SERUM PARAMETERS OF LIVER FIBROSIS

(SERUM PARAMETERS BIJ LEVERFIBROSE)

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Proefschrift

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Voor Patricia en Céline

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VOORWOORD

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LIST OF ABBREVIATIONS

AP	antipyrine
CCl4	carbontetrachloride
CCLI	combined clinical and laboratory index
CP	Child-Pugh
CSL	Copenhagen Study Group for Liver Diseases
ICG	indocyanine green
IL-1	interleukin-1
LP-1	laminin P 1 fragment
mRNA	messenger ribonucleic acid
NAG	amino-acetyl-β-D-glucosaminidase
PIIIP	aminoterminal peptide of procollagen type III
PBC	primary biliary cirrhosis
PDGF	platelet-derived growth factor
pN-collagen	para-amino-collagen
RIA	radioimmunoassay
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α

AIM OF THE THESIS

Chronic liver disease is often associated with deposition of fibrous tissue, a process which together with the destruction of normal liver and liver cell regeneration, leads to the condition called cirrhosis. Cirrhosis is known to be associated with a reduction in life expectancy. In recent years there has been an increasing interest in the pathogenesis of cirrhosis and in the possibilities reversing the process of fibrogenesis. Liver biopsy is the present "gold standard" for detecting the presence of fibrosis and cirrhosis, but is not suitable for evaluating the dynamics of pathological fibrogenesis. The aim of this thesis was to study serological markers of connective tissue metabolism as non-invasive tests of liver fibrosis and cirrhosis.

We studied procollagen type III aminoterminal peptide and the P1 fragment of laminin as possible serum markers of fibrosis in alcoholic cirrhosis, primary biliary cirrhosis and after liver transplantation.

The value of procollagen type III aminoterminal peptide for predicting histological progression of primary biliary cirrhosis was investigated.

A new radioimmunological technique, based on a so-called "coated tube" method, for determination of procollagen type III aminoterminal peptide was tested and compared with the conventional radioimmunoassay.

The clinical value of N-acetyl-β-D-glucosaminidase as a marker of fibrosis and liver disease was evaluated.

Concomitantly with the investigation of these serum markers of fibrosis, we performed a study on the prognosis of patients with alcoholic liver cirrhosis. The main reason for performing the study was to determine what the prognosis of alcoholic cirrhosis was in The Netherlands, and to determine whether features which have been described to have prognostic implications in other countries also apply in the local population.

Chapter I INTRODUCTION

Fibrosis is frequently encountered in chronic liver diseases (Popper and Udenfriend 1970, Chen and Leevy 1975, Popper and Piez 1978). Fibrosis is defined as an excessive accumulation of connective tissue within the organ. Fibrogenesis is defined as the new formation of fibrous tissue. The "extracellular matrix" is the ground substance of the mesenchyme and the basement membrane of epithelia and blood vessels, formed by the non-cellular components of the framework that supports the parenchymal and non-parenchymal cells of organs like kidney and liver. The composition of the extracellular matrix of the normal and fibrotic liver and the cells involved in the production of the extracellular matrix will be discussed. Some etiologic varieties of liver fibrosis are mentioned. The synthesis and degradation of collagen, the main component of the extracellular matrix is dealt with. Parameters of liver fibrosis in liver tissue and serum are discussed. Continuing fibrosis may lead to an irreversibly scarred liver, called liver cirrhosis, which is caused by an abnormal reconstruction of the preexisting lobular architecture (Popper 1977).
A cirrhotic liver is characterized by fibrous bands around nodules of surviving but structurally altered parenchyma, which result from regeneration (Scheuer 1979). Patients with liver cirrhosis generally have a shortened life expectancy. Complications of this condition such as portal hypertension and liver insufficiency are the major causes of death in these patients (Schlichting et al. 1983). Characteristics, prognosis and therapeutic options of alcoholic and primary biliary cirrhosis, the two main diseases studied in this thesis, will be briefly summarized.

I.1 THE EXTRACELLULAR MATRIX OF THE NORMAL LIVER

The extracellular matrix of the normal liver is composed of many different types of molecules. The main biochemical components of this matrix are collagen, elastin and matrix glycoproteins. They are embedded in a hydrated gel of proteoglycan chains. Basement membrane, a specialized part of the matrix contains laminin.
Elastin, fibronectin and the proteoglycans will be discussed briefly.
Elastin serves to provide elasticity. It is normally found in the vessel walls of the central and portal tracts. It can be demonstrated on light microscopy

