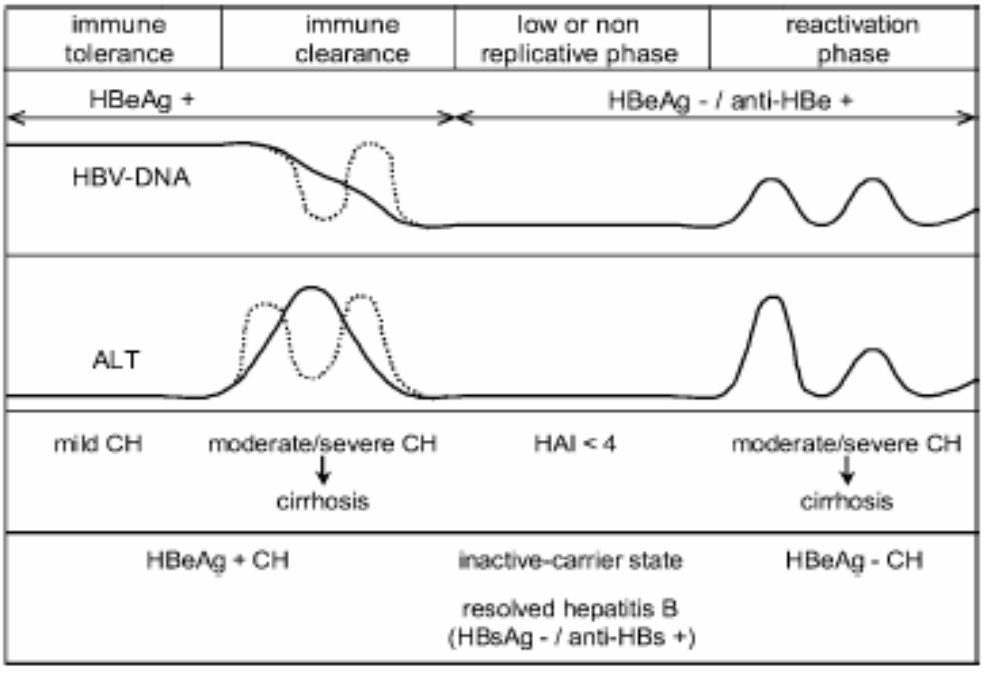


**Figure 2. Hepatitis B virus infection [Fattovich 2003].**
Dotted lines show fluctuating transition from replicative to the nonreplicative phase. After HBeAg seroconversion, reactivation of HBV replication along with the disease activity are notable.

The symptomatic period of this stage for the patients with acute infection lasts approximately 3-4 weeks. In the third stage, the host can target the infected hepatocytes and the HBV. Viral replication no longer occurs, and HBeAb can be detected. The HBV DNA levels are lower or undetectable, and aminotransferase levels are within the reference range. HBsAg is still present. In the fourth stage, the virus cannot be detected and antibodies to various viral antigens have been produced. Resolution of hepatitis B includes loss of hepatitis B surface antigen (HBsAg) and the development of an antibody to HBcAg (anti-HBs) [Fattovich 2003, Pyrsopoulos 2002].

The production of antibodies against HBsAg provides protective immunity and can be detected in patients who have recovered from HBV infection or in those who have been vaccinated. An antibody to HBcAg is detected in almost every patient with previous exposure to HBV. The immunoglobulin M (IgM) subtype is indicative of acute infection or reactivation, while the immunoglobulin G (IgG) subtype is indicative of chronic infection. This marker is not sufficient to detect the activity of the disease. An antibody to HBeAg is suggestive of a nonreplicative state, and the antigen has been cleared [Pyrsopoulos 2002].

The pathogenesis and clinical manifestations of hepatitis B are caused by the interaction of the virus and the host immune system. The host immune system attacks the HBV and causes liver injury. Activated $CD4^+$ and $CD8^+$ lymphocytes recognize various HBV-derived peptides located on the surface of the hepatocytes, and an immunologic reaction occurs. Impaired immune reactions (eg, cytokine release, antibody production), or relatively tolerant immune status results in chronic hepatitis. In particular, a restricted T cell–mediated lymphocytic response occurs against the HBV-infected hepatocyte [Pyrsopoulos 2002, Rehermann 2003].

### 1.3.3. DIAGNOSIS, PREVENTION AND THERAPY

Recommendations for the recognition, diagnosis, and management of patients chronically infected with the hepatitis B virus (HBV) include evaluation of patients with chronic HBV infection, prevention of HBV infection, role of HCC surveillance and treatment of chronic hepatitis B [Lok and McMahon 2001]. Although widespread

immunization and improved blood testing significantly decreased new HBV infections, the large number of infected individuals remains a serious health problem [Esteban 2002]. Prevention programs for hepatitis B involve the reduction of chronic HBV infection and HBV-related chronic disease. Practices involving donor screening, services promoting counseling for risk reduction along with effective infection control should reduce the potential risk for HBV transmission. Nevertheless, immunization is certainly the single most effective prevention [Alter 2003].

The goals of therapy in hepatitis B e antigen (HBeAg)-negative chronic hepatitis are to suppress and abolish viral replication that represents the main cause of liver necroinflammation and fibrosis. At present, interferon alfa (IFN-α), lamivudine and adefovir are available medications for chronic hepatitis B treatment. All these treatments have limited long-term efficacy [Hadziyannis et al. 2003, Fontana 2003, Marcellin 2002, Manns 2002].

Hepatitis B vaccine has a good record of safety and effectiveness, though it has been underutilized in high-risk adults despite long standing recommendations [Rich et al. 2002]. HBV vaccines induce protective levels of antibody to HBsAg (anti-HBs) in 95% of children and 90% of adults. Long-term serologic monitoring shows that infections occur in 1%-20% of vaccinated individuals, though without clinical disease or chronic infection [Lok et al. 2001].

### 1.3.4. MMPS AND HBV INFECTION

The application of molecular diagnostic assays like polymerase chain reaction allows detection of low levels of HBV DNA in serum. Most patients with chronic HBV infection including HBeAg negative, HBe antibody (anti-HBe) positive patients with normal aminotransferase levels have detectable serum HBV DNA [Lok 2003]. Research studies on matrix metalloprotease (MMP) expression in hepatitis B infected patients mostly show variations in enzyme levels that are observed in studies on MMP expression and active enzyme levels. Studies of serum active MMP concentrations within the group of cirrhotic patients showed an increase in serum proMMP-2 concentrations [Murawaki et al. 2002]. Analysis of hepatocytes from HCC patients

monitored the upregulation of MMP-9 expression and the elevated plasma levels of active enzyme in correlation of MMP-9 to the HBV infection [Chung et al. 2002]. Hayasaka and his colleagues also measured elevated plasma levels of MMP-9 in HCC subjects compared to the normal controls. Simultaneously, increased MMP-2 activity and decreased MMP-9 activity in groups of HBsAg and HBeAg positive carriers was detected by Kuo and the co-workers. The majority of the findings indicate the fluctuations of MMPs RNA, DNA or active enzyme levels in relation to the presence of the HBV and chronic liver infections giving rise to the better understanding and management of this liver disorder while leaving questionable points to be answered.

**SECTION 4**

## 1.4. HEPATITIS C

### 1.4.1. EPIDEMIOLOGY

Hepatitis C is a global health problem involving approximately 3% of the world's population. Infections caused by hepatitis C virus affect about 170 million people, being one of the leading causes of end-stage liver disease worldwide [WHO 1999]. Although representative prevalence data on epidemiology of hepatitis C infections are obscure and not easily accessible from many countries, available data indicate that in both developing and developed countries viral hepatitis remains an important public heath problem (Table 1) [Kim 2002, Wasley and Alter 2000].

**Table 1. Anti-HCV prevalence in various WHO regions**

| WHO region | Total population (million) | Percentage prevalence (%) | Number of people with anti-HCV (million) | Number of countries with no data available |
|---|---|---|---|---|
| Africa | 602 | 5.3 | 31.9 | 12 |
| America | 785 | 1.7 | 13.1 | 7 |
| Eastern Mediterranean | 466 | 4.6 | 21.3 | 5 |
| Europe | 858 | 1.0 | 8.9 | 19 |
| Southeast Asia | 1500 | 2.2 | 32.3 | 3 |
| Western Pacific | 1600 | 3.9 | 62.2 | 11 |
| Total | 5811 | 2.9 | 169.7 | 57 |

Among the people infected with hepatitis C, approximately 75% become chronic with a predisposition to the potential risk of serious long-term clinical disorders like

cirrhosis and hepatocellular carcinoma [Kim 2002]. HCV patients are usually adults with chronic HCV infection (for at least 6 months) with an increased serum level of transaminases, detectable serum HCV RNA, and histologic evidence for liver injury in the absence of cirrhosis [Zein and Zein 2002].

HCV is a blood-related pathogen that may be transmitted through parenteral exposure to contaminated blood or body fluids. Transmission is mostly associated with intravenous drug use and blood transfusion before 1990 [NIH 1997]. It is less commonly associated with vertical or sexual transmission [Majid and Gretch 2002]. Additional risk factors for hepatitis C transmission include the use of inadequately sterilized medical equipment, high-risk sexual behaviors, organ transplantation, hemodialysis, as well as the social practices of body piercing, tattooing and intranasal cocaine use. Perinatal transmission is not a common risk factor, except from mothers with co-infection with HIV [NIH 2002, Kim 2002, Yen et al 2003].

### 1.4.2. VIRAL PERSISTENCE

The HCV genome encodes a large polyprotein of 3011 amino acids. It is a single-stranded RNA virus belonging to the Flaviviridae family [Majid and Gretch 2002]. It contains RNA structural elements and genetic information encoding the HCV proteins. The genomic RNA penetrates the host cell where ribosome binding and translation occurs. Genomic RNA is replicated by virally encoded enzymes into a complementary antigenomic RNA, which serves as a template for the synthesis of progeny RNAs. Details of the HCV replication cycle are not known. It is believed that HCV RNA replication takes place in the cytoplasm of the infected cell. Positive-sense viral RNA acts as a template for the translation of the encoded polyprotein. Simultaneously, it serves as a transcriptional template for the production of negative sense vRNA to produce a double-stranded replicative form (RF).

The replicative form then acts as a template for production of multiple copies of nascent positive-strand RNA through replicative intermediate (RI) [Gowans 2000]. It is an error-prone process that generates a mixed population of RNA sequences (quasispecies). The RNA-dependent RNA polymerase, the main enzyme in HCV replication, lacks proofreading capabilities and thus generates a large number of

mutant viruses known as quasispecies. In persons who are infected, HCV may produce approximately a trillion new viral particles each day in a steady state of viral replication. Over time, mutation and selection occur causing population change and evolution drift towards varied genotypes [Dhawan 2002, Branch 2000].

The general condition of chronic hepatitis is viral persistence. Practice shows that the mechanism of viral persistence varies in the same individual and is accompanied by the presence of different viral quasispecies [Alter and Seeff 2000]. A single patient can have more than 20 strains of HCV at a single point of time making it extremely difficult for the host immune system to attack and neutralize it. Studies involving inoculations of chimpanzees with a mixture of known infectious inocula and human sera obtained from the infected person show that humans develop antibodies to neutralize HCV. Human antibodies are incapable of preventing the emergence of the viral variants that can maintain the infection [Farci et al. 1992].

After initial exposure, HCV RNA can be detected in blood within 1 to 3 weeks and is present at the onset of symptoms. Antibodies against HCV are detected by enzyme immunoassay (EIA) in only 50% to 70% of patients when symptoms appear due to the long interval between infection and the appearance of anti-HCV. In chronically infected patients, the possibility of detection increases to more than 90% after 3 months. Within an average of 4 to 12 weeks, liver cell injury is manifested through the elevation of serum alanine aminotransferase (ALT) [NIH 2002].

### 1.4.3. INFECTION

Despite the generally mild clinical presentation, HCV infection leads to persistent infection, chronic hepatitis in most cases, which may progress to cirrhosis and hepatocellular carcinoma (HCC) [Alter and Seeff 2000]. The HCC development may take as long as 30 years (Figure 1). Severe progression of hepatitis C to cirrhosis occurs in approximately 20% of patients who have chronic infection. The rate and chance of progression varies with certain factors, including sex, alcohol use, concomitant hepatitis, age, and several other factors. Once cirrhosis occurs, the risk of hepatocellular carcinoma is approximately 1-4% per year [Dhawan 2002].

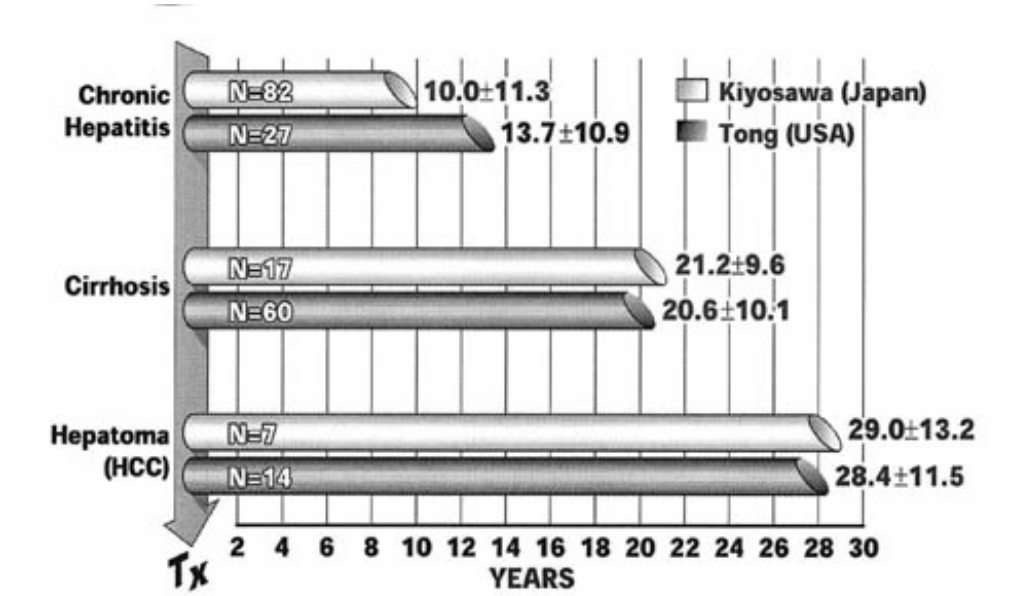


**Figure 1. Progression course for HCV–related disease [Alter and Seeff 2000].**

Acute infection can be severe but rarely is fulminant. Symptoms are uncommon but can include malaise, weakness, anorexia, jaundice, hepatomegaly (eg, hepatocellular carcinoma), and portal hypertension. After several weeks as ALT levels decline, the appearance of symptoms usually drops [NIH 2002, Dhawan 2002]. In resource-limited regions, the high cost of the enzyme immunoassay method becomes an obstacle in anti-HCV detection in the blood donation screening practice. Recombinant immunoblot assay (RIBA), which identifies antibodies that react with individual HCV antigens, is another method currently used for HCV diagnosis. Genomic RNA testing by means of RT-PCR when used for confirmation of serological results and infection detection give promising results in advanced diagnostics [WHO 1999].

### 1.4.4. THERAPIES

The natural targets of HCV are hepatocytes and, possibly, B lymphocytes [Dhawan 2002]. Immune mediated-liver disease is thought to be initiated by HCV-specific liver infiltrating T-cells that are amplified by antigen-nonspecific cells. Viral clearance is associated with the development and persistence of strong virus-specific responses by cytotoxic T lymphocytes ($CD8^+$ T cells) and helper T cells ($CD4^+T_H$) (Figure 2) [Rehermann 2000].

Therapeutic approaches of hepatitis C viral infection include therapies against HCV RNA, protein based therapies and immune based therapies. Therapies against HCV RNA are based on inhibition of viral RNA translation. Protein-based therapies of hepatitis C virus are directed to suppress HCV replication involving protease and helicase inhibitors. They represent improvement in the understanding of post translational mechanisms of HCV polyprotein (Figure 3). Immune-based therapies are related to cytokines and T-cell based approaches. A number of these, like HCV immunoglobulins, interleukin-2, interleukin-10 and several T-cell vaccines, are at different stages of development, promising to give good results in the treatment of HCV [Rehermann 2000].