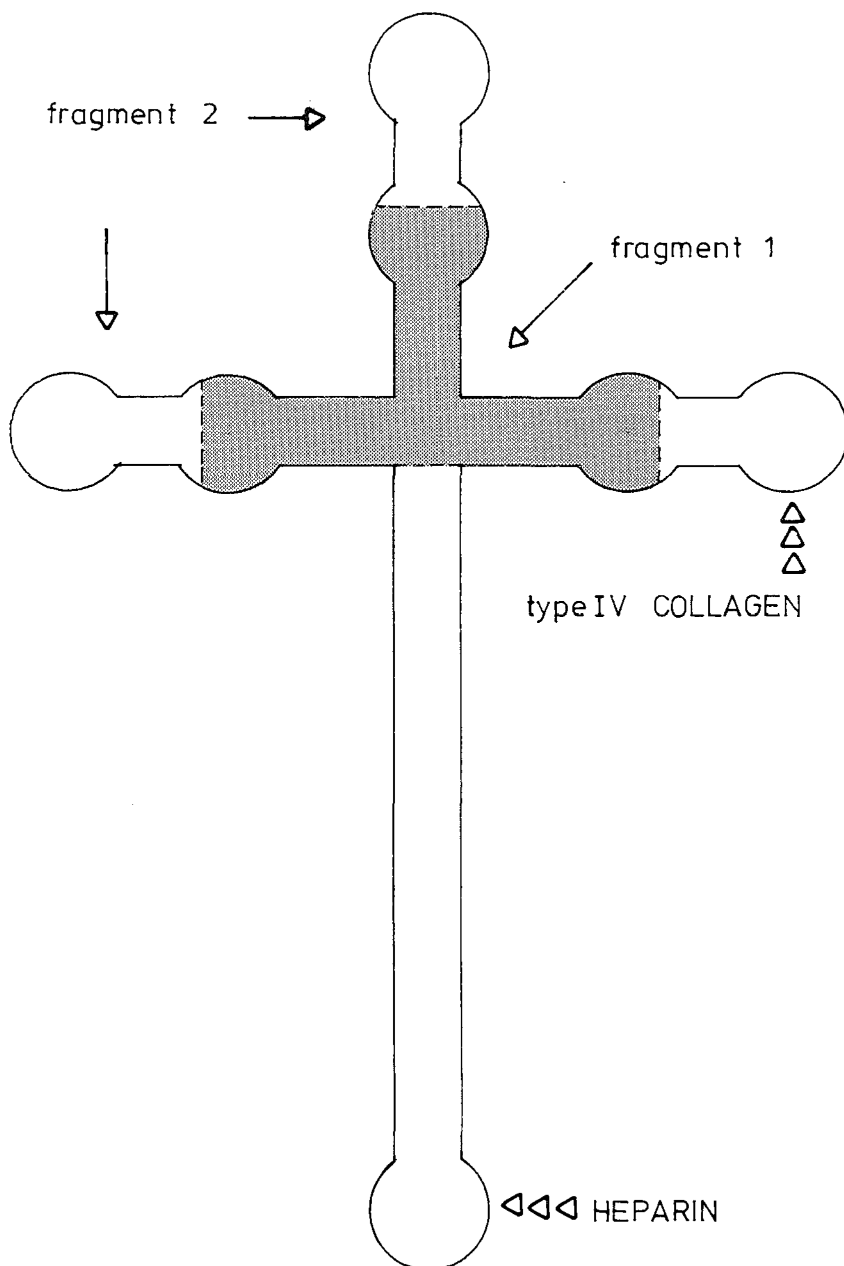
by orcein, resorcin and Victoria blue stains (Bancroft and Stevens 1977). **Fibronectin** is a non-collagenous glycoprotein manufactured by hepatocytes, endothelial cells, Kupffer cells (Rojkind and Perez-Tamayo 1983) and by hepatic lipocytes (Ramadori et al. 1987, Bissell and Choun 1988). Fibronectin is present in several forms. One type is plasma (soluble) fibronectin, others are cellular (insoluble) forms. The fibronectin in the space of Disse in normal liver probably is plasma fibronectin passively associated with the matrix (Oh et al. 1981), together with the cellular fibronectin produced by lipocytes and sinusoidal endothelial cells. Fibronectin is detectable along sinusoids and around portal structures. In the extracellular space fibronectin interacts with many macromolecules, including collagens, glycosaminoglycans and cell-surface receptors and is also associated with basement membranes (Engvall et al. 1978, Ruoslahti et al. 1981). In this way it promotes adhesion of cells (platelet aggregation, fixation of fibrin in wounds), chemotaxis of fibroblasts, phagocytosis, cell proliferation and migration (Hynes 1982, Martinez-Hernandez 1984, Clement 1986, Biagini and Ballardini 1989). Apart from fibronectin and laminin the matrix contains other glycoproteins including coagulation factors, growth factors and proteinases. Their function in the matrix is not yet clear (Bissell and Roll 1989).
Proteoglycans consist of a protein backbone (core protein) covalently linked with at least one glycosaminoglycan. Glycosaminoglycans (formerly called mucopolysaccharides) are long unbranched polysaccharide chains composed of repeated dissaccharide units. Some examples are chondroitin-sulphate, hyaluronic acid, dermatan-sulphate, heparin and heparan-sulphate. In the liver heparan-sulphate is secreted by both hepatocytes (Kjellen 1980, Stow 1985) and lipocytes (Arenson 1987), with the latter cells producing dermatan-sulphate and chondroitin-sulphate as well (Stow 1985). The proteoglycans are hydrophylic, forming hydrated gels and thereby contributing to the extracellular matrix turgor, effectively filling the extracellular space. In normal liver heparan sulphate is the main glycosaminoglycan. It has a filtration-like activity in basement membranes; it seems to be anchored on the hepatocyte surface and interacts with laminin and fibronectin. Heparan sulphate is detectable sinusoids and portal basement membranes. Proteoglycans may have a role in informing the cells about their microenvironment (Heinegard and Sommarin 1987, Rojkind 1988, Biagini and Ballardini 1989).
Basement membranes or basal laminae are thin layers of specialized extracellular matrix, separating parenchymal cells from connective tissue. Their ultrastructure consists of a network of irregular, fuzzy strands referred to as "cords". The cords are composed of collagen type IV, laminin, heparan-sulphate, entactin and fibronectin (Leblond and Inoue 1989), the

first two being the major components (Kefalides et al. 1979). Laminin will now be discussed in detail.

LAMININ

Laminin is a large non-collagenous glycoprotein (100 nM, MW 900 kD), which is a normal component of the surface of the hepatocyte and is present in the extracellular matrix in high concentrations (Timpl et al. 1979, Rojkind and Ponce-Noyola 1982). With immunohistochemical techniques using antibodies to laminin this glycoprotein was demonstrated in all basement membranes in the portal field, but not in the sinusoidal lining of the parenchyma of the normal human liver (Hahn et al. 1980). Laminin was suggested to play an important role in the organization of liver plates during regeneration. Partial resection of the liver leads to growth of cells that presumably are in contact with normal matrix, resulting in orderly restoration of the normal hepatic mass. In this situation laminin was found to be present in the sinusoidal areas (Carlsson et al. 1981). Ekblom et al. (1980) found laminin during embryogenesis in association with the appearance of epithelial cells and suggested a role in morphogenesis.
The laminin molecule has a cross-shaped structure (see figure I.1). The three short arms of the cross terminate in distal globules.

Figure I.1
Structure of laminin.



The region is the site of interaction with type IV collagen in the basement membrane. The center of the cross is a disulphide bonds rich area, which is resistent to proteolysis. This region is involved in cell attachment because it interacts with a cell plasma membrane receptor. Laminin is a molecular link of epithelial cells to the basement membrane. The long arm of the cross begins by an α-helicoidal region and terminates by a globular region. This end piece interacts with glycosaminoglycans. In the basement membrane laminin has structural and functional roles. Besides the above mentioned cellular attachment, it has a role to play in cellular differentiation, migration and proliferation (Lissitzky et al. 1986).
Hepatic laminin is produced primarily by lipocytes (Arenson et al. 1987), but also by endothelial and bile duct epithelial cells (Milani et al. 1989).

COLLAGEN

Collagen is a family of extracellular fibrous proteins. Collagen is the most common protein of the organism, representing about 30% of body proteins. At least 13 structurally different types have been identified (Burgeson 1988). Characteristic for the collagen molecules is the triple-shaded helical structure, composed of three identical collagen polypeptides, the so-called α-chains. The function of such triple-helical structures is to provide strength and resist extension.
In the currently used classification the collagen molecules are categorized according to molecular weight and length of the helical domain of the α-chains (Miller 1985). In this classification three groups of collagens are discriminated.
Group 1 collagens (I, II, III, V and XI) are the most rigid types. Their long helical regions have the same length (295 nm). According to current models this length is required for packing of collagen molecules into a fibril, and indeed these five collagen types combine to form fibrils with periodic banding visualized by electron microscopy. These collagen types constitute a large proportion of the collagen in tissues and are probably responsible for the tensile strength of the matrix.
Group 2 collagens (IV, VI, VII, VIII) generally do not form fibrils, have helical domains separated by non-helical domains and are more susceptible to cleavage by proteolytic enzymes.
Group 3 collagens (IX, X, XI, XII) have a different structure compared to group 2 collagens and have chains of low molecular weight (Miller 1985, Burgeson 1988). The function of these minor collagens is not known but maybe they act limiting on the fibril diameter or mediate interaction of fibrils with other matrix components.

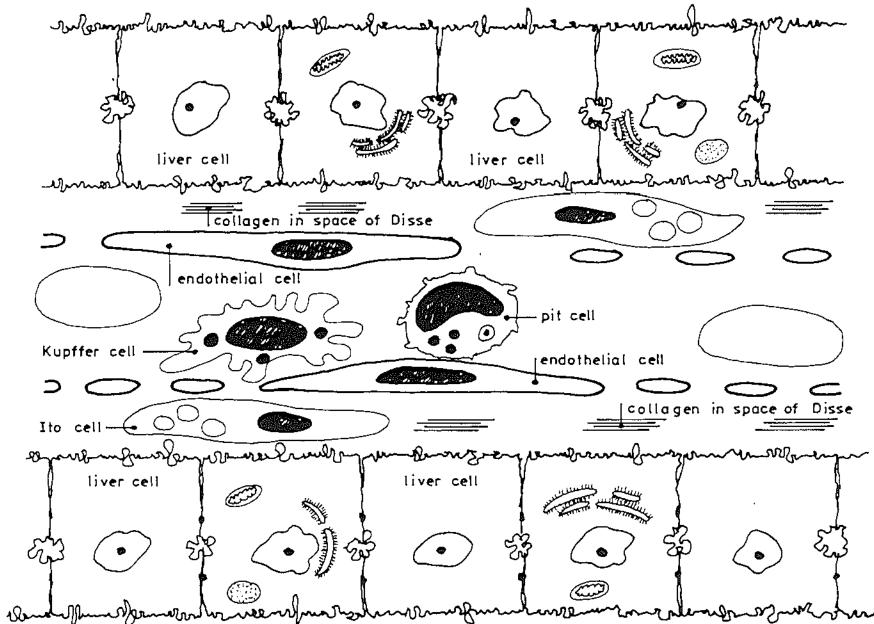
The normal liver contains 5.5 +/- 1.6 mg of collagen/gram of wet liver tissue (Rojkind et al. 1979); that means 5-10% of the total protein content (Schuppan et al. 1988).
The collagens present in the liver tissue are type I, III, IV, V and VI collagens. The types I and III account for 70-80% of the total amount in equal percentages (Rojkind and Perez-Tamayo 1983). Type IV collagen is found in basement membranes of portal vessels, lymphatics, nerves, bile ducts and central veins. The presence along the sinusoids between endothelial lining and liver cell plates is less definite (Hahn et al. 1980, Rojkind and Ponce-Noyola 1982). Collagen type V could be demonstrated around smooth muscle cells in vessels and bile ducts and in sinusoids; type VI collagen appears to be codistributed with collagen type I and III (Schuppan et al. 1988).

I.2 CELLS INVOLVED IN COLLAGEN PRODUCTION

It has been established that hepatocytes occupy approximately 80% of the total hepatic volume and represent about 80% of the total number of cells as well. The rest is made up by sinusoidal and connective tissue cells (20-30%) and bile duct cells (around 2%). In the normal liver there are several types of mesenchymal cells present: endothelial cells, Kupffer cells, fat-storing "Ito" cells, fibroblasts and pit cells (Wisse 1970, Wisse et al. 1976, Knook and Wisse 1982). These cells are also called sinusoidal lining cells. They are found in close relationship to the structure of the sinusoid capillaries. Fibroblasts are present in the connective tissue of the capsule, the portal spaces and the adventitia of the hepatic veins (Rojkind and Perez-Tamayo 1983). The rare pit cells have characteristic dense core granules resembling pips (Wisse 1976). These cells are almost certainly natural killer lymphocytes (Bouwens and Wisse 1988).
Endothelial cells form the lining of the sinusoidal space in the liver. They lack a basement membrane and have a sieve-like structure in their extensions, making communication between the sinusoidal space and the space between endothelial cells and the surfaces of the hepatocytes (Disse's space) possible (Wisse 1972). They act as a selective barrier and have an active metabolic function (Wisse et al. 1985). Via the production of factors like prostaglandin E2, they may modify the function of other cells (Irving et al. 1984).
Kupffer cells and their function have recently been reviewed by Wardle (1987). Apart from phagocytosis and defence of the liver against bacteria, endotoxaemia and viral infections, which is probably their main function, Kupffer cells have various other important effects. They control

proliferation and regeneration of hepatocytes and produce lymphokine mediators that direct protein synthesis by the hepatocytes. Like other macrophages they are able to synthesize mediators that can cause transformation of connective tissue cells, chemotaxis or proliferation (Rojkind and Kershenobich 1986, Wardle 1987, Schuppan et al. 1988). In figure I.2 a schematic representation of the sinusoidal cells of the liver is given.

Figure I.2
Schematic representation of the sinusoidal cells of the liver.