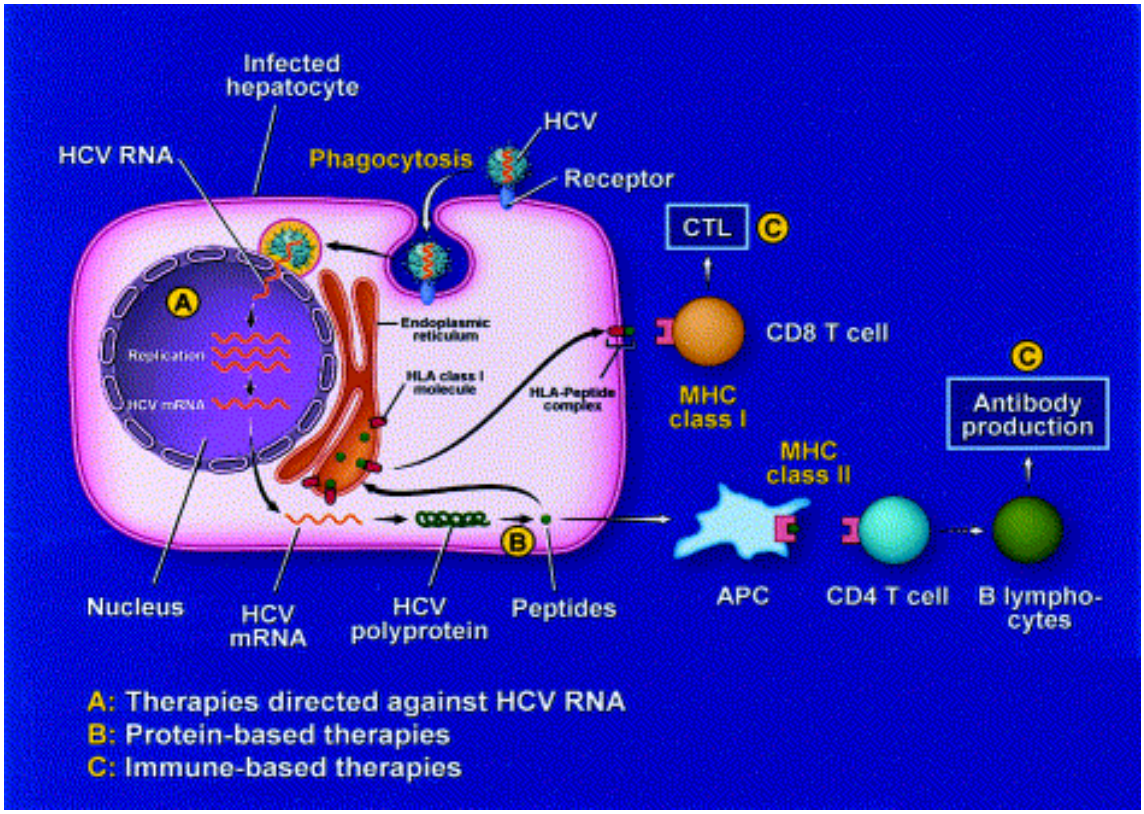


**Figure 2. Hepatitis C viral (HCV) infection targets [Zein and Zein 2002].**
HCV, hepatitis C viral infection; APC antigen-presenting cell; CTI, cytotoxin T- lymphocyte; HLA, human leukocyte antigen; MHC, major histocompatibility complex; mRNA, messenger RNA.

Interferon-α (IFN-α) in combination with the nucleoside analog ribavirin is the most effective up-to-date treatment of HCV infection. Interferons are low-molecular-weight proteins produced by leukocytes (interferon–α), fibroblasts (interferon-β), and T lymphocytes (interferon-γ). The action of IFN-α involves several mechanisms of

antiviral effects designed to reduce inflammation during fibrosis, cirrhosis and hepatocellular carcinoma in chronically infected patients [Zein and Zein 2002, WHO 1999]. Therapy with IFN-α in combination with ribavarin is quite expensive though leads to lasting elimination of the virus and to cure of the disease in approximately 40% of patients [Foster 1999].

In most persons infected with HCV, viremia persists, and is accompanied by variable degrees of hepatic inflammation and fibrosis. Studies suggest that 50% or more of the hepatocytes may be infected with HCV [Dhawan 2002]. The lack of a vigorous T-lymphocyte response and the high propensity of the virus to mutate appear to promote a high rate of chronic infection [NIH 2002].

### 1.4.5. MMPs IN HCV MEDIATED FIBROGENESIS

Chronic infection with hepatitis C virus typically induces injury and inflammation of the liver, thus promoting fibroproliferation [Schuppan et al. 2001]. The rate at which fibrosis progresses from person to person in chronic hepatitis C varies over time. In the majority of patients with chronic hepatitis C, fibrosis does not develop, or progresses so slowly that after decades of infection no change of the liver architecture is detectable on the liver biopsy. Only a minority of individuals has a rapid progression of fibrosis with an early development of cirrhosis along with an end-stage liver disease, portal hypertension, and hepatocellular carcinoma. Current knowledge of the factors that are responsible and that correlate with more rapid progression of fibrosis is limited. Being implicated in extracellular matrix synthesis and remodeling, MMPs are believed to play an essential role in its developing pathway [Marcellin et al. 2002].

In a number of studies involving the relation of MMPs to chronic HCV infections, increased expression levels of MMP-2, MMP-9 were measured in the liver during chronic hepatitis C [Lichtinghagen et al. 2001]. Hepatic mRNA expression data identified in groups of MMPs during the course of chronic hepatitis C showed steadily increasing mRNA expression of MMP-2 and transiently elevated expression for MMP-9 with disease progression [Lichtinghagen et al. 2003]. Strong MMP-2

expression was obtained in hepatic tissue from patients with chronic liver disease. Circulating levels of MMP-2 showed a close correlation to the hepatic enzyme expression, indicating a possible role of MMP-2 in serving as a marker for fibroproliferation in chronic liver diseases [Boeker et al.2002]. Prospective studies on the correlation of various MMPs as fibrosis markers and the development of their application in diagnosis should be further refined.

**SECTION 5**

## 1.5. NON-ALCOHOLIC STEATOHEPATITIS (NASH)

### 1.5.1. PREVALENCE AND EPIDEMIOLOGY

Non-alcoholic steatohepatitis is a condition characterized by elevated plasma liver enzyme levels, hepatomegaly, and liver biopsy findings similar to alcoholic steatohepatitis in the absence of alcohol abuse. It encompasses a broad clinicopathologic spectrum ranging from simple steatosis to statohepatitis that may progress to cirrhosis and end-stage liver disease [Yu and Keeffe 2002].

NASH has been reported as the most common liver disease in the United States along with NAFLD [Yu and Keeffe 2002]. 7%-11% of patients undergoing liver biopsy in the United States and Canada, and 1.2% of patients in Japan are found to have histologic diagnosis of NASH [Reid 2002]. Although NASH is generally considered to be a benign disease, it occasionally may progress to cirrhosis. Recent studies have shown that almost 20% of patients with NASH progress to cirrhosis over a decade [Yu and Keeffe 2002].

Ludwig and colleagues first described NASH in 1980 in patients whose liver histology was almost identical to alcoholic hepatitis with a different epidemiologic and clinical profile and without history of significant alcohol consumption [Ludwig et al. 1980, Sheth et al. 2003]. This condition has been designated by several terms including fatty liver hepatitis, nonalcoholic Laënnec's disaease, diabetic hepatitis, alcohol-like liver disease, pseudoalcoholic hepatitis, steatonecrosis, and non-alcoholic fatty liver disease (NAFLD) [Angulo 2002].

Patients with NASH are mostly obese, middle-aged women, many of whom have diabetes mellitus 2 (non-insulin dependent), hypercholesterolemia, or hypertriglyceridemia [Sheth et al. 1997, Marchesini et al. 2003]. Most of these are asymptomatic patients who do not consume alcohol in quantities known to cause liver injury. A number of metabolic conditions, surgical procedures and drug treatments were connected to NASH. Clinical and biochemical evaluations do not represent accurate methods for diagnosing NASH due to the incapability of differentiating these patients from asymptomatic patients with alcoholic hepatitis [Marleau 1998, Angulo 1999].

**Table 1. Conditions associated with non-alcoholic steatohepatitis**

| | |
|---|---|
| Nutritional Abnormalities | Obesity* |
| | Total parenteral nutrition |
| | Rapid weight loss |
| Metabolic Diseases | Diabetes mellitus* |
| | Hypertriglyceridemia* |
| | Abetalipoproteinemia |
| | Hypobetalipoproteinemia |
| | Weber-Christian disease |
| | Limb lipodystrophy |
| Drugs | Synthetic estrogens |
| | Corticosteroids |
| | Amiodarone |
| | Methotrexate |
| | Perhexiline |
| | Diltiazem, nifedipine |
| Surgery | Jejunoileal bypass |
| | Gastropexy |
| | Extensive small bowel loss |
| | Billiopancreatic diversion |
| Occupational exposure | Environmental toxins, e.g. hydrocarbons |

*Conditions associated with insulin resistance syndrome and seen most often in NASH.

Common findings for NASH include laboratory abnormalities expressed by a 2-4-fold elevation of serum aminotransferase levels, hepatomegaly, and histological findings identical to acoholic steatohepatitis which involve moderate to severe macrovesicular steatosis, lobular hepatitis with necrosis or ballooning degeneration and /or fibrosis [Reid 2001]. The ratio of aspartate aminotransferase to alanine aminotransferase is usually less than 1. In patients with non-alcoholic fatty liver disease, this ratio increases as fibrosis advances, leading to a loss of diagnostic accuracy [Angulo 2002]. Moreover, the diagnosis of NASH is followed by negative evaluation for chronic hepatitis C virus infection (antibody against hepatitis C virus) and hepatitis B virus infection (hepatitis B surface antigen) (Table 1) [Marleau 1998].

### 1.5.2. HISTOLOGY AND PATHOGENESIS

Histologically NASH is indistinguishable from liver damage resulting from alcohol abuse, and the diagnosis depends on the evidence of negligible alcohol consumption. However, the frequency of specific histologic findings varies in the two conditions. Characteristic findings in non-alcoholic steatohepatitis include macrovesicular steatosis, mild lobular inflammation, mixed inflammatory cell-filtration, hepatocyte degeneration, ballooning, necrosis, Mallory bodies and fibrosis (Table 2) [Angulo 2002]. Analysis of liver biopsies of over 50 patients in one series demonstrated steatosis, hepatocyte ballooning, and portal inflammation to be the most common histologic features of NASH [Yu and Keeffe 2002].

A number of conditions can be used as diagnostic criteria associated with NASH (Table 1). Primarily, these include obesity, which is reported in 40%-100% of patients with NASH, type 2 diabetes mellitus, hyperglycemia, and glucose intolerance, which have been described in 20%-75% of adult patients with NASH [Reid 2001, Yu and Keeffe 2002]. Hyperlipidemia and insulin resistance syndrome (IRS) were found in a large portion of patients, while hypertriglyceridemia, hypercholesterolemia, were present in 20%-81% of patients with NASH [Ludwig et al. 1980, Bacon et al. 1994, Angulo 1999]. Jejunoileal bypass was associated with a 40% incidence of liver function abnormalities postoperatively and with severe NASH and hepatocellular failure in up to 6% of patients [Reid 2001, Chitturi and Farrell 2001].

The combination of steatosis, infiltration by mononuclear cells or polymorphonuclear cells (or both) and hepatocyte ballooning and spotty necrosis are common features of non-alcoholic steatohepatitis. In most patients with this type of non-alcoholic fatty liver disease, fibrosis was detectable [Angulo 2002]. In obese patients, weight loss by fasting affects the level of insulin by reducing it. Paradoxically, in obese patients who had intestinal bypass surgery for weight reduction an elevation of insulin levels occurs due to the continuation of taking large amounts of food. Increased amounts of insulin perpetuate the accumulation of free fatty acids [Sheth et al. 1997].

**Table 2. Histologic features of non-alcoholic steatohepatitis**

| | |
|---|---|
| Spectrum | Steatosis- steatohepatitis- steatohepatitis with fibrosis-cirrhosis |
| Steatosis | Macrovesicular; mild to severe |
| Steatohepatitis | Iflammation: usually mild, lobular, and mixed mononuclear and neutrophilic<br>Hepatocyte degeneration: ballooning and Mallory bodies |
| Fibrosis | Initially pericellular |
| Cirrhosis | In up to 20% over 10 years |

The distinction between NASH and steatosis alone cannot be reliably made due to the absence of correlation between inflammation and the serum aminotransferase levels. Liver biopsy remains the main tool for diagnosing NASH [Clark et al. 2002, Neuschwander-Tetri and Caldwell 2003]. It excludes other causes of liver disease, allows the distinguishing of steatosis from NASH, estimates prognosis based on degree of fibrosis, determines progression of fibrosis over time. However, the role of liver biopsy is controversial. Arguments against this method are related to risks and costs of the biopsy and lack of effective therapy [Neuschwander-Tetri 2000].

The pathogenesis of NASH is poorly understood. Little is known about the factors involved in its progression from steatosis to steatohepatitis to fibrosis and cirrhosis [Younossi et al. 2002]. Nevertheless, hepatic fat accumulation, lipid peroxidation, oxidative stress with formation of free radicals, activated steallate cells, abnormal

patterns of cytokine production, and decreased synthesis or secretion of very-low-density lipoprotein represent most possible causes of this etiology [Marleau 1998, Younossi et al. 2002, Yu and Keeffe 2002].

A fatty liver without inflammation is a common histological finding in patients with protein-energy malnutrition, obesity, acute starvation, carbohydrate overload, and corticosteroid therapy. In obese patients, greater stores of free fatty acids (FFA) are detectable in adipose tissue. These patients show resistance to insulin and have normal or elevated levels of insulin, and higher plasma levels of free fatty acids. Accumulation of FFA may be responsible for liver dysfunction due to the high reactivity that can impair membrane integrity, cause mitochondrial swelling, and increase lysosomal fragility, thereby contributing to necro-inflammation [Marleau 1998].

Type 2 diabetes mellitus or insulin resistance influences the increase of adipose tissue lipolysis and free fatty acid production. As a consequence of obesity, the synthesis of free fatty acids (FFA) and triglyceride increases raising their levels in the liver. Simultaneously, insulin inhibits the oxidation of free fatty acids in the liver [Marleau 1998]. When the liver fails in triglyceride export due to the saturation of the liver, an excess of free fatty acid forms, contributing to the "fatty liver" formation. The presence of steatosis itself is not a main cause of liver injury [Yu and Keeffe]. Accumulation of excess fat in hepatic parenchyma is related to insulin resistance and seems to be the cause of oxidative stress [Younossi et al. 2002]. Toxic levels of free fatty acids amplify oxidative stress, initiating formation of free radicals. In this way, formed hydrogen peroxide and superoxide generate abnormal lipid peroxidation, initiating hepatocyte injury, inflammation, and fibrosis [Yu and Keeffe]. It is proposed that the fatty liver is injured by reactive oxygen species, generated from mitochondrial and/or other hepatocellular pro-oxidant pathways during lower antioxidant defense. Inflammation may be secondary to oxidative stress or injury, or a primary event resulting from some factor which gives rise to some other mobilizer of proinflammatory injurious cytokines [Chitturi and Farrell 2001].