In 1972 smooth muscle-like cells were demonstrated in human cirrhotic liver by electron microscopy, later this was confirmed (Bhatal 1972, Rudolph et al. 1979). These cells, known as myofibroblasts, seem to originate from fat-storing "Ito" cells that are present in the space of Disse of the normal liver (Mak et al. 1984). The fat-storing cells, also called lipocytes, have several large lipid droplets in their cytoplasm. They are supposed to play a role in fat metabolism and are vitamin A storage sites (Hendriks et al. 1985). There is growing evidence that fat-storing cells are involved in fibrogenesis (Senoo et al. 1984, Friedman 1985, Takahara 1988).

All the cells present in the liver have the ability to produce collagen (McGee and Patrick 1972, Guzelian et al. 1981, Voss et al. 1982, Clement et al. 1986). In tissue cultures they synthesize collagen molecules. However, one must take into consideration that a tissue culture resembles more closely a wound healing process than a physiological situation. Rojkind and Ponce-Noyola (1982) suggested that cells separated from their matrix would have to synthesize some or all of the components of the matrix in order to survive in culture. On the contrary, it is possible that collagen production is ascribed to cells that in fact do not synthesize it, due to impure cultures (Maher et al. 1986).

More important is the question which cell or cells *in vivo* are the major collagen producing cells.

Patrick and McGee (1967) and McGee and Patrick (1972) suggested in studies, using electron microscopy and autoradiography, that the fibroblast is the principle collagen producing cell. The question which liver cells are the most important in collagen synthesis is still a matter of some controversy. The hepatocyte and the perisinusoidal fat-storing cell are the two cell types that have received most attention (Guzelian et al. 1981, Senoo et al. 1984, Friedman et al. 1985, Chojkier 1986, Shiratori 1986, Takahara et al. 1988). The fat-storing cell probably may undergo transformation to a fibroblast, loosing fat and vitamin A while transforming. As this fibroblast ultrastructurally contains smooth muscle, it has been termed a myofibroblast (Ballardini et al. 1983, Mak et al. 1984, Clement et al. 1986, Shiratori et al. 1986, Rojkind and Kershenobich 1987).

It is possible that previous studies with "pure" hepatocytes may have been unknowingly contaminated with fat-storing cells giving rise to false results (Maher et al. 1986). On the whole the fat-storing cell seems to be the most reliable candidate for interstitial collagen production (Friedman et al. 1985).

Nevertheless it is possible that more cell types produce collagen. In that case future studies may elucidate on the relative contribution of the

different cell types to the total amount of collagen. In table I.1 a summary of the synthetic activities of liver cells is given.

Table I.1
Synthetic activities of hepatic cells in culture (C) and *in vivo* (V).

Cells	Hepatocytes		Ito cells		Myofibroblasts		Endothelial cells	
	C	V	C	V	C	V	C	V
Collagen type I	+	+	+	+	+	+?	+	+
(Pro)collagen type III	+	+	+	+	+	+?	+	+
Collagen type IV	+	+	+	+	+	+?	+	+
Collagen type V	+		–		+	+?	+	
Collagen type VI			–					
Laminin	+		+	+	+	+?	+	+
Proteoglycans	+		+		+	+?		
Elastin							+	

Modified after Biagini and Ballardini (1989)

I.3 EXTRACELLULAR MATRIX OF THE FIBROTIC LIVER.

Connective tissue staining methods such as Gordon and Sweets' reticulin and Picro-Sirius' collagen have made it possible to observe accumulation of extracellular matrix in liver biopsies on light microscopy (Vyberg et al. 1987). Advances in biochemical and immunological methods and in electron microscopy have contributed to a deeper insight into the changes involved.
In fibrotic liver all types of collagen are increased (Rojkind et al. 1976, Schuppan et al. 1988). The total collagen content may increase from 4 to 7 fold (Rojkind and Dunn 1979, Rojkind and Kershenobich 1981). In early fibrosis (<20 mg of collagen/g of wet tissue) type I and type III collagen were estimated to increase in equal amounts. In cirrhotic livers containing >20 mg of collagen/g of wet tissue, the ratio type I/type III collagen increased, which was ascribed to a larger content of type I

collagen. These findings were independent of the etiology of the cirrhosis (Rojkind et al. 1979).
Wick et al. (1978) reported that elevated production of type III collagen preceeded that of type I collagen in fibrotic liver disease. A decrease in the ratio type I/type III collagen due to a relative increase of collagen III was also suggested by other studies (Wu et al. 1982, Olds et al. 1985, Kucharz 1987). In advanced cirrhosis type I collagen is the predominant type (Seyer et al. 1977, Rojkind et al. 1979).
Other matrix components like fibronectin, laminin and proteoglycans have also been found to be increased in fibrosis (Hahn et al. 1980, Bianchi et al. 1984, Martinez-Hernandez 1985, Murata et al. 1985).
Accumulation of basement membrane collagen in Disse's space was shown by electron microscopy (Grimaud et al. 1980). This accumulation of collagen in the perisinusoidal space is known as capillarization of the sinusoids and has been associated with hepatocellular dysfunction due to decreased exchange possibilities of metabolites (Schaffner and Popper 1963) and increased resistance to portal venous flow (Orrego et al. 1981).

I.4 METABOLISM OF COLLAGEN

SYNTHESIS OF COLLAGEN

Comprehensive reviews on biosynthesis and degradation of collagen were published in recent years (Fleischmayer 1985, Laurent 1987, Schuppan et al. 1988, Bissell and Roll 1989). The biosynthesis of collagen leads from the transcription of a collagen gene via various posttranslational steps (during which modulation and regulation may take place) to the functional collagen fiber. Many steps in this process have been identified and they are summarized in figure I.3.
The first steps in the synthesis of the main collagens (type I and type III) take place intracellularly. The transcription of collagen gene in the nucleus results in the formation of messenger ribonucleic acid (mRNA). In the rough endoplasmic reticulum in the cytoplasm a pre-procollagen chain is formed containing a signal peptide that serves to direct the chain into the Golgi apparatus. Here cleavage of this signal peptide is performed giving rise to the procollagen α-chain consisting of a NH2-domain, a COOH-domain and the 300 nm long helical domain. The next steps involve a hydroxylation (by prolyl- and lysylhydroxylase), a glycosylation (by hydroxylysyl galactosyl transferase and galactosyl hydroxy-lysyl glucosyl transferase) and formation of disulphide bonds between 3 α-chains in the

triple helical procollagen molecule.
The procollagen molecule is transported to the extracellular space in secretory vacuoles of the Golgi apparatus. When the procollagen molecule leaves the cell the extension peptides are cleaved by N- and C-proteases and the molecules in the tissue then assemble to form collagen fibrils.
The type III procollagen molecule is first trimmed at the carboxyterminal end and, only later and incompletely, at the aminoterminus (see figure I.4). The collagen molecule containing the uncleaved aminoterminal propeptide is called pN-collagen (Fessler et al. 1981). Thus in the tissues some of the type III collagen is in fact still in the pN-collagen form.
The non-helical extension peptides are assumed to play an important role in the regulation of the synthesis of collagen (Pagliaro et al. 1979, Horlein et al. 1981, Wu et al. 1986, Aycock et al. 1986). Both propeptides retard fibril formation by preventing side-to-side aggregation of collagen helices; cleavage of the propeptides of collagen may limit the rate of fibril formation (Bissell et al. 1990).

The collagen molecules are firstly assembled into microfibrils. Strong intra-molecular and inter-molecular cross-linkages are formed in the course of time, giving strength and resistance to proteolysis.
Microfibrils may aggregate to fibrils and fibers, as can be shown by light microscopy. Collagen type I fibers are thick and are found in dense connective tissue. Collagen type III fibers are thinner and present in loose connective tissue (Grimaud et al. 1980).

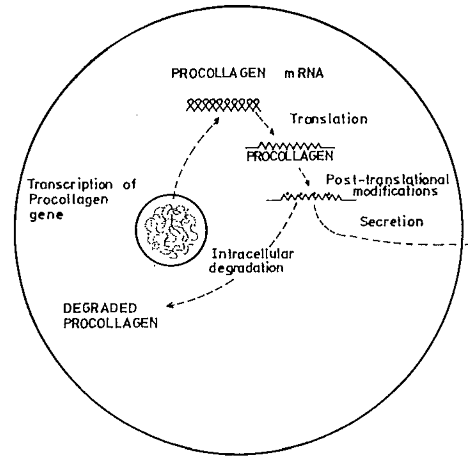
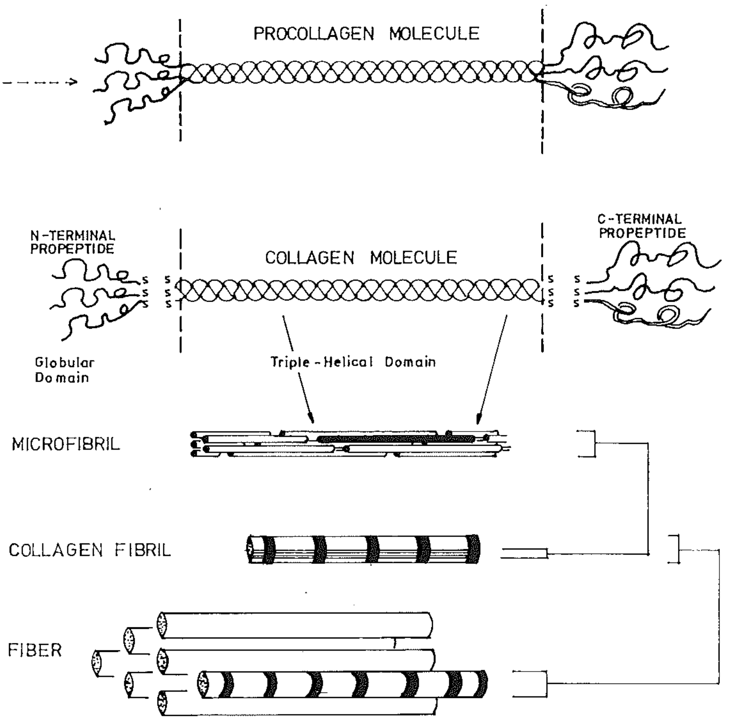


Figure I.3
Synthesis of collagen.



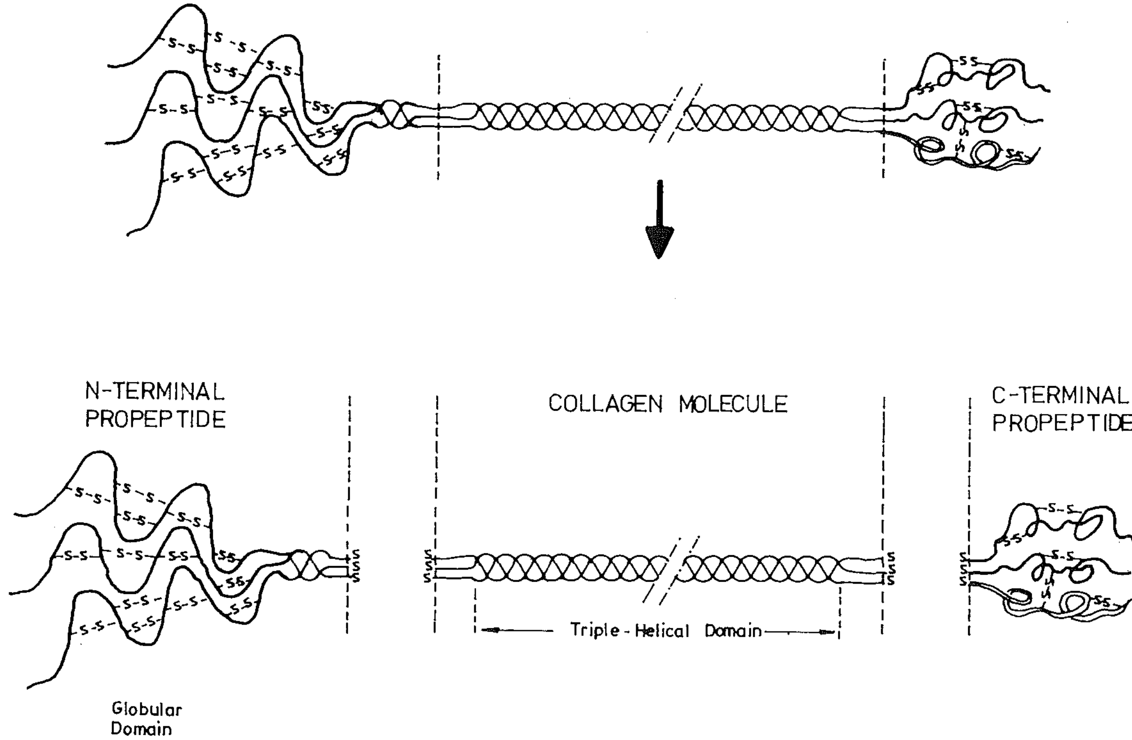


Figure I.4
Schematic representation of the structure of the procollagen molecule.