The "multi-hit" theory about the role of oxidative stress in the pathogenesis of NASH suggests that the first "hit" involves the accumulation of excess fat in the hepatic

parenchyma and the second "hit" is connected to the imbalance between pro-oxidant and antioxidant processes in the liver [Younossi et al 2002]. The latter may result from the induction of cytochrome P450 (CYP2E1), mitochondrial release of reactive oxygen species (ROS), $H_2O_2$ release from peroxisomal oxidation of fatty acids and cytokines released from activated inflammatory cells. Accordingly, oxidative stress has a dual role by directly producing liver injury and indirectly activating recruitment of the inflammatory response that perpetuates injury and fibrosis (Figure 1) [Chitturi and Farrell 2001].

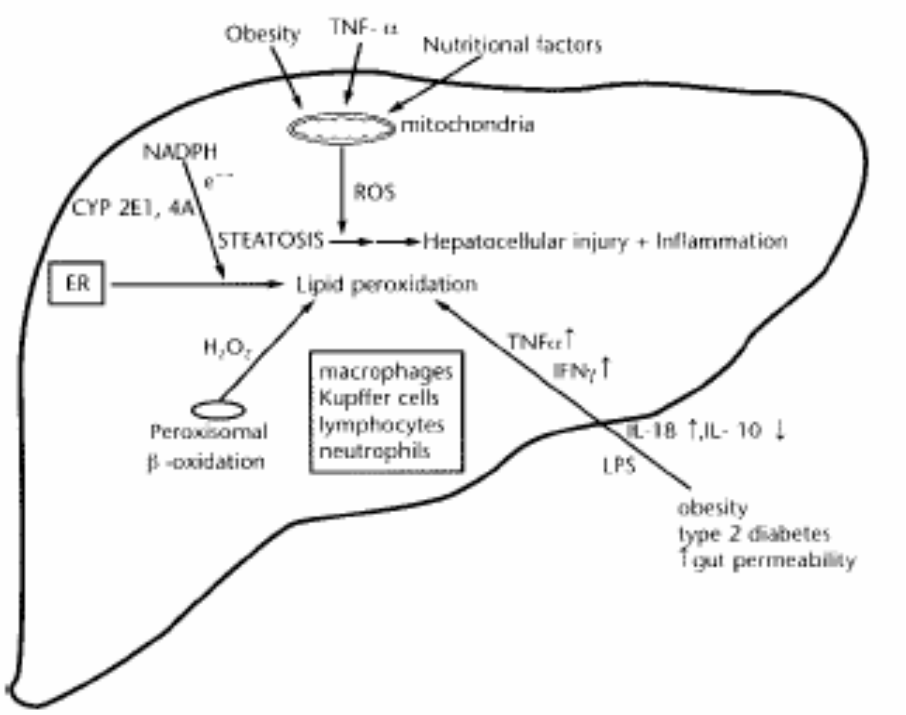


**Figure 1. Inflammation in NASH [Chitturi and Farrell 2001].** NADPH, reduced nicotinamide adenine dinucleotide phosphate; ER, endoplasmic reticulum; CYP, cytochrome; TNF-α, tumor necrosis factor-α; IFNγ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; ROS, reactive oxygen species.

The source of inflammation in human NASH remains unclear. Animal studies have shown that endotoxin (lipopolysaccharide) can produce focal necroinflammatory change in the fatty liver of mice and rats probably through TNF-α release [Hotamisligil and Spiegelman 1994]. Moreover, lipopolysaccharides have been shown to be responsible for the oxidative stress that can stimulate hepatic inflammation through the expression of TNF-α, other cytokines and chemokines (Figure 1) [Chitturi and Farrell 2001].

### 1.5.3. DIAGNOSIS AND THERAPY

Insulin resistance was found to be an important factor in the development of NASH in number of patients with this condition. It can lead to hyperinsulinemia, thus initiating and promoting steatosis. High levels of insulin possibly block mitochondrial fatty acid oxidation, leading to accumulation of triglycerides and fatty acids in the liver [Reid 2001]. The molecular pathogenesis of insulin resistance seems to be multifactorial, involving various mediators in the action of insulin [Diehl 1999, Angulo 2002].

A fatty liver, without hepatitis, is more frequently diagnosed than NASH. Even though a fatty liver alone is considered to be nonprogressive, some patients with NASH develop progressive liver disease and cirrhosis. Fatty accumulation alone is considered to be benign. However, it is not well understood why only a subgroup of patients with NASH have a progressive course [Matteoni et al. 1999]. It is possible that only some causes of steatosis can provoke histologic progression to cirrhosis or that additional insults are necessary for this outcome [Diehl 1999].

Hepatitis and cirrhosis occur much less often than steatosis. The relationships between steatosis, steatohepatitis, and fibrogenesis have not been established. It is not clear whether the accumulation of fat in the liver is responsible for the inflammation or whether inflammation induced by some stimulus causes hepatocyte dysfunction resulting in steatosis. Steatosis is not always associated with inflammation and fibrosis, and a fatty liver may be a secondary occurrence and not the initiating factor in the pathogenesis of NASH [Marleau 1998, Schaffner and Thaler 1986].

The natural history of NASH is unknown and its natural history varies according to its histologic type [Falck-Ytter et al 2001]. There is uncertainty about the finding of simple steatosis development in some patients, steatohepatitis and progressive disease development in others [Diehl 1999]. Patients with hepatic steatosis appear to have a benign clinical course without histologic progression when followed for up to 19 years; whilst patients with other histologic forms may have histologic progression associated with hepatic steallate cell activation and unfavorable clinical effects of liver injury [Falck-Ytter et al 2001].

Fibrosis in NASH involves more advanced and severe liver injury. Cross-sectional studies of 673 liver biopsies showed 66% with diagnosed fibrosis, 25% with severe fibrosis (septal fibrosis and cirrhosis) and 14% with well-established cirrhosis [Angulo 2002]. The prediction of fibrosis in patients with NASH is not well supported due to the incoherence between clinical features and existing laboratory data. Nevertheless, investigations show that older age, obesity, and diabetes mellitus are independent predictors of the degree of fibrosis [Angulo 1999]. Some research work suggested an increase of hepatic iron as a possible risk factor for fibrosis. Contradictory findings on this behalf in NASH have been accredited to the heterogeneity in patient selection, study design and sample size [Reid 2001].

The therapy for NASH has not been proven, though weight reduction and an exercise program in obese patients along with treatment of diabetes mellitus, lipid disorder and discontinuation of potentially hepatotoxic drugs are recommended [Angulo and Lindor 2001]. Experimental therapies involve ursodeoxycholic acid, vitamin E, hepatoprotective agents, and use of phlebotomy [Yu and Keeffe 2002, Reid 2001]. Better histological prognosis factors are required in steatohepatitis. A greater understanding of the factors controlling fibrogenesis would lead to new therapeutic approaches and to the management of steatohepatitis [Burt 2001].

No study has attempted to ascertain matrix metalloproteinase expression in NASH. Few studies have addressed NASH indicating its connection to progressive fibrosis and eventual cirrhosis [Yu and Keeffe 2002]. To achieve more information about this recent clinical entity, expression of MMPs in NASH was studied with the hypothesis to describe its expression pattern and to compare it to the same in viral liver diseases.

# CHAPTER 2

# Methodology

**SECTION 1**

## 2.1. PATIENTS, MATERIALS AND METHODS

### 2.1.1. PATIENTS

Needle liver biopsy specimens were obtained from 63 patients. 47 biopsies were taken from patients with chronic viral hepatitis (14 cases with chronic hepatitis B, 33 cases with chronic hepatitis C). Sixteen biopsies were taken from patients with non-viral liver disease (non-alcoholic steatohepatitis, diagnosed according to standard histological criteria). Grading and staging of liver biopsies was done as proposed by Ishak et al. [Ishak et al. 1995]. Ten liver samples taken at operation for cholecystectomy were used as normal controls. All samples were taken during diagnostic liver biopsies. After biopsy, specimens were immediately frozen in liquid nitrogen and kept at –80°C until extraction of total RNA.

**SECTION 2**

## 2.2. MATERIALS

Reagents and equipment used routinely are listed below. Details of specific purchases are given in the appropriate section.

### 2.2.1. CHEMICALS

All chemicals were analytical quality grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA), Merck (Inc. Whitehouse Station, NJ, USA) and Invitrogen (Carlsbad, CA, USA) except:
30% acrylamide, Alter Chem (Athens, Greece)

100 bp DNA Ladder, Promega (Madison, WI, USA)

Primers, MWG Biotech AG (Ebersberg, Germany)

Sea Kem LE agarose, FMC Bioproducts (Philadelphia, PA, USA)

TEMED- Fluka (Buchs, Switzerland)

### 2.2.2. ENZYMES

Enzymes were supplied with appropriate buffers and dNTPs from Invitrogen (Carlsbad, CA, USA) and Roche (Basel Switzerland).

### 2.2.3. STOCK SOLUTIONS

In further described experiments the following buffers (10X) have been used:

| | |
|---|---|
| 30 % Acrylamide: | ready solution 29:1 |
| 10% Acrylamide, resolving gel (60 ml): | 30 % acrylamide 16 ml |
| | TBE 5X 6 ml |
| | APS 0.6 ml |
| | TEMED 24 µl |
| | $H_2O$ to 60 ml |
| Acrylamide, stacking gel (10 ml): | 10 m of 10% acrylamide resolving sol. |
| | APS 150 ml |
| | TEMED 50 µl |
| $AgNO_3$ (1 L): | 1g $AgNO_3$ |
| | 1 L $H_2O$ |
| 10% Ammonium persulfate (APS): | ammonium persulfate 1g |
| | $H_2O$ to 10 ml |

The solution may be stored at 4º C for several weeks.

| | |
|---|---|
| 1M DTT: | 15.45 g DTT |
| | 100 ml H2O |
| | Store at -20°C |
| EDTA (0.5 M): | 186.1 g EDTA |
| | $H_2O$ to 800ml |
| | NaOH to adjust to pH 8.0 |

| | |
|---|---|
| Ethidium bromide (10mg/ ml): | 1g EtBr |
| | 100 ml of $H_2O$ |

Stirring on magnetic stirrer for several hours to dissolve the dye.
Keep in dark bottle at room temperature.

| | |
|---|---|
| Fixing solution (1 L): | 100 ml Ethanol (absolute) |
| | 50 ml Acetic acid (glacial) |
| | 850 ml $H_2O$ |
| Gel loading buffer (6X): | 30% glycerol in water, |
| | 0.25% xylene cyanol FF |
| | 0.25% bromophenol blue |
| $Na_2CO_3$ (0.5 N): | 18.75 g $Na_2CO_3$ |
| | 2 L $H_2O$ |
| TBE-Tris borate (5X): | 54g Tris base |
| | 27.5g Boric acid |
| | 20 ml 0.5M EDTA (pH 8.0) |

Different buffers are in use for PCR reaction and their role is to stabilize the pH and provide necessary cofactors and stabilizing substances for the effectiveness of PCR.

### 2.2.4. EQUIPMENT

Thermal cycler Gene Amp PCR System 2400, Perkin-Elmer/Applied Biosystems Division (Norwalk, CT, USA).
Spectrophotometer U-2001, UV/Vis, Hitachi Instruments Inc (Hitachi Instruments Inc, Tokyo, Japan).
Herolab E.A.S.Y. camera and UV lamp illuminator Herolab GmbH (Wiesloch, Germany).
Digital graphic printer UP860CE (Sony Electronics Inc, NJ, USA)

## SECTION 3

## 2.3. METHODS

### 2.3.1. TOTAL RNA EXTRACTION FROM HUMAN TISSUES

Total RNA was isolated from frozen tissues using the commercial solution, Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The above-mentioned solution is a mono-phasic solution of phenol and guanidine isothiocyanate. Total RNA extraction was done by a slight modification of the method proposed by Chomczynski and Sacchi [Chomczynski and Sacchi 1987] based on cell lysis using guanidine isothiocyanate, and the removal of lipids, proteins and DNA using acidic phenol. RNA isolation steps include tissue sample homogenisation in Trizol, phase separation with chloroform, RNA precipitation with isopropyl alcohol, RNA wash with ethanol and redissolvement of RNA in water. RNA is recovered after the tissue homogenization and lysis in Trizol, where cell disruption and dissolving occurs while maintaining RNA integrity. Incubation at 15° to 30° C for 5 min provides complete dissociation of nucleoprotein complex, and the addition of 1/10 of chloroform volume allows separation of the solution into an aqueous and organic phase. RNA recovery by precipitation occurs after the addition of an equal volume of isopropyl alcohol to the aqueous phase. RNA wash with 75% ethanol follows, as well as redissolving of RNA in RNase-free water. Digestion of RNA samples with DNaseI (Invitrogen, Carlsbad, CA, USA) was performed in order to discard genomic DNA.

All the aqueous solutions used during RNA isolation, as well as the $H_2O$ used for its final resuspension, have previously been treated with 0.1 % (w/v) DEPC (diethyl pyrocarbonate) for 7 hours at room temperature for tissue RNase deactivation. DEPC is deactivated by autoclaving the solution. The quality and the quantity of the isolated RNA, e.g. purity and concentration are measured by spectrophotometry at 260 and 280 nm (spectrophotometer U-2001, UV/Vis, Hitachi Instruments Inc, Tokio, Japan). The concentration of extracted RNA was calculated on the basis that a 40 μg/ml solution of single stranded RNA would have an $OD_{260}$ measurement of 1. RNA was stored in a small volume of diethyl pyrocarbonate treated $dH_2O$ at –70º C. 1% agarose gel electrophoresis and ethidium bromide staining were used to examine RNA integrity [Maniatis, Fritsch, Sambrook 1982].

### 2.3.2. RNA ELECTROPHORESIS ON AGAROSE GEL

For sample preparation for RNA electrophoresis approximately 5µg of RNA was resuspended in 50µl RNase-free water. 1 µg of RNA was applied on agarose gel for RNA identification.

Electrophoresis through gels containing 1% agarose (w/v) in 0.5X TBE was used to separate and analyse RNA molecules. To prepare 1% agarose gel 1g of agarose powder (Sea Kem LE, FMC Bioproducts, Philadelphia, PA, USA) was mixed with 100 ml of the electrophoresis buffer (0.5 X TBE) to the desired concentration, then heated in a microwave oven until completely melted. Ethidium bromide (final concentration 0.5 mg/ml) was added to the gel to facilitate visualization of RNA after electrophoresis. After cooling the solution to about 60° C, gel was poured into a casting tray containing a sample comb (10/15 tooth) and allowed to solidify (polymerise) at room temperature. After the gel has polymerised, the comb was removed and the gel was inserted horizontally into the electrophoresis chamber and submerged in the buffer. Samples containing 1 µg of RNA were mixed with 6X gel loading buffer and pipetted into the sample wells, the lid and power leads were placed on the apparatus, and a current (5V/cm) was applied. Gel was viewed and RNA bands were detected on a shortwave UV transilluminator and photographed using Herolab E.A.S.Y. camera (Herolab GmbH, Wiesloch, Germany), Sony digital graphic printer type UP860CE, Sony glossy, black and white film (Sony Electronics Inc, NJ, USA) [Sharp et al. 1973].

### 2.3.3. REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR is a sensitive method for the detection and analysis of RNAs present in low quantities or rare mRNA transcripts. RNA cannot serve as a template for PCR and therefore must be reversely transcribed into cDNA. Powell et al. first described a combined technique of a reverse transcriptase PCR in which reverse transcription (RT) is coupled with PCR amplification of the resulting cDNA [Powell et al 1987].

cDNA synthesis

For first strand cDNA synthesis, 1-3 µg of total RNA is reversibly transcribed in a 20 µl reaction volume containing 4 µl of 5x First-Strand buffer, 50 ng random hexamers, 0.2 mM dNTPs, 0.1M DTT and 200 U Reverse Transcriptase, (SuperScript II RT, Invitrogen, Carlsbad, CA, USA); incubated for 50 min at 42° C according to the instructions of the manufacturer. Reaction product is further processed with RNase H (2 U/µl) for the digestion of total RNA which did not transcribe to cDNA. After the digestion, reaction product is directly used for amplification of genomic sequences in PCR reactions (table 1).