DEGRADATION OF COLLAGEN

The breakdown of collagen is a complex process involving intracellular and extracellular events as well as interactions between cells and extracellular matrix.
Degradation of procollagen may occur before the molecule is secreted to the extracellular space. This intracellular breakdown can be subdivided into basal and enhanced degradation. In physiological situation a certain percentage of the synthesized collagen is broken down within the collagen-producing cell. It has been suggested that the basal degradation takes place in the Golgi-apparatus, while in disease states (like ascorbate deficiency and production of defective proteins) there is enhanced degradation in the lysosomes (Berg et al. 1980). It has been estimated that up to 15% of all synthesized collagen molecules may be degraded intracellullarly (Bienkowski 1984, McAnulty and Laurent 1987). This may regulate the amount of extracellular collagen and eliminate defective molecules. For the extracellular breakdown of collagen, either before or after incorporation of the collagen molecule into a fibril a specific collagenase is necessary. This enzyme cleaves the triple helical polypeptide; the collagen-derived peptides then can be broken down by other proteases. Many different collagenases have been identified. Fibroblasts are known to release collagenase and degrade extracellular collagens, but other cells may play a role, particularly in disease states. Cells like macrophages and neutrophils are capable of phagocytosing collagen as well as releasing proteases which can degrade collagens extracellularly or agents capable of stimulating fibroblast-mediated degradation (Laurent 1987, Bissell et al. 1990). In the normal tissue the biosynthesis and degradation of collagen are in balance resulting in a steady total amount of collagen.

I.5 MECHANISM OF FIBROSIS

Wound healing and tissue repair involve a complex series of biological events which include inflammation, cellular migration, production of collagen and tissue remodelling. These events are influenced and regulated by the inflammatory response which follows immediately after tissue injury. A major component of inflammation is the inflammatory cell, which infiltrates the damaged tissue to remove dead tissue. During this process these cells are activated to secrete a variety of substances such as enzymes, growth factors and cytokines. Many of these substances affect the biological activities of fibroblasts and activate them to proliferate,

migrate into the wound site and produce matrix constituents; once tissue integrity is restored the numbers of fibroblasts and their activities return to normal values (Narayan et al. 1989). In human liver the inflammatory reaction after hepatocellular injury is supposed to take place in a similar way (Rojkind and Perez-Tamayo 1983).
When the inflammatory process becomes chronic, liver fibrosis is a frequent finding (Popper and Udenfriend 1970, Popper and Piez 1978).

Hepatic fibrosis is defined as an excessive accumulation of connective tissue within the organ. There are many different causes of liver fibrosis. The Criteria Committee of the International Association for the Study of the Liver recognized 12 different etiologic varieties of liver fibrosis, given in their recommendations for the classification of diseases of the liver and the biliary tract (Leevy et al. 1976). Some of these varieties will be discussed briefly.

A. Infectious and inflammatory liver fibrosis.
   Viral hepatitis, especially the form caused by hepatitis B virus is one of the most important frequent causes of liver fibrosis in the world (Popper 1977, MacSween and Scothorne 1979). Although in most of the cases this form of infectious hepatitis will heal without any further complication a small fraction will develop a chronic active hepatitis with fibrosis and end with cirrhosis of the liver. Other examples of inflammatory liver fibrosis are the fibrosis due to chronic autoimmune hepatitis and in primary biliary cirrhosis.
B. Toxic liver fibrosis.
   The fibrosis caused by alcohol is probably the most frequent cause of hepatic fibrosis in the world (Orrego et al. 1981). Chronic overindulgence in alcohol results in a variety of liver changes, almost all of them associated with various degrees of fibrosis (Rojkind and Dunn 1979, Hahn et al. 1980, Edmonson 1980, De la Hall 1985). The lesions accompanied by fibrosis caused by alcohol are sclerosing hyaline necrosis (Edmonson 1980), interstitial fibrosis (Galambos 1972, Nakano and Lieber 1982) portal fibrosis (Goldberg et al. 1977, Morgan et al. 1978) and cirrhosis (Rubin and Lieber 1975, Christofferson and Poulsen 1979). Other toxic substances have also been reported to cause liver fibrosis. In studies with animals fibrosis has been induced by several toxic substances such as carbon tetrachloride, D, L-ethionine and dimethylnitrosamine (Zimmerman 1978).
C. Cholestatic liver fibrosis.
   In chronic inflammatory disease of the extrahepatic biliary tract, and especially with the occurrence of occlusive episodes due to stones,

there is a characteristic periportal deposition of collagen in an "Onion peel" fashion (Foulk and Baggenstoss 1975, Popper and Stern 1979). This form may or may not be accompanied by inflammation; in children, mechanical obstruction of the biliary tract results in portal fibrosis in the absence of inflammatory infiltration and the same has been observed experimentally in rats (Carlson et al. 1977).

D. Other causes of liver fibrosis.
A relatively large group of cases of human cirrhosis occurs without a known or discoverable cause; they are known as cryptogenic. Some of them are probably due to viral infections (e.g. non A - non B hepatitis viruses), others to immunological disorders or other subtle mechanisms.

Hepatic fibrosis may be the result of increased collagen production, decreased degradation or both processes, occurring simultaneously or successively.

Increased synthesis: The synthesis of collagen may be influenced at several levels of the production process e.g. the transcription, the translation and the post-translational phase. The levels of procollagen mRNA are increased in hepatic tissue of ethanol-fed baboons (Zern et al. 1985), in CCL4-induced liver fibrosis in rats (Panduro et al. 1986) and in humans in alcohol-and virus-induced liver fibrosis (Annoni et al. 1990). Three factors that increase procollagen mRNA levels *in vitro*, probably by stimulating procollagen gene transcription, have been identified. Transforming growth factor β (TGF-β) is the best characterized profibrogenic factor (Roberts et al. 1986, Ignotz et al. 1986). TGF-β, a product of hematopoietic cells, stimulates fibrogenesis in experimental animals and may play a role in hepatic fibrosis (Czaja et al. 1987, Czaja et al. 1989).
Acetaldehyde, the first metabolite of ethanol, stimulates procollagen gene transcription in cultured human fibroblasts (Brenner and Chojkier 1987). This effect could be responsible for the stimulation of the collagen production by acetaldehyde in baboon liver myofibroblasts (Savolainen et al. 1984) and rat liver transitional lipocytes (Shiratori et al. 1986). Acetaldehyde is supposed to be able to induce fibrosis and cirrhosis in the absence of inflammation (Popper and Lieber 1980, Nakano et al. 1982). A third fibrogenic factor, isolated from fibrotic rat liver, was found to increase procollagen mRNA transcription in cultured cells (Raghow et al. 1984, Choe et al. 1987).
Increased production of collagen may also result from increased number of collagen-synthesizing cells. Factors produced by inflammatory cells in tissue injury or fibrogenesis e.g. platelet-derived growth factor (PDGF) and

TGF-β, have been shown to induce a rise in proliferation, migration and transformation of collagen-producing cells (Stavenow et al. 1981, Senior et al. 1985, Roberts et al. 1986).
Activated monocytes including macrophages are able to produce factors that promote fibroblast growth, including PDGF, fibroblast growth factor, interleukin 1 (IL-1) and tumor necrosis factor- α (TNF-α) (Nathan 1987).
Kupffer cell-derived factors were reported to stimulate proliferation and collagen formation by hepatic lipocytes in rats given a high-fat diet and ethanol (Matsuoka et al. 1990).
Lymphokines, released by sensitized T-cells, cause fibroblast proliferation and stimulation of collagen production in fibroblast culture (Wahl 1985).
It is evident that the route followed in this cytokine-mediated fibrosis is different from the route in fibrosis due to direct fibrogenic agents as discussed above. However, the end result is the common pathway from fibrosis to cirrhosis in both cases.

Decreased degradation: degradation of procollagen may occur intra- or extracellularly. As mentioned before, up to 15% of all synthesized collagen molecules may be degraded intracellularly. Theoretically, when this process stops it may result in an increase of collagen production of 15%. An increase could also result from an interruption of extracellular degradation. In alcohol-induced liver fibrosis in baboons the collagenase activity was increased in early fibrosis, however in fully developed cirrhosis the activity was decreased (Maruyama et al. 1982). It has been suggested that in advanced liver disease the presence of excess collagen in the Disse space and fibrous septa disturbes the interactions of the epithelial and mesenchymal cells. In this situation the epithelium would not be able to stimulate the stromal cells to produce collagenase, resulting in reduced collagenase activity and excess collagen deposition (Rojkind and Perez-Tamayo 1983).

I.6 ASSESSMENT OF FIBROSIS

The evaluation of fibrosis ideally should indicate the total quantity of matrix, the rate of matrix deposition, the localization of matrix within the liver and the relative amounts of various matrix components.
The histological examination of a liver biopsy is still the most direct method for diagnosing and quantitating fibrosis. The use of specific stains and antibodies for the individual matrix components can give insight in the composition of the matrix in the liver biopsy. However, it is not possible to differentiate between recently deposited and older collagen, so

one cannot determine by histology whether the fibrotic process is still going on. In other words the liver biopsy only gives a static impression of the dynamic process of liver fibrosis. To obtain more insight in the activity of the collagen biosynthesis the activity of enzymes involved has been measured in liver tissue. Prolyl hydroxylase and galactosylhydroxylysyl glucosyltransferase activities were reported to correlate well with actual hepatic collagen synthesis (Kuutti-Savolainen et al. 1979a, Mann et al. 1979, Kuutti-Savolainen et al. 1979b).
In general, liver fibrosis and cirrhosis are not distributed homogeneously in the liver, which can lead to sampling error, resulting in a biopsy that is not representative for the whole liver (Scheuer 1970). A guided biopsy can diminish the problem of sampling error (Pagliaro et al. 1983).
Furthermore liver biopsy has a certain morbidity and small mortality (Sherlock et al. 1984, Minuk et al. 1987, Hederstrom et al. 1989) and the number of biopsies that can be taken in the individual patient is limited. Because of these drawbacks other non-invasive methods to diagnose fibrosis have been studied. Galambos and Wills (1978) studied the relationship between paired liver tests and biopsies and found a poor association between the conventional liver tests (serum bilirubin, serum aspartate aminotransferase, serum alkaline phosphatase and serum albumin) and the lesions in the liver biopsy.
Fasting serum bile acids determinations appear to be useful in the diagnosis of alcoholic liver disease with and without cirrhosis (Skrede et al. 1978, Tobiasson and Boeryd 1980, Joelsson et al. 1984) and in the diagnosis of anicteric cirrhosis (Douglas et al. 1981, Greenfield et al. 1986) as in these conditions serum bile acid concentrations were elevated in the presence of normal conventional liver tests. Serum bile acids were reported to be of prognostic significance in cirrhosis (Mannes et al. 1986). However, for the diagnosis and follow-up of liver fibrosis they are not useful (Einarsson et al. 1985). Raised serum bile acids reflect the disturbances in portal circulation by the fibrosis, rather than the fibrosis itself.

Because the conventional liver tests have not proved to be useful in the diagnosis and follow-up of liver fibrosis and because of the limitations of the liver biopsy other methods for investigation of hepatic fibrosis have been studied. Imaging techniques like ultrasonography, radioisotopic liver scanning or computerized tomography may suggest the presence of cirrhosis indirectly from findings such as prominence of the caudate lobe associated with atrophy of the right lobe (Harbin et al. 1980) or signs of portal hypertension. They do not detect early fibrosis (Ralls et al. 1986, Medhat et al. 1988).