**Table 1. Summary of the protocol for cDNA (first strand) synthesis**

| | | |
|---|---|---|
| RNA + Primer | | |
| 1 cycle | 70° C for 10 min | Sample denaturation |
| Place on ice | ⇓ | |
| | Random hexamers+ cDNA synthesis mix<br>RT polymerase | |
| | ⇓ | |
| | 25° C for 10 min | Primer annealing |
| 1 cycle | 42° C for 50 min | cDNA synthesis |
| | 70° C for 15 min | Reaction termination |
| | ⇓ | |
| | RNase H | |
| 1 cycle | 37° C for 20 min | RNA template removal |
| | ⇓ | |
| | PCR amplification | |

### 2.3.4. POLYMERASE CHAIN REACTION (PCR)

The Polymerase Chain Reaction (PCR) is a method by which within a short time, not exceeding 2-3 hours, the amplification in an exponential way of specific DNA sequences is accomplished thus making possible their study and further manipulation [Saiki et al. 1985].

The template DNA is incubated in Taq DNA polymerase containing buffer, mixture of deoxyribonucleotides and the primer pair. Initially, thermal DNA denaturation occurs at 95°C for 5 minutes and then 30-35 cycles at 95°C (denaturation), 55-60°C (primer hybridization) and at 72°C (polymerization) follow, each step lasting for approximately 30 seconds. In this way, the number of DNA copies exponentially increase according to the formula of $N=n(1+e)^c$ , where N= final product quantity, n= initial template quantity, e=reaction efficiency and c= PCR cycle number.

High reaction efficiency makes possible the amplification of the sequences, even when small number of copies are present or when DNA has been partially damaged. The specificity of PCR reaction relatively to the sequence to be amplified depends on the primers. It has been statistically found that DNA of at least 20 base pair long is unique in the genome. Consequently, in order to achieve specific amplification the primers must be 20bp long. Indeed, this is the average length of the primers used in the vast majority of PCR applications. This provides the thermodynamic stability so that at about 55°C, DNA chains are able to reassemble.

The achievement of satisfactory polymerization reactions that influence the efficiency and the reaction specificity depends on the following factors

1) choice of suitable primers
2) concentration of Mg ions, which is necessary for polymerase action
3) determination of the exact temperature for the primers to reassemble.

With a given primers pair, the standardization of the PCR reaction conditions includes experiments in which the temperature of the reassembling and the Mg ion concentration are independently changed.

For quantification of the PCR results it is necessary to stop the reaction while this is still at the exponential phase. In this way we obtain linear correlation between the final product concentration and the initial substrate concentration. Usually, pilot studies are undertaken in order to construct the standard curve.

Even more reliable is the coamplification of the target sequence with stable quantity of substrate in which the sites recognised by the primers are identical to the target sequence. In this way competition between the two sequences that are coamplified is achieved, so that target sequence is expressed as a ratio of target sequence and the band density of the competitor.

PCR conditions

Amplification of cDNA was performed in a 20 μl reaction volume containing 1 μg cDNA, 0.4 μM of each primer, 0.2 mM dNTPs, 2 μl 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500mM KCl), 1.5 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase (Ampli Taq, Roche, Basel, Switzerland). All reagents except the template and enzyme were premixed to ensure standard conditions and to avoid contamination of the reaction with unwanted DNA. The oligonucleotide primers and the annealing temperatures used for each gene, were as follows: MMP-2 (sense) 5' ATG GGG AAT CGG TTG AAG G 3 ', (antisense) 5' AAT TGC ATT TCC TGA CAG AAG 3 ', annealing temperature 58º C (Table 2). PCR primers, which amplify a 195 bp region, were derived from the MMP-2 sequence in UniSTS database (#19916). Primers for MMP-9 (sense) 5' GAG AAG AGA GGG CCC AGC 3 ', (antisense) 5' ACG TGA CCT ATG ACA TCC TGC 3 ', annealing temperature 54º C. PCR primers, which amplify a 177 bp region, were derived from the MMP-9 sequence in UniSTS database (#88486). Primers for MMP-10 (sense) 5' GGG GGA AGA CAG ATA TGG GT 3 ', (antisense) 5' CTG TTC AGT GCA ATT CAA AAG C 3 ', annealing temperature 58º C. PCR primers, which amplify a 185 bp region, were derived from the MMP-10 sequence in UniSTS database (#155979). Primers for MMP-11 (sense) 5' ACG CCA GTA GTC CCT GC 3 ', (antisense) 5' ACG GTG AAA AGC CAG TCC T 3 ', annealing temperature 63º C. PCR primers, which amplify a 134 bp region, were derived from the MMP-11 sequence in UniSTS database (#9683). All primers were purchased from MWG Biotech AG (Ebersberg, Germany). 1pg of each nucleotide

was used per reaction. The PCR program was carried out at 94º C for 30 sec for denaturation, annealing temperature depending on each set of primers for 30 sec and 72º C for 30 sec for primer elongation. Cycles were preceded by incubation for 5 min at 94º C to ensure full denaturation and followed by incubation at 72º C after the final cycle to ensure full extension of the product (Table 3). Samples were amplified through 31-35 consecutive cycles.

**Table 2. Sequences of amplification primers**

| Gene | Amplification primers | Annealing temperature |
|---|---|---|
| MMP-2 | sense: 5' ATG GGG AAT CGG TTG AAG G 3 '<br>anti-sense: 5' AAT TGC ATT TCC TGA CAG AAG 3 ' | 58° C |
| MMP-9 | sense: 5' GAG AAG AGA GGG CCC AGC 3 '<br>anti-sense: 5' ACG TGA CCT ATG ACA TCC TGC 3 ' | 54° C |
| MMP-10 | sense: 5' GGG GGA AGA CAG ATA TGG GT 3 '<br>anti-sense: 5' CTG TTC AGT GCA ATT CAA AAG C 3 ' | 58° C |
| MMP-11 | sense: 5' ACG CCA GTA GTC CCT GC 3 '<br>anti-sense: 5' ACG GTG AAA AGC CAG TCC T 3 ' | 63° C |
| β-actin | sense: 5' CAA GGC CAA CCG CGA GAA GAT G 3'<br>anti-sense: 5' CCG GCC AGC AGG TCC AGA 3' | 61° C |
| β2-microglobulin | sense: 5' AAA GAT GAG TAT GCC TGC CG 3'<br>anti-sense: 5' ACT CAA TCC AAA TGC GGC 3 ' | 57° C |

The PCR reactions were performed on a DNA thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer Applied Biosystems (Foster City, CA, USA). Preliminary experiments had established the conditions in which amplification reaction remained in the exponential phase (data not shown) and thus the results could be used for quantitation of the template. 10 μl of the PCR product was electrophosed through the 8% polyacrylamide gel, silver stained [Maniatis, Fritsch, Sambrook 1982] and the intensity of the bands was analysed by computational analysis system.

DNA synthesis

**Table 3. Protocol summary for DNA (second strand) synthesis**

| | | |
|---|---|---|
| 1 cycle | 95° C for 5 min | Initial denaturation |
| | ⇓ | |
| | 95° C for 30 sec | Denaturation |
| 30-35 cycles | 42-65° C for 50 min | Annealing |
| | 72° C for 30 sec | Extension |
| | ⇓ | |
| 1 cycle | 94° C for 5 min | Final extension |
| | ⇓ | |
| | 4° C | Reaction termination |

The quantity and the quality of RNA samples was normalised after the amplification of 214 bp of β actin RNA, using the primers (sense) 5' CAAGGCCAACCGCGAGAAGATG 3', and (antisense) 5' CCGGCCAGCAGGTCCAGA 3' with annealing temperature of 61° C. For semi-quantitation of MMP-9 samples 123 bp sequence of β2 microglobulin (β 2m) RNA were amplified using the primers (sense) 5' AAAGATGAGTATGCCTGCCG 3' and (antisense) 5' ACTCAATCCAAATGCGGC 3 ' with annealing temperature 57° C. PCR products were semi-quantitatively analysed by densitometric analysis of template versus β actin. The RNA levels of each gene were expressed as a ratio of the intensity of the bands in diseased tissues versus the corresponding level of β2 microglobulin.

### 2.3.5. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Electrophoresis through agarose or polyacrylamide gels is the standard method to separate, identify, and purify DNA fragments. The choice of the gel depends on the size of DNA to be electrophoresed and the expected band separation.

Polyacrylamide gel electrophoresis is highly effective in separation of small DNA fragments. Its high resolving power allows separation fragments that differ in size by as little as 1 bp. Polyacrylamide gels are run in a vertical configuration in a constant electric field. In presence of the free radicals supplied by ammonium persulfate (APS), stabilized by TEMED, a chain reaction occurs in which monomers of acrylamide are polymerized into long chains. Addition of methylenebisacrylamide initiates cross-linking of the chains and gel formation. 1 molecule of cross-linker is included for every 29 monomers of acrylamide (1:29)

PAGE gel preparation conditions

Nondenaturing PAGE gel (10% w/v) is used for the separation and purification of fragments of double-stranded DNA. It is poured and run in 1X TBE (10.8 g/l Tris pH 8.0, 0.002 EDTA) at low voltage (1-8 V/cm) to prevent denaturation of small fragments of DNA by heat generated by electric current passage. Polymerisation of the gel is achieved by the addition of 25 µl of TEMED and 10% solution of ammonium persulfate (APS) for 60 ml of acrylamide solution.

In order to be electrophoresed, the DNA sample is resuspended in a loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water. The location of PCR products is determined directly by silver staining.

### 2.3.6. DNA SILVER STAINING IN PAGE GEL

The method is applied when DNA is electrophoresed in polyacrylamide gel and it offers high sensitivity staining. The process relies on differential reduction of silver ions that are bound to the side chains of amino acids [Switzer et al. 1979, Oakley et al.

1980, Ochs et al. 1981]. Its advantage is that it dyes double stranded DNA as well as single stranded DNA, RNA and proteins. Very clean and specific DNA needs to be used in this method. Silver staining is usually applied for electrophoresis of PCR products.

Acrylamide gel is placed on a shaker in fixing solution containing 10% ethanol and 0.5% v/v acetic acid for 10 min. The addition of silver nitrate 0.1 % (w/v) dyes the gel by silver ions bonding to the nucleic acid during 20 min in the dark. Washing the salts with water and the addition of the developing solution containing 0.4 N NaOH and 0.15% (v/v) formaldehyde allows bands detection on gel. Termination of the reaction is achieved by the addition of sodium carbonate 0.75% (w/v).

### 2.3.7. DENSITOMETRIC ANALYSIS

Acrylamide gels are transferred to the scanning device (Agfa Snap Scan 1212u, Agfa Geveaert N.V, Mortsel, Belguim) for the gel picture transfer to the computing device. Integrated density, ID=(Mean OD-Background OD)* pixels; OD= optical density) of DNA bands is defined by computational programs and is used as a parameter for DNA quantification.

Densitometry was used for semi-quantitation of the expression level of each metalloprotease. The density of the DNA fragment obtained during RT-PCR of MMP-2, 9, -10, -11 was expressed as a ratio of the density of actin/β2-microglobuline to the PCR product density. Quantity of each RNA in normal liver specimens was assigned a value of 100%.

### 2.3.8. STATISTICAL ANALYSIS

Results were expressed as mean $\pm$ SE. Non-parametric tests (Kruskal-Wallis and Mann-Whitney) were performed to examine differences of the mean metalloprotease expression amongst diseases. The significance level was set at $p< 0.05$.

# CHAPTER 3

## Results

**SECTION 1**

## 3. RESULTS

During liver fibrosis the pathological accumulation of extracellular matrix occurs as a consequence of the modifications that follow the synthesis and degradation of matrix proteins. Matrix metalloproteinases (MMPs) specifically degrade extracellular matrix (ECM) and are involved in tissue remodeling during fibrotic and inflammatory processes [Takahara et al. 1997]. A large number of data indicates the importance of MMPs in the processes of ECM and basement membrane degradation, though the role of specific matrix-degrading enzymes is not yet clear [Uria and Lopez- Otin 2000, Nagase 1999]. Evidence for the involvement of MMPs in various disease processes that take place in the liver has been provided by a number of studies [Arthur 1990, Emonard and Grimaud 1990].

The activity of these endopeptidases is tightly co-ordinated at various levels including transcription, secretion, proenzyme activation and inhibition [Benyon et al. 1996]. This study was carried out to obtain more information on the role of MMPs in the cascade of cellular and molecular events, which alter liver pathohistology. For that purpose expression levels of metalloproteases were assessed on transcriptional level. In particular, to establish the presence of MMP-2, -9, -10, -11 total RNA in the examined tissues from patients with hepatitis B, hepatitis C and non-alcoholic steatohepatitis (NASH) reverse transcription polymerase chain reaction (RT-PCR) was performed.

### 3.1. PATIENTS AND CONTROLS

The patients involved in this study were 63 adults (22 males and 41 females) of median age 48 (range 22-77). Diagnosis of liver diseases and histology is given in Table 1.

Ten liver samples taken at operation for cholecystectomy were used as healthy controls. All other samples were taken during diagnostic liver biopsies. The Ethics Committee of the University Hospital approved the protocol.

**Table 1. Diagnosis and histology of patient liver samples**

| Histologic diagnosis | Ishak score |
|---|---|
| Chronic mild hepatitis | Ishak score up to 3, 18 male and 10 female patients |
| Chronic moderate hepatitis | Ishak score up to 5, 11 male and 8 female patients |
| Chronic severe hepatitis | None |
| Cirrhosis | None |
| Non Alcoholic Steatohepatitis | None, 12 male and 4 female patients |

### 3.2. EXPRESSION OF MMP-2, -9, -10, -11 IN CONTROLS

Currently, a small number of research studies have assessed MMP expression in healthy livers [Giambernardy et al. 1998, Lichtinghagen et al. 1995, Lichtinghagen et al. 1998, Lichtinghagen et al. 2003, Terada et al. 1996]. We aimed to verify the expression of all studied MMPs in control liver tissues. RT-PCR amplification achieved the detection of size specific products corresponding to total RNA fragments for each studied MMP.

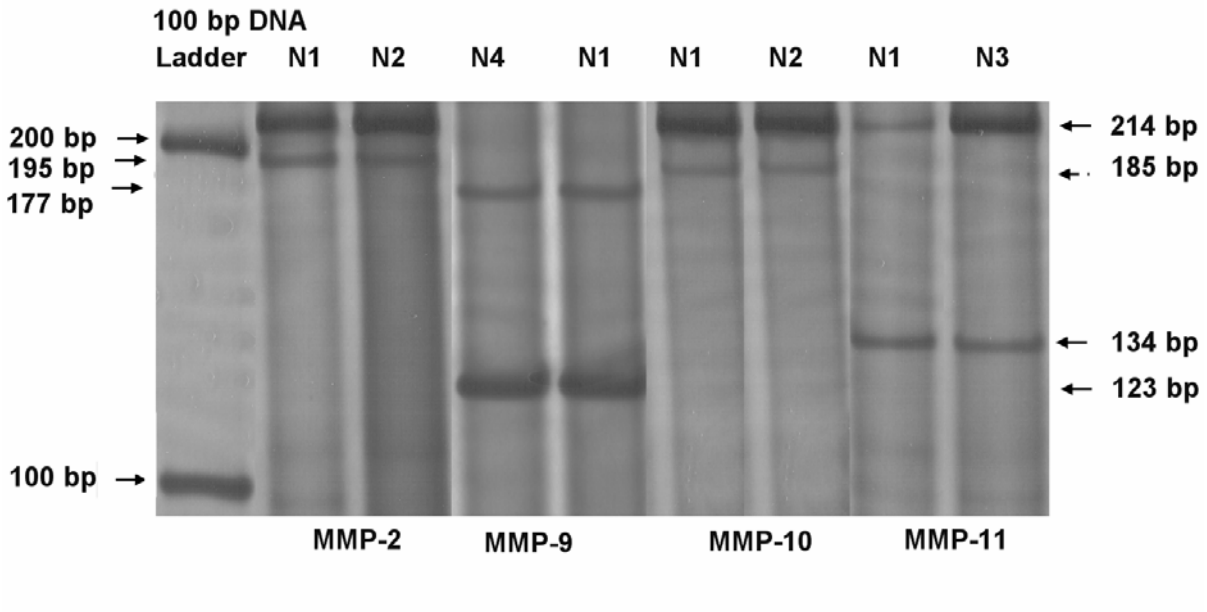


**Figure 1. Representative examples of PCR assays for MMP-2, -9, -10 and -11 expression in controls.** Bands shown refer to MMP-2, 195 bp; MMP-9, 177 bp; MMP-10, 185 bp; MMP-11, 134 bp; β-actin, 214 bp and β2-microglobuline 123bp.

Figure 1 shows amplification products for all studied MMPs obtained after RT-PCR reaction of the appropriate RNAs from normal liver tissues. β αctin (214 bp) and β2 microglobulin (123 bp) were used as internal controls. Molecular mass marker was 100bp DNA Ladder. 100 bp and 200 bp band sizes are shown.

Overal, RT-PCR detected the presence of total RNA for all MMPs studied and showed that both livers from patients and healthy controls yielded amplification products of the appropriate sizes for MMP-2, MMP-9, MMP-10 and MMP-11 genes (Figure 1-5).

### 3.3. EXPRESSION OF METALLOPROTEINASES IN PATIENTS

#### 3.3.1. MMP-2

Various studies have shown the implication and presence of gelatinase A in chronic viral liver diseases [Takahara et al.1997, Koulentaki et al. 2002, Boeker et al. 2002, Kuo et al. 2000, Milani et al. 1994]. Our goal was to verify its expression in diseased livers and define the expression levels in viral hepatitis and non-alcoholic steatohepatitis.

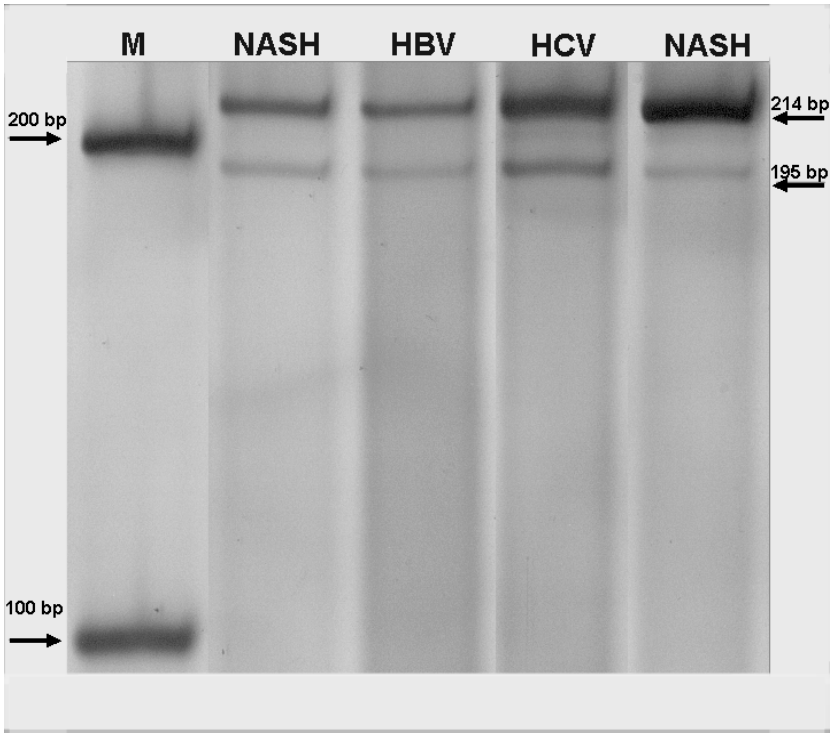


**Figure 2. Representative examples of PCR assays for MMP-2 expression in patients**.

Semi quantitative identification of MMP-2 transcripts in total RNA using RT-PCR technique resulted in gene expression. Results revealed the presence of fragments, which are size specific only for total RNA of MMP-2 gene (195 bp product) in both patients and controls. Molecular mass marker was a 100bp DNA Ladder. β-actin (214 bp) was used as an internal control (Figure 2).

### 3.3.2. MMP-9

Research studies have shown the implication and presence of gelatinase B in a large number of cases of chronic viral liver diseases [Lichtinghagen et al. 1998, Koulentaki et al. 2002, Kuo et al. 2000]. We aimed to confirm the expression of MMP-9 in patients and to identify the expression level in viral hepatitis and non-alcoholic steatohepatitis patients.

The expression of MMP-9 transcripts was identified after application of RT-PCR. Both groups of patients with hepatic disorders were analysed. Results demonstrate the presence of size specific fragments for total RNA of MMP-9 gene (177 bp) in all patient groups. β2-microglobulin (123 bp) was used as an internal control. Molecular mass marker was a 100bp DNA Ladder (Figure 3).

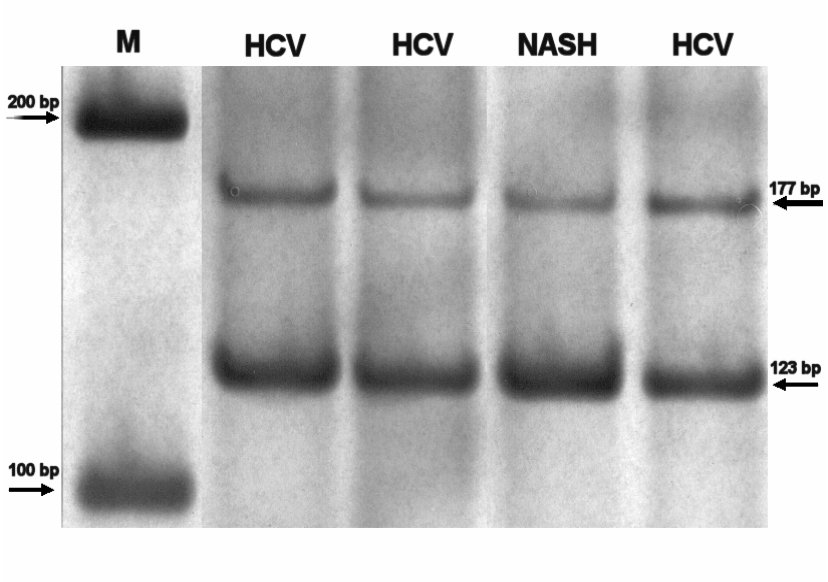


**Figure 3. Examples of RT-PCR assays for MMP-9 expression in patients.**

### 3.3.3. MMP-10

Research studies of stromelysin 2 expressions are not abundant. Nevertheless, several reports show its presence in hepatocelular carcinoma associated with chronic viral liver diseases [Knittel et al.1999, Bodey et al.2000, Lichtinghagen et al. 1998, Lichtinghagen et al. 1995]. With this analysis we confirmed the expression of MMP-10 and defined its level in all patients.

Our results demonstrate the presence of fragments for MMP-10 gene (185bp). Gene expression was obtained in patients with chronic viral HBV, HCV, non-viral and steatohepatitis. β-αctin (214 bp) was used as an internal control. 100bp DNA Ladder served as molecular mass marker (Figure 4).

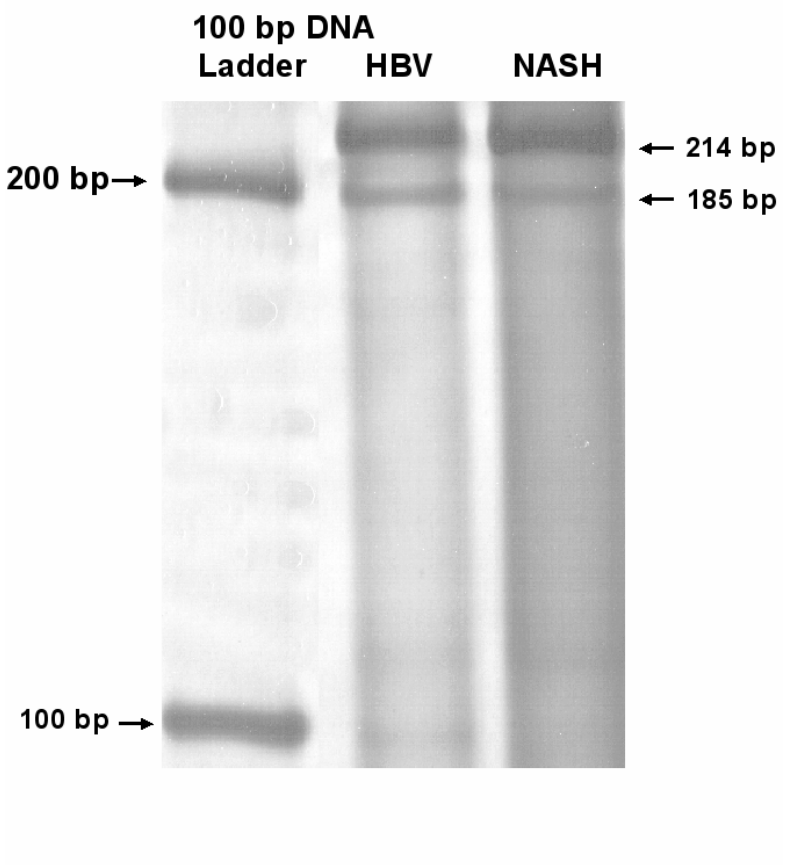


**Figure 4. Representative examples of RT-PCR assays for MMP-10 expression in patients.**

### 3.3.4. MMP-11

Expression of stromelysin 3 was studied in very few research works most of which examined cirrhosis [Lichtinghagen et al. 1998, Geisler et al. 1997].

Our findings show gene expression for MMP-11. 134 bp PCR products yielded after RT-PCR with primers specific for MMP-11 gene (Figure 5). β- actin (214 bp) was used as an internal control. 100bp DNA Ladder served as molecular mass marker. Size specific products of RT-PC confirm the presence of MMP-11 total RNA from viral and non-viral patients.

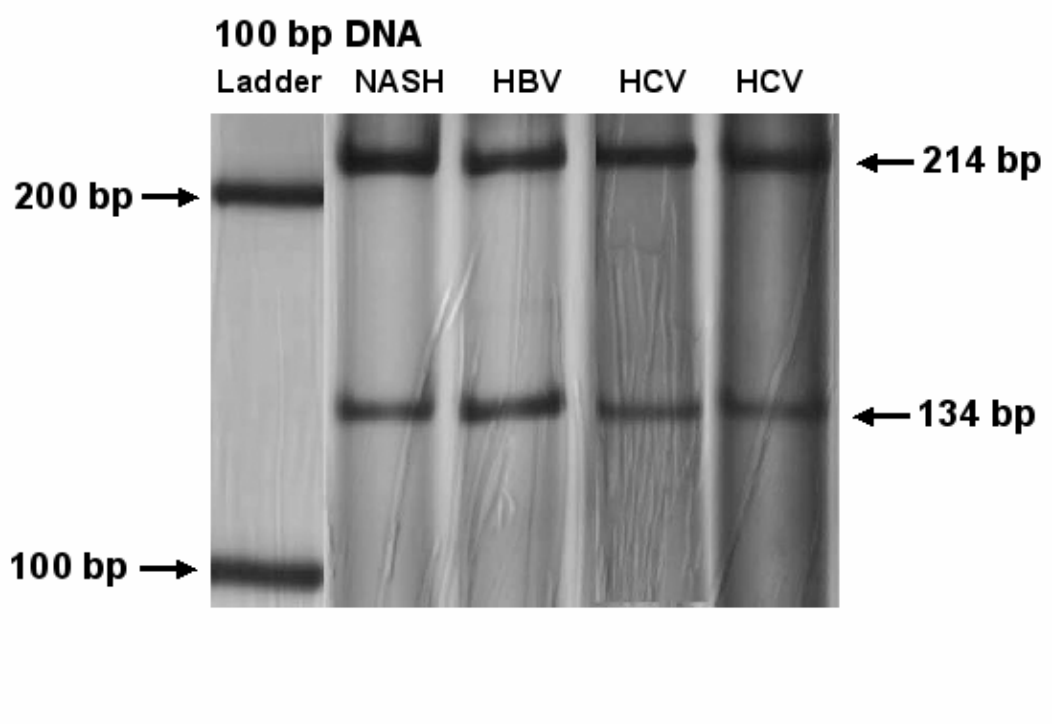


**Figure 5. Representative examples of RT-PCR assays for MMP-11 expression in patients.**

### 3.3.5. EXPRESSION LEVELS FOR STUDIED MMPS

Expression levels for studied metalloproteases were defined based on the percentage of their expression in patient samples (Table 2). The mean of normal samples was taken as normal expression and samples over these levels were considered as overexpressing the enzyme, while samples below this level were considered as underexpression.

**Table 2. Percentage of expressed metalloproteinases in patients. Over and under expression are defined in comparison to the control samples**

| Expression | MMP-2 | MMP-9 | MMP-10 | MMP-11 |
|---|---|---|---|---|
| Overexpressed | 66,1 % | 57,4% | 29,8% | 13,5% |
| Underexpressed | - | 0,7% | 66,7% | 57,7% |
| Normal | 33,9% | 1,9% | 3,5% | 28,8% |

Analysis of MMP expression in all patient groups showed an overexpression for MMP-2 in most patients (66.1%). MMP-9 expression gave similar results, showing overexpression in 57.4% of the patients. Unlike MMP-2, a considerable number of cases (40.7%) demonstrated MMP-9 underexpression (Table 2). By contrast, most patients displayed MMP-10 and MMP-11 underexpression (66.7 % and 57.7 % of the patients, respectively).

## SECTION 4

### 3.4. METALLOPROTEINASE EXPRESSION IN PATIENTS AND CONTROLS

### 3.4.1. MMPS EXPRESSION IN NASH AND CONTROLS

Non-alcoholic steatohepatitis was examined on MMP expression. MMP expression findings in NASH were compared with values in healthy controls (Figure 6). Statistical analyses did not find significant differences.

**Table 3. Mann-Whitney analysis of metalloproteinase expression in NASH and controls**

| OD MMP/OD internal control | Patiens/Controls | Mean ± SD | Median (Range) | U (p)[(a)] |
|---|---|---|---|---|
| OD mmp-2/OD a | NASH | 0,322±0,266 | 0,259 (0,072-0,972) | U= 18,0 Z=-1,325 (p=0,185) |
| | Controls | 0,131±0,046 | 0,114 (0,099-0,197) | |
| OD mmp-9/OD b2m | NASH | 1,161±0,831 | 1,014 (0,075-2,729) | U= 6,0 Z=-1,637 (p=0,102) |
| | Controls | 0,288±0,042 | 0,267 (0,259-0,336) | |
| OD mmp-10/OD a | NASH | 0,508±0,323 | 0,423 (0,077-1,297) | U= 21,0 Z=-0,178 (p=0,859) |
| | Controls | 0,479±0,020 | 0,470 (0,465-0,502) | |
| OD mmp-11/OD a | NASH | 0,337±0,334 | 0,303 (0,033-1,240) | U= 12,0 Z=-1,456 (p=0,145) |
| | Controls | 0,429±0,090 | 0,396 (0,362-0,560) | |

[(a)] Mann-Whitney, non-parametric test.

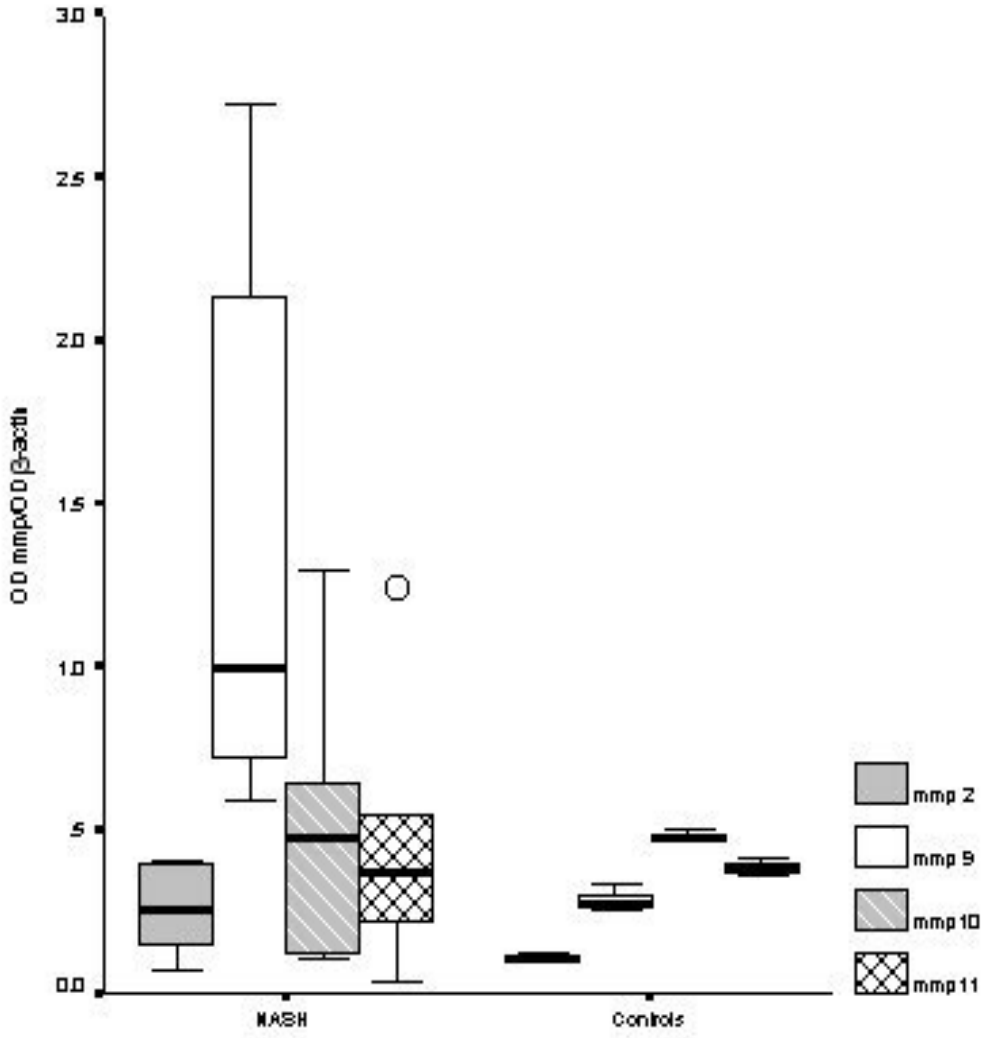


**Figure 6. MMP expression in NASH and controls.**

### 3.4.2. MMPs EXPRESSION IN HBV AND CONTROLS

The expression of metalloproteases in HBV and healthy controls was assessed by Mann-Whitney analysis. No statistically significant differences were observed on MMPs expression (Table 4).

**Table 4. Mann-Whitney analysis of metalloproteinase expression in HBV and controls**

| OD MMP/OD internal control | Patiens/Controls | Mean ± SD | Median (Range) | U (p)[(a)] |
|---|---|---|---|---|
| | **HBV** | 0,384±0,321 | 0,260 (0,072-1,083) | U= 14,0 |
| **OD mmp-2/OD a** | **Controls** | 0,131±0,046 | 0,114 (0,099-0,197) | Z=-1,216 (p=0,224) |
| | **HBV** | 0,516±0,711 | 0,100 (0,075-2,139) | U= 9,0 |
| **OD mmp-9/OD b2m** | **Controls** | 0,288±0,042 | 0,267 (0,259-0,336) | Z=-1,215 (p=0,224) |
| | **HBV** | 0,320±0,357 | 0,152 (0,077-1,282) | U= 6,0 |
| **OD mmp-10/OD a** | **Controls** | 0,479±0,020 | 0,470 (0,465-0,502) | Z=-1,702 (p=0,089) |
| | **HBV** | 0,303±0,370 | 0,128 (0,033-1,140) | U= 13,0 |
| **OD mmp-11/OD a** | **Controls** | 0,429±0,090 | 0,396 (0,362-0,560) | Z=-1,344 (p=0,179) |

[(a)] Mann-Whitney, non-parametric test.

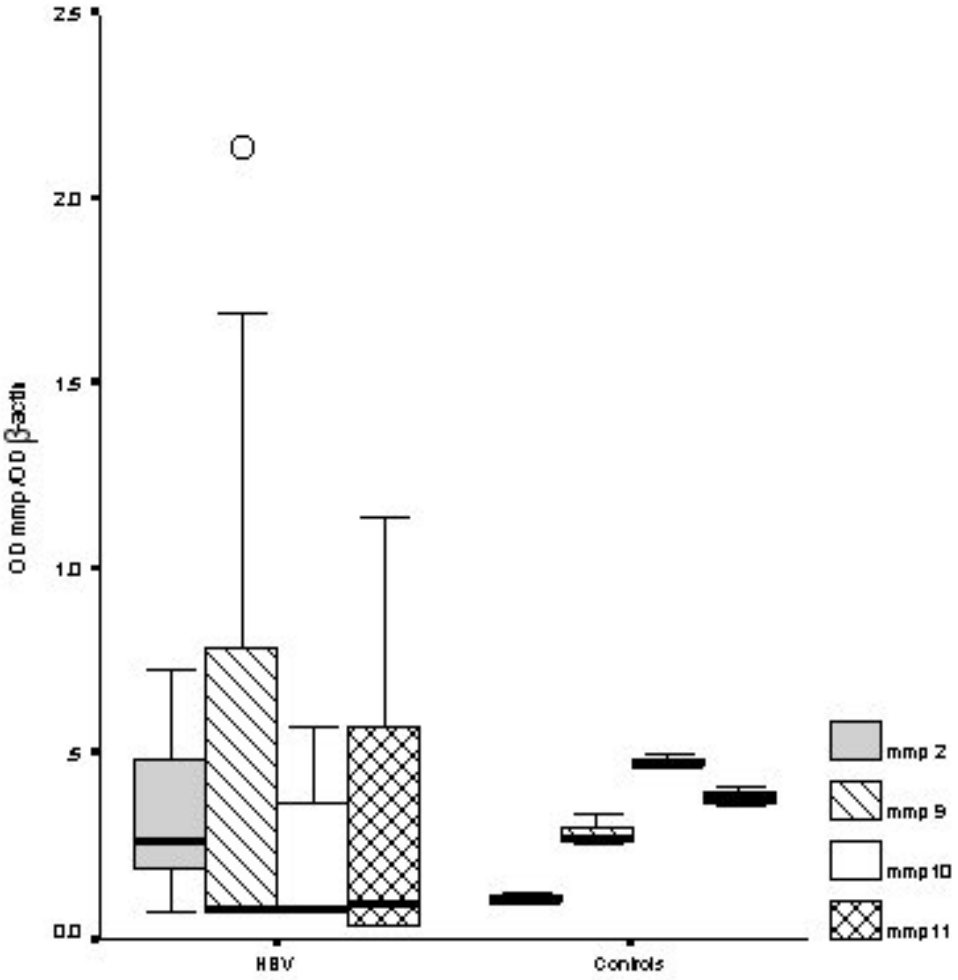


**Figure 7. MMP expression in HBV and controls.**

### 3.4.3. MMPS EXPRESSION IN HCV AND CONTROLS

In patients with hepatitis C virus infection the expression of metalloproteases was measured and compared to its expression in healthy controls. Mann-Whitney analysis revealed statistically significant differences for MMP-2 expression (U=10, p=0,009) (Table 5).

**Table 5. Mann-Whitney analysis of metalloproteinase expression in HCV and controls**

| OD MMP/OD internal control | Patiens/Controls | Mean ± SD | Median (Range) | U (p)[(a)] |
|---|---|---|---|---|
| | **HCV** | 0,490±0,505 | 0,284 (0,072-2,388) | U= 10,0 |
| **OD mmp-2/OD a** | **Controls** | 0,131±0,046 | 0,114 (0,099-0,197) | Z=-2,621 (p=0,009) |
| | **HCV** | 0,698±0,716 | 0,453 (0,075-2,452) | U= 30,0 |
| **OD mmp-9/OD b2m** | **Controls** | 0,288±0,042 | 0,267 (0,259-0,336) | Z=-0,559 (p=0,576) |
| | **HCV** | 0,390±0,338 | 0,336 (0,077-1,585) | U= 21,0 |
| **OD mmp-10/OD a** | **Controls** | 0,479±0,020 | 0,470 (0,465-0,502) | Z=-1,161 (p=0,246) |
| | **HCV** | 0,391±0,286 | 0,223 (0,033-1,021) | U= 31,0 |
| **OD mmp-11/OD a** | **Controls** | 0,429±0,090 | 0,396 (0,362-0,560) | Z=-1,043 (p=0,297) |

[(a)] Mann-Whitney, non-parametric test.

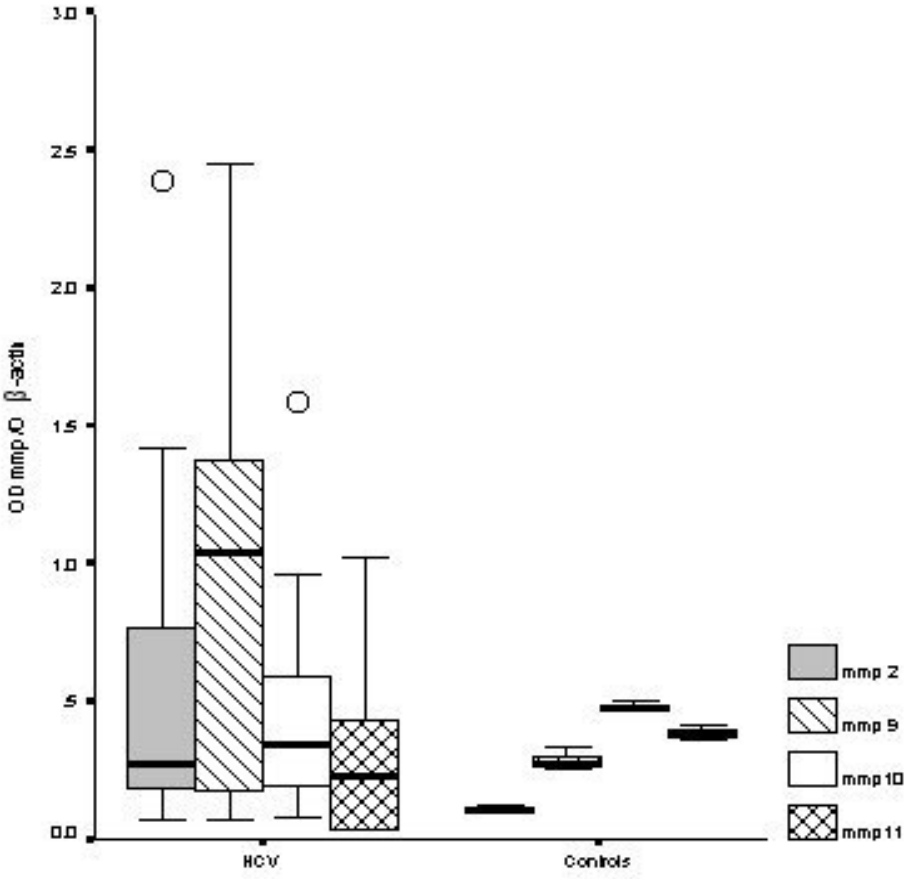


**Figure 8. MMP expression in HCV and controls.**

**SECTION 1**

### 3.5. QUANTIFICATION OF METALLOPROTEINASES IN VIRAL HEPATITIS AND NASH

Multiple studies were carried out on metalloprotease research in hepatic diseases [Boeker et al. 2002, Koulentaki et al. 2002, Kuo et al 2000, Lichtinghagen et al. 1998]. This is not the case with NASH even though it has been recognised as an important pathological entity. For a better understanding of the connection and relationship between these two entities we quantified and compared MMPs expression in relation to them.

Cases of HBV and HCV related chronic viral hepatitis were grouped together. Estimation of differences of MMP RNA quantities between viral hepatitis (HCV+HBV) and steatohepatitis (NASH) was made using Mann-Whitney test. Descriptive statistics and results of non-parametric analysis are presented in Table 6.

**Table 6. Mann-Whitney analysis of MMP expression between viral hepatitis and non-alcoholic steatohepatitis**

| OD MMP/ OD internal control | Patiens | Mean ± SD | Median (Range) | U (p)[(a)] |
|---|---|---|---|---|
| OD mmp-2/OD a | HBV+HCV | 0,457±0,455 | 0,284 (0,072-2,388) | U= 287 Z=-0,854 (p=0,393) |
| | NASH | 0,322±0,266 | 0,259 (0,072-0,972) | |
| OD mmp-9/OD b2m | HBV+HCV | 0,642±0,710 | 0,410 (0,075-2,452) | U= 136 Z=-1,859 (p=0,050) |
| | NASH | 1,161±0,831 | 1,014 (0,075-2,729) | |
| OD mmp-10/OD a | HBV+HCV | 0,368±0,341 | 0,327 (0,077-1,585) | U=189 Z=-1,910 (p=0,049) |
| | NASH | 0,508±0,323 | 0,423 (0,077-1,297) | |
| OD mmp-11/OD a | HBV+HCV | 0,295±0,311 | 0,214 (0,033-1,140) | U= 184,5 Z=-0,764 (p=0,445) |
| | NASH | 0,337±0,334 | 0,303 (0,033-1,240) | |

[(a)] Mann-Whitney, non-parametric test.

### 3.5.1. MMP-2

MMP-2 showed slightly higher levels in viral hepatitis compared to non-viral hepatitis. No significant difference was found by t-test and the Mann- Whitney test (U=287, p=0.393).

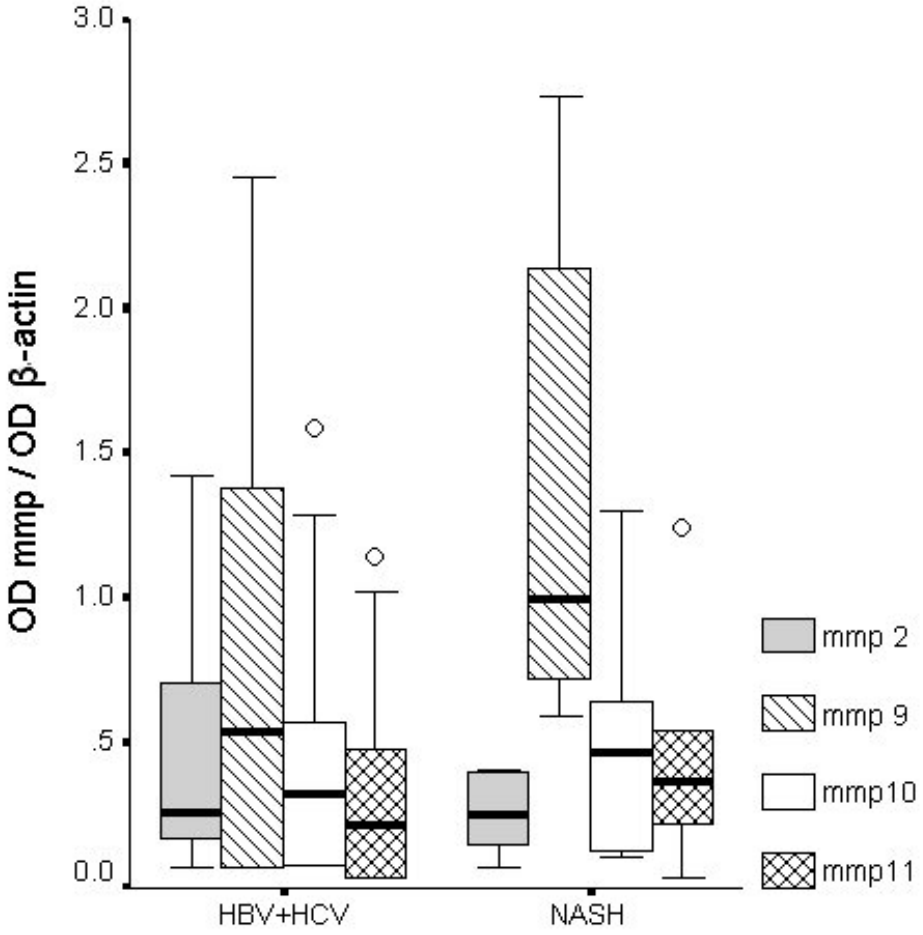


**Figure 9. Boxplot for MMP-2,-9,-10,-11 expression in viral and non-viral hepatitis.**

### 3.5.2. MMP-9

Comparative study of expression levels between viral hepatitis and non-viral hepatitis showed a statistically significant difference on MMP-9 expression (t=--2,062, df=48, p=0.045). Mann-Whitney test showed a significant difference on MMP-9 expression (U=136, p=0.050) and MMP-10 expression (U=189, p=0.049). Considerable increase of MMP-9 gene expression in non-alcoholic steatohepatitis compared to viral hepatitis patients is shown on Figure 9.

### 3.5.3. MMP-10

Mann-Whitney test showed a significant value of U=189, p=0.049 for MMP-10 expression in comparative study of expression levels between viral and non-viral hepatitis. Figure 9 demonstrates a major increase in MMP-10 gene expression in non-alcoholic steatohepatitis compared to viral livers.

### 3.5.4. MMP-11

MMP-11 exhibited a slightly higher level of expression in viral hepatitis compared to non-viral hepatitis. No significant difference was found by t-test and the Mann-Whitney test (U=184.5, p=0.445). Expression of MMP-11 in the viral liver disorders and non-alcoholic steatohepatitis is displayed in Figure 9.

## SECTION 6

### 3.6. QUANTIFICATION OF MMPS ACCORDING TO VIRAL AETIOLOGY AND HISTOLOGICAL STAGING

Patients with chronic viral HBV and HCV were analysed and the expression of MMPs was analysed in each hepatitis carrier group and compared to the controls. Patients were divided into two study groups based on histological staging according to Ishak [Ishak et al. 1995]. The first group included patients with the early disease stages (stages 1-3) and the second group encompassed patients with the later disease stages (stages 4-5). Staging according to Ishak expresses the degree of fibrosis while grading expresses the degree of inflammation.

### 3.6.1. EXPRESSION IN HBV

Expression of MMPs in HBV was studied and differences were examined using Anova and Kruskal-Walis test (Table 7). A differential expression pattern was identified between HBV and HCV related disease. In HBV patients MMP-2, -10 and 11 exhibit higher expression levels in later stages of the disease development. By contrast, MMP-9 expression was increased in the initial stages following a descending

pattern towards later hepatitis stages. These differences did not reach a statistical significance (Figure 10).

**Table 7. Kruskal-Wallis test of MMP expression in HBV based on disease stages**

| OD MMP/ OD internal control | Patients/Controls | Mean ± SD | Median (Range) | U (p)[(b)] |
|---|---|---|---|---|
| | **HBV stage 1-3** | 0,505±0,391 | 0,478 (0,072-1,083) | $\chi^2$= 2,544 |
| **OD mmp-2/OD a** | **HBV stage 4-5** | 0,281±0,229 | 0,478 (0,072-0,589) | (p=0,280) |
| | **Controls** | 0,131±0,046 | 0,159 (0,099-0,197) | |
| | **HBV stage 1-3** | 0,816±0,905 | 0,458 (0,075-2,139) | $\chi^2$= 2,951 |
| **OD mmp-9/OD b2m** | **HBV stage 4-5** | 0,216±0,280 | 0,075 (0,075-0,776) | (p=0,229) |
| | **Controls** | 0,288±0,042 | 0,267 (0,259-0,336) | |
| | **HBV stage 1-3** | 0,303±0,484 | 0,077 (0,077-1,282) | $\chi^2$= 3,093 |
| **OD mmp-10/OD a** | **HBV stage 4-5** | 0,338±0,214 | 0,408 (0,077-0,577) | (p=0,243) |
| | **Controls** | 0,479±0,020 | 0,470 (0,465-0,502) | |
| | **HBV stage 1-3** | 0,260±0,288 | 0,160 (0,033-0,848) | $\chi^2$=1,873 |
| **OD mmp-11/OD a** | **HBV stage 4-5** | 0,364±0,493 | 0,035 (0,033-1,140) | (p=0,392) |
| | **Controls** | 0,429±0,090 | 0,396 (0,362-0,560) | |

[(b)] Kruskal-Wallis, non-parametric test.

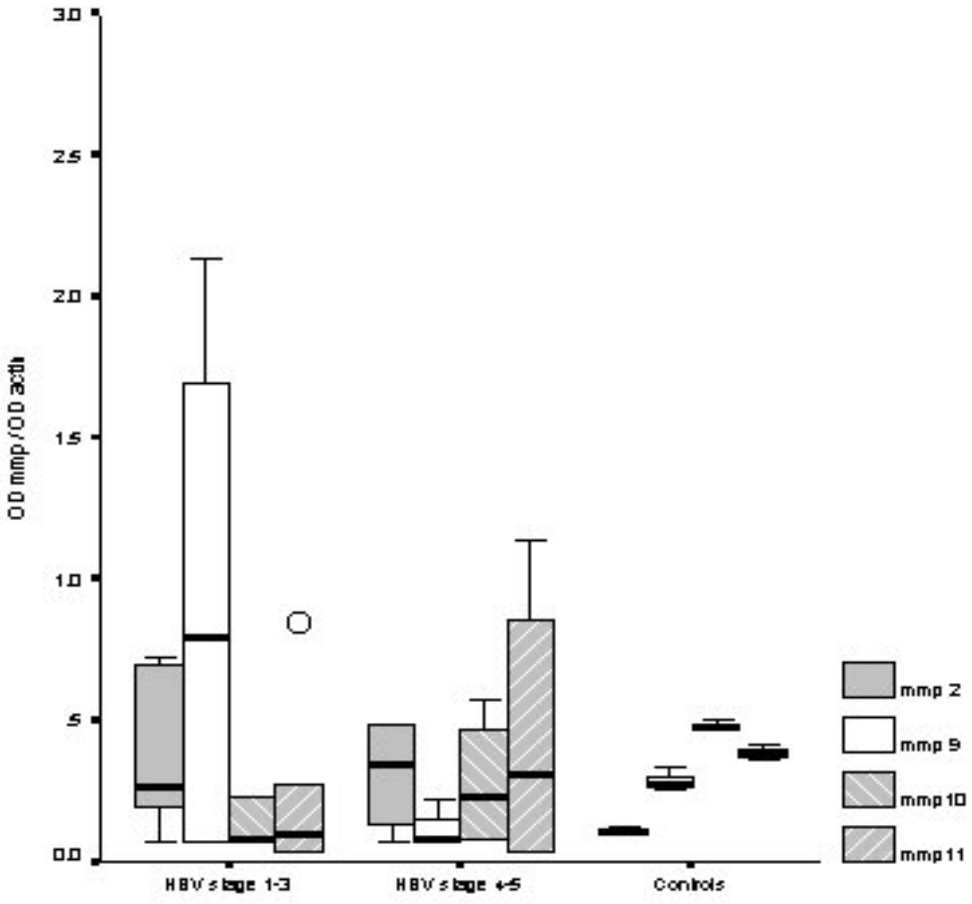


**Figure 10. MMPs expression according to disease staging in chronic HBV.**

### 3.6.2. EXPRESSION IN HCV

In HCV related liver disease metalloproteinases showed a different expression pattern. A stronger presence of gelatinases in final disease stages was detected, while the opposite was observed for the stromelysins. Stromelysins followed a decreasing pattern during disease development. Statistical significance between HCV patients and control samples was identified for MMP-2 expression in the final stages of the disease ($p<0.05$) (Figure 11).

**Table 8. Kruskal-Wallis test of metalloproteinase expression in HCV based on disease stages**

| OD MMP/ OD internal control | Patients/Controls | Mean ± SD | Median (Range) | U (p)[(b)] |
|---|---|---|---|---|
| | **HCV stage 1-3** | 0,456±0,376 | 0,292 (0,072-1,421) | $\chi^2$= 6,214 |
| **OD mmp-2/OD a** | **HCV stage 4-5** | 0,565±0,739 | 0,266 (0,072-2,388) | (p=0,045) |
| | **Controls** | 0,131±0,046 | 0,159 (0,099-0,197) | |
| | **HCV stage 1-3** | 0685±0,637 | 0,630 (0,075-2,344) | $\chi^2$= 0,328 |
| **OD mmp-9/OD b2m** | **HCV stage 4-5** | 0,718±0,870 | 0,434 (0,075-0,452) | (p=0,849) |
| | **Controls** | 0,288±0,042 | 0,267 (0,259-0,336) | |
| | **HCV stage 1-3** | 0,440±0,366 | 0,336 (0,077-1,585) | $\chi^2$= 2,491 |
| **OD mmp-10/OD a** | **HCV stage 4-5** | 0,309±0,288 | 0,252 (0,077-0,966) | (p=0,288) |
| | **Controls** | 0,479±0,020 | 0,470 (0,465-0,502) | |
| | **HCV stage 1-3** | 0,325±0,292 | 0,400 (0,033-1,021) | $\chi^2$= 2,822 |
| **OD mmp-11/OD a** | **HCV stage 4-5** | 0,210±0,275 | 0,033 (0,033-0,770) | (p=0,244) |
| | **Controls** | 0,429±0,090 | 0,396 (0,362-0,560) | |

[(b)] Kruskal-Wallis, non-parametric test.

A statistically significant increase on MMP-2 expression in different stages of HCV was observed using Kruskal-Wallis test ($\chi^2$=6,21, df=2, p=0,045) (Table 8).

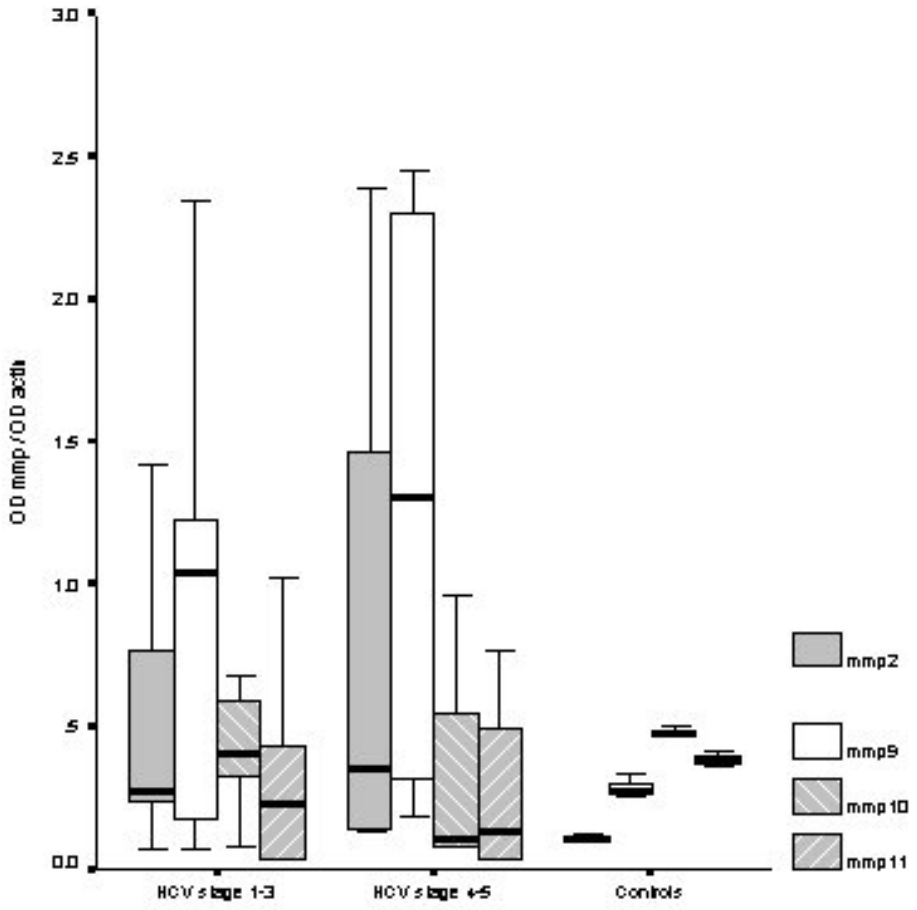


**Figure 11. MMPs expression according to disease staging in chronic HCV.**

# CHAPTER 4

# Discussion

**SECTION 1**
# 4. DISCUSSION

Liver fibrosis occurs as a result of an excessive accumulation of extacellular matrix components. Loss of normal liver cell function due to the over-accumulation of extracellular matrix (ECM) components alters ECM metabolism in the fibrotic liver. Of the several families of ECM-degradative enzymes, matrix metalloproteinases (MMPs) are the most important [Kossakowska et al. 1998]. This group of neutral proteinases requiring zinc ions for their activity emerge as a mediator of normal and disease related tissue remodelling processes. Increasing information about these enzymes is becoming available concerning their primary structure, activation of proenzymes and modulation of active enzyme by tissue inhibitors [Geisler et al 1995]. In contrast, their expression and regulation at the RNA level in normal and diseased tissues is poorly understood.

## 4.1. OVERALL EXPRESSION OF STUDIED MMPS

Our results revealed general overexpression of gelatinases and underexpression of stromelysins in all patient groups. In non-diseased human livers, a considerable number of studied specimens were expressing MMP-2 and MMP-11, while a very small number was expressing MMP-10 and MMP-9. The observed variance in metalloproteinases mode of expression in patient groups and controls could be a result of the effect of hepatic inflammation on MMPs action underlying the inter-individual diversity between these enzymes that was previously mentioned in similar studies [Lichtinghagen et al 1998].

### 4.1.1. EXPRESSION OF METALLOPROTEINASES IN CONTROL SAMPLES

The metalloproteinases network has not been adequately studied in human liver fibrosis. Most studies have been focused on the involvement of these enzymes in hepatocellular cancer development and metastasis [Bodey et al. 200, McKenna et al. 2000, Giannelli et al. 2002, Theret et al. 2001, Terada et al. 1996]. No research findings are available on the role of metalloproteinases in non-alcoholic steatohepatitis (NASH). Few studies have assessed their expression in chronic hepatitis with conflicting results reporting a reduction in MMP-9 circulating levels in

some of the studies and elevation of mRNA levels in other [Lichtgehagen et al. 1999, Kuo et al. 2000, Lichtinghagen et al. 2001, Lichtinghagen et al. 2003]. In the present study we attempted to identify possible expression differences in viral and non-viral liver disease of different aetiologies and also to see the expression of key metalloproteinases in the different fibrotic stages of viral liver diseases.

#### 4.1.1.1. MMP-2, -9, -10 AND -11

The expression of matrix metalloproteinases was assessed by limited number of studies in normal and non-diseased liver specimens. Few research studies measured MMP-2 gene expression in normal human liver detecting it in small quantities [Geisler et al. 1997, Takahara et al. 1997]. MMP-9 was identified in healthy human liver in traceable amounts when measured by antibodies raised against recombinant proteins [Geisler et al. 1997, Lichtinghagen et al. 1995]. MMP-10 was hardly detectable in normal liver specimens, while minor expression of MMP-11 was observed in fibroblasts [Giambernardi et al. 1998, Lichtinghagen et al. 1995].

Our analyses revealed presence of all studied metalloproteinases in human liver controls. This finding agrees with the report of Lichtinghagen et al. who showed presence of MMP-2,-9,-3,-10,-11 and MMP-7 in healthy human liver [Lichtinghagen 1995]. Presence of MMPs in normal liver tissues, indicate their role in physiological processes occurring in human liver [Lichtinghagen 1995].

## SECTION 2

### 4.2. EXPRESSION OF MMPS IN VIRAL HEPATITIS, NON-ALCOHOLIC STEATOHEPATITIS AND THEIR COMPARISON

#### 4.2.1. MMP-2

Numerous studies have assessed gelatinase A expression in liver disease. A research study that involved patients with fibrosis detected steady levels of MMP-2 gene transcripts in cultured human fat-storing cells [Milani et al. 1994]. Arthur and his colleagues showed the MMP-2 gene expression in human hepatic stellate cells [Arthur et al 1992]. Several research studies have detected the presence of RNA in

human liver fibrosis [Benyon et al. 1996, Lichtinghagen et al. 2003, Lichtinghagen et al. 2001] and in $CCl_4$ induced fibrosis [Takahara et al. 1995].

When HCV and HBV related chronic hepatitis were grouped together and compared to the controls, a significant increase in the MMP-2 expression was observed. Results from experimental animal and human studies agree with our findings. Lichtinghagen et al. have detected a 14 fold increase in MMP-2 mRNA expression levels in chronic hepatitis C and HCV induced cirrhosis when compared to the controls [Lichtinghagen et al.2003, Lichtinghagen et al.2001]. Benyon et al. [Benyon et al. 1996] have reported a 3-4 fold increase of MMP-2 expression levels in cholestatic fibrotic liver disease. Theret et al. measured a 7.8 fold increase of MMP-2 mRNA in metastatic liver when compared with normal liver specimens [Theret et al. 1998]. Northern blot analysis done by Milani et al. showed an increase in the MMP-2 expression in fibrosis of viral aetiology [Milani et al. 1994], which was subsequently confirmed by others [Takahara et al. 1997]. In carbon tetrachloride-induced fibrosis, a temporal analysis also showed an increased expression of MMP-2 as fibrosis progressed [Takahara et al. 1995]. Elevated activity of MMP-2 has been previously addressed to inflammation process in several studies that involved a role of MMPs in fibrogenesis [Kumagai et al. 1999, Esteve et al. 1998, McMillan et al. 1995].

The possible explanation for this finding might be addressed to the enhanced activity of MMPs due to the ongoing liver remodelling during pathogenesis. Further research is required to outline the role of MMP-2 in degradation of fibrotic matrix in the liver.

### 4.2.2. MMP-9

A considerable number of studies reported the importance of MMP-9 involvement in extracellular tissue remodeling in the liver. Expression of gelatinase B gelatinolytic activity has been detected in hepatic tissues from patients with viral and alcoholic cirrhosis [Lichtinghagen et al. 1995, Hayasaka et al. 1996], hepatitis B and hepatitis C [Kuo et al. 2000].

The present results of metalloproteinases expression by RT-PCR in viral hepatitis (hepatitis B and C) and non-alcoholic steatohepatitis revealed significant increase of

MMP-9 in NASH when compared to viral hepatitis. Similar findings of increased MMP-9 plasma levels in patients with chronic hepatitis were reported in the study of Hayasaka and his co-workers [Hayasaka et al. 1996]. Also, a significant increase in MMP-9 activity was observed in subjects from the HCV and HBV positive patients and it was correlated to the influence of the virus on the collagenase IV activity in serum. Kuo and his colleagues observed significantly lower activity of MMP-9 when compared to healthy control in all groups of hepatitis virus carriers such as HBV, HCV, HCC and Ci [Kuo et al. 2000]. Most of the research works that studied viral hepatic disorders found an increase in MMP-9 levels in studied patient groups. In NASH, there are not currently available research findings related to metalloproteinase expression. We suggest that this increase might be a compensatory mechanism occurring as a result of the overaccumulation of type IV collagen previously reported in fibrotic livers [Tsutsumi et al. 1993]. Our findings may explain the controversial results reported so far and demonstrate the need for careful histological classification of patients included in different studies.

### 4.2.3. MMP-10

The interest to study MMP-10 expression comes from the reports that find this enzyme upregulated in viral liver diseases [Bodey et al. 2000, Lichtinghagen et al. 1995]. In a study, which involves rat liver HSC and Kupffer cells, myofibroblasts and hepatocytes, MMP-10 was found expressed predominantly by stellate cells [Knittel et al. 1999]. Its expression has mostly been investigated in HCC and a very few research works examined the presence of stromelysin 2 in chronic liver diseases [Bodey et al. 2000, Lichtinghagen et al. 1995].

A characteristic increase of MMP-10 expression in non-alcoholic steatohepatitis was observed in our study when compared to viral hepatitis B and C. Increased levels of MMP-10 mRNA have been reported in regenerating rat liver after $CCl_4$ injury [Herbst et al. 1991]. Inconsistent expression of MMP-10 has also been reported in both non-diseased livers and liver tumours [Lichtinghagen et al. 1995], while strong expression of the protein was identified in the extracellular matrix around neoplastic cells in hepatocellular carcinoma [Bodey et al. 2000]. A possible explanation to our findings could lie in different mechanisms of MMPs activation process that might exist

between non-alcoholic and viral hepatitis. The differential expression pattern of MMP-10 that we observed in hepatitis B and C might support this hypothesis. Further studies in NASH should shed more light to this finding.

In our viral hepatitis patients we identified MMP-10 expression with an increasing pattern in advanced fibrosis of HBV cases and a decreasing pattern in advanced HCV fibrosis when compared to the early fibrosis. The reason for this discrepancy is not clear.

#### 4.2.4. MMP-11

The variation in the mRNA expression values and enzymatic levels of MMP-11 reported in a few studies made on human liver prompted an interest for its assessment [Geisler et al. 1997, Lichtinghagen et al. 1998].

In our study, MMP-11 exhibited a slightly higher level of expression in viral hepatitis compared to non-viral hepatitis without statistically significant differences. Data on MMP-11 expression in viral fibrosis are very few. A transient increase in early HCV fibrosis, followed by a decline in later stages, has recently been described [Lichtinghagen et al.2003]. Increased mRNA levels of MMP-11 were reported in a case of cirrhosis. No results are available regarding its expression in viral and non-viral liver disease [Lichtinghagen et al. 1998]. Findings of Geisler et al. and Lichtinghagen et al. are in agreement with our findings stressing the role of the MMP-11 in liver pathophysiology [Geisler et al. 1997, Lichtinghagen et al 1998].

No statistical difference was found in either HBV or HCV fibrosis in the present study, although a tendency towards reduced levels was a uniform finding irrespective of the virus or the fibrotic stage.

## SECTION 3

### 4.3. EXPRESSION OF METALLOPROTEINASES IN INDIVIDUAL DISEASES

Each of the studied hepatic disorders is recognized as liver aethiology with specific inflammation pattern and clinical manifestation. For this reason, studied diseases were discussed individually regarding the action of MMPs during fibrosis.

#### 4.3.1. MMPS EXPRESSION IN NASH

Currently, research findings on metalloproteinases expression in patients with non-alcoholic steatohepatitis are not available. In order to learn more about this recent pathologic entity, we examined the MMP expression in NASH and compared it to the expression in healthy controls.

Considering that the development of non-alcoholic steatohepatitis involves some clinical manifestations characteristic for all type of hepatitis like elevated plasma liver enzyme levels, hepatomegaly, hepatic fat accumulation, activated steallate cells, abnormal patterns of cytokine production and liver biopsy findings similar to alcoholic steatohepatitis that may progress to cirrhosis and end-stage liver disease [Yu and Keeffe 2002], the dissimilarity in the expression levels of studied MMPs was expected to be found when compared to the controls. However, statistical analyses did not find a significant difference.

#### 4.3.2. MMPS EXPRESSION IN HBV

In the present study, when the patients were analysed according to the viral aetiology and disease staging using the Ishak's system the results were not uniform. In fact, MMP-9 was significantly reduced in late stage HBV. A study by zymography on serum levels of MMP-2 and MMP-9 also reported that patients with chronic hepatitis B had lower levels of MMP-2 and MMP-9 compared to patients with hepatitis C [Kuo et al. 2000]. Kuo et al. reported decreased serum activity of MMP-9 in the presence of HBeAg in the liver tissue that was not evident in early disease stage.

One can speculate on a possible explanation to such findings referring it to the active viral replication process.

### 4.3.3. MMPS EXPRESSION IN HCV

In the present study, we found markedly increased MMP-2 mRNA concentration in HCV fibrotic liver when compared with healthy controls. Increased levels of MMP-2 RNA were detected in a number of research studies. An overexpression of MMP-2 was detected by immunohistochemistry and RNAase protection assay when human cirrhotic patients were compared with controls [Benyon et al. 1996]. Upregulation of MMP-2 gene was identified in a study on fat-storing human cell cultures using *in situ* hybridization in diseased liver [Milani et al. 1994]. Elevated MMP-2 expression levels were reported after $CCl_4$-induction in rat livers [Takahara et al. 1995], and in a study of human fibrotic livers by Northern blot analysis [Takahara et al. 1997]. An increased expression of gelatinase A was also reported during the process of lipocyte activation, which possibly takes place during the fibroproliferation process [Arthur et al. 1992, Lichtinghagen 1995].

Our findings correspond to the observations made by Murawaki et al. who reported increased RNA levels in patients with chronic liver injury caused by HCV infection. It remains to be clarified whether the increased expression of MMP-2 represents a compensatory mechanism against the excessive deposition of type IV collagen. However, it is likely that the enhanced expression of MMP-2 may contribute to the alteration of normal liver matrix in the perisinusoidal space, thus affecting liver cell function and favouring progression of the fibrotic process as suggested by Milani et al. [Milani et al 1994].

Our results regarding late HCV stages showed a different profile, with values of MMP-9 relatively increased. This increase did not reach statistical significance in patients with chronic viral hepatitis. Our findings in HCV agree with the findings of Lichtinghagen et al. [Lichtinghagen et al.2003, Lichtinghagen et al. 2001] who also observed increase in MMP-9 mRNA expression in patients with advanced HCV fibrotic stages.

## SECTION 4

### 4.4. EXPRESSION OF MMPS IN VIRAL HEPATITIS ACCORDING TO HISTOLOGICAL STAGING (FIBROSIS)

The analysis within subgroups of patients involved the separation of individuals with early disease stages based on Ishak's classification (stages 1-3) from those with advanced fibrosis (stages 4-5). A different expression pattern for metalloproteinases was identified.

#### 4.4.1. MMPS EXPRESSION IN RELATION TO DISEASE STAGES IN HBV

Between HBV and HCV related patients, a differential expression pattern was identified. In HBV patients MMP-2, MMP-10 and MMP-11 exhibited higher expression levels in the later fibrotic stages. By contrast, MMP-9 expression was increased in the initial stages following a descending pattern towards later hepatitis stages. The differences did not reach statistical significance.

Similar findings were reported by Kuo and his co-workers in the studies made on chronic hepatitis B and C patients, hepatitis B carriers and HCV antibody positive patients [Kuo et al. 2000]. Variability in the MMP-2 and MMP-9 activities among virus carriers and chronic hepatitis patients were identified. It is suggested that individual response of these parameters on the viral infection might indicate a difference between pathological mechanisms in HCV and HBV.

#### 4.4.2. MMPS EXPRESSION IN RELATION TO DISEASE STAGES IN HCV

When analysed according to viral aethiology and disease staging based on Ishak's classification our patients exhibited increased RNA expression levels of MMP-2 in late HCV stages.

Our results were consistent with the data of Kuo et al., who observed significant variations in MMP-2 activity when measured during different stages in HCV patients [Kuo et al. 2000]. A research study by Murawaki and his colleagues did not identify significant difference between chronic hepatitis C group and controls. In chronic

hepatitis C patients, an elevated serum of MMP-2 activities was reported [Ebata et al. 1997, Murawaki et al. 1999, Walsh et al. 1999]. Increased concentrations of serum MMP-2 levels were monitored in patients with liver cirrhosis, and not in patients with chronic hepatitis.

Although increased MMP-2 expression or enzyme activity might serve as a good explanation for the decomposition of extracellular matrix in hepatic tissue, they are in conflict with the fibrotic histology in cirrhosis tissue. It has been suggested that the pathological significance of ECM of a disease stage is the result of the metalloproteinases activity of the previous stage. The hypothesis has been made using the example of hepatitis B virus which shows that the significantly upraised MMP-2 expression levels in HBV carriers might enhance liver inflammation and cause the occurrence of chronic hepatitis. Alternatively, the extremely low activity of MMP-2 in the chronic B patients could help the progression into cirrhosis, at which stage metalloproteinases activity increases and might lead to cancerous pathology [Kuo et al. 2000].

It is questionable whether this kind of hypothesis can be applied to hepatitis C virus due to the differences in viral pathology. Thus, when speaking of metalloproteinases function the mechanism pathway seems possible.

# CHAPTER 5

# Conclusion

## SECTION 1
## 5. CONCLUSION

Liver fibrosis is a complex and dynamic process that changes liver architecture in a response to progressing liver injury. The collagen synthesis and breakdown shift the balance towards accumulation of fibrillar collagens creating hepatic dysfunction. Metalloproteinases seem to be main mediator of these changes.

The role of these Zn-depended endopeptidases is studied in various pathogenic disorders without adequate accent on human liver fibrosis. Most studies have been focused on the involvement of metalloproteinases in hepatocellular cancer development and metastasis. To understand their participation during viral and non-viral liver diseases, the expression pattern of metalloproteinases MMP-2, -9, -10, -11 was studied in patients with non-alcoholic steatohepatitis (NASH), hepatitis B (HBV) and hepatitis C (HCV) infections.

Although our findings are descriptive, based on semi quantitative RNA studies, a differential expression pattern exists between viral and non-viral chronic liver diseases, and also between late stage HBV and HCV related diseases. These differences might indicate the divergence in the involvement of metalloproteinase in various fibrolytic processes.

The only careful examination of various metalloproteinases so far is a comprehensive study of MMP gene expression according to the different stages of fibrotic HCV chronic liver disease done by Lichtinghagen et al. [Lichtinghagen et al. 2003]. This study was able to demonstrate a differential expression of the examined metalloproteinases and describe three basic patterns: an increasing expression with fibrosis progression (MMP-1,MMP-2, MMP-7 and MMP-14), a transient elevation pattern in early fibrosis (MMP-9, MMP-11 and MMP-13) and no change in the expression pattern (MMP-3). Our results indicate that apart from fibrotic stages, the viral aetiology should also be taken into account when MMP expression is examined. As a further addition to these findings our results on MMPs expression in NASH indicate that a different pattern emerges in this non viral potentially fibrotic liver disease.

The present study is the first to show an extensive variance in MMP-9 and MMP-10 mRNA expression between non-alcoholic steatohepatitis and chronic viral hepatitis B and C. Significantly higher levels of MMP-9 and MMP-10 were found in NASH when compared to chronic viral hepatitis.

In conclusion, although the various forms of chronic hepatitis have similar clinical manifestations, biochemical abnormalities and histologic characteristics, the participation of metalloproteinases in the process of fibrosis may be different in viral and non-viral disease. Despite the detailed studies on substrate specificities, the exact role of the different metalloproteinases in liver fibrosis is not known. Our results from NASH patients may appear to indicate that in the process of NASH induced fibrolysis the expression of MMPs is patterned in a different manner as compared to viral fibrolysis. This may be of importance when looking for diagnostic targets and therapy in the case of hepatic fibrosis. The analysis of MMPs with the use of highly sensitive methods during various stages of fibrosis should shed more light to the role of MMPs in the complex cascade of the events following this process.

## 5.1 FUTURE STUDIES

Future research work related to the role of MMPs in liver fibrosis should be addressed to the quantitative analysis of MMP and TIMP mRNA expression by real time-PCR in both chronic viral and non-viral diseases. The possibility to follow the changes in MMP and TIMP expression during each PCR cycle would offer real picture of the enzyme's variations during fibrotic process. Also, it would show the variation of mRNA levels for both MMPs and TIMPS during each fibrotic stage. This kind of studies would gradually improve our understanding of liver fibrogenesis.

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