PROCOLLAGEN TYPE III AMINOTERMINAL PEPTIDE

In early hepatic fibrosis the synthesis of collagen type III is increased. During the production the procollagen molecule is firstly trimmed at the carboxyterminal by a specific peptidase and subsequently and not completely at the aminoterminus (see chapter I.4). The aminoterminal peptide of type III procollagen (PIIIP) used for establishing the radioimmunoassay has been prepared from fetal calf skin (Rohde et al. 1979). In the commercially available radioimmunoassay the labeled peptide is allowed to react with a rabbit antiserum against the peptide but also against the collagen molecule with the unsplit aminoterminal peptide (RIAgnost® PIIIP tachysorb, Behringwerke, Marburg, Germany).
The presence of material antigenically related to the aminoterminal peptide of type III procollagen has been reported in human serum, urine, ascitic fluid (Rohde et al. 1979), bile (Raedsch et al. 1980), pulmonary lavage fluid (Low et al. 1983), synovial fluid (Gressner and Neu 1984a), cerebrospinal fluid (Gressner and Neu 1984b), seminal fluid (Gressner and Neu 1984c) and amniotic fluid (Pierard et al. 1984).
In most cases the inhibition curve in the radioimmunoassay given by the samples does not parallel that of the standard fetal calf aminoterminal segment. This is the result of the presence of several antigenic forms with different inhibitory capacities. Apart from the antigen that gives the same slope in the inhibition curve as the standard aminoterminal peptide and which probably represents the aminopeptide that is split off during the conversion of type III pN-collagen to type III collagen, there are smaller and larger forms. The smaller forms are thought to originate from degradation of the propeptide, while the larger may be derived from the breakdown of pN-collagen type III (Niemela et al. 1982, Rojkind 1984, Davis and Madri 1987).

Only a few reports concerning the radioimmunoassay based on antibody Fab fragments have been published. This assay does not distinguish the intact aminopropeptide from its globular fragment. Elevated serum levels of the aminopeptide were found in alcoholic hepatitis and liver cirrhosis. This radioimmunoassay was suggested to be able to discriminate between simple fatty liver and perivenular fibrosis in alcoholic liver disease (Rohde et al 1983, Sato et al. 1986).

Risteli et al. (1988) developed an equilibrium-type radioimmunoassay for the aminoterminal peptide of procollagen type III in which the problem of the non-parallelism between the standard and human serum samples has been overcome. Interference from degradation products of the propeptide

in serum is diminished due to selection of the antiserum and the reaction conditions.

Recently a new radioimmunological procedure, in which the antibody raised against PIIIP is coated on the test tubes, has become available (RIAgnost® PIIIP coated tube, Behringwerke, Marburg, Germany).
The value of these last two tests remains to be established.

The serum levels of PIIIP vary with age, being very high in young infants and children and decreasing gradually to the adult values. There is no significant difference in PIIIP levels between the sexes (Trivedi et al. 1985). During the third trimester of about half of all normal pregnancies the PIIIP level in the maternal serum is elevated due to the rapid rate of collagen synthesis and breakdown in the growing uterus (Risteli et al. 1987).
A great number of clinical studies correlating PIIIP levels with liver tests and histologic features has been performed to assess the clinical value of PIIIP in liver disease. Elevated levels were found in about 80% of individuals with a broad scale of liver disorders, including alcoholic liver disease particularly alcoholic hepatitis (Rohde et al. 1979, Savolainen et al. 1984, Tanaka et al. 1986), acute and chronic viral hepatitis (Annoni et al. 1986, Bentsen et al. 1987, Chang et al. 1989), primary biliary cirrhosis (Babbs et al. 1988, Niemela et al. 1988, Mutimer et al. 1989), hepatocellular carcinoma and metastatic liver cancer (Bolarin et al. 1982, Hatahara et al. 1984). Serum concentrations of PIIIP were reported to be of prognostic significance in primary biliary cirrhosis (Eriksson and Zettervall 1986, Niemela et al. 1988, Babbs et al. 1988).
Various studies have addressed the relationship between PIIIP levels and liver histology; correlation of PIIIP levels and established fibrosis was reported by several authors (Frei et al. 1984, Sato et al. 1986b, Tanaka et al. 1986, Babbs et al. 1989, Gabrielli et al. 1989), while others found a correlation with inflammation and necrosis (Rohde et al. 1979a, Colombo et al. 1985, Surrenti et al. 1987) or fibrogenesis (Heredia et al. 1985, Galambos et al. 1985, Torres-Salinas et al. 1986, Bentsen et al. 1987). In our opinion these processes cannot be separated, as increased fibroplastic activity may be triggered by inflammation and necrosis.
PIIIP has also been reported to correlate with hepatic prolyl hydroxylase (Savolainen et al. 1983, Torres-Salinas et al. 1986), the rate-limiting enzyme in collagen synthesis (Fessler and Fessler 1978) and with routine liver tests in primary biliary cirrhosis (Savolainen et al. 1983) and in alcoholic liver disease (Tanaka et al. 1986).
In patients with alcoholic cirrhosis or alcoholic hepatitis serum PIIIP levels were correlated with the severity of fibrosis and were useful in monitoring

progression to cirrhosis in early cases (Rohde et al. 1979a, Savolainen et al. 1984, Torres-Salinas et al. 1986). Correlations of PIIIP with the severity of fibrosis were also described in primary biliary cirrhosis, chronic active hepatitis and cirrhosis (Tuderman et al. 1977, Kuutti-Savolainen et al. 1979a, Niemela et al. 1983, Savolainen et al. 1984, Frei et al. 1984, Lu et al. 1986). In a recent study the integrated value of serum PIIIP over time predicted hepatic stainable collagen in a model of dietary cirrhosis in the rat (Ruwart et al. 1989).
Unfortunately many patients with inactive cirrhosis, chronic persistent hepatitis or fatty liver have normal values, which makes the test unsuitable for discriminating between those patients and normal subjects (Niemela et al. 1983, Frei et al. 1984, Torres-Salinas et al. 1986). This fact taken into account, it will be clear that determination of PIIIP can not replace the liver biopsy in the differential diagnosis of a liver disease.
Elevated PIIIP levels have been reported in several non-hepatic diseases including infectious diseases (Bolarin et al. 1984), diabetes mellitus with macroangiopathy (Triolo et al. 1989), rheumatoid arthritis (Hrslev-Petersen et al. 1988) and renal insufficiency (Hahn and Schuppan 1983).

The physiology of PIIIP is not fully understood. In liver disease, the concentration of PIIIP in the hepatic vein is higher than in the renal vein and in the arterial circulation, pointing to the liver as a source (Gressner et al. 1986).
Substantial biliary excretion of the peptide in humans was reported in one study (Raedsch et al. 1983). However, the influence of bile duct obstruction on the PIIIP levels is unknown. The fact that in renal failure the PIIIP levels are increased in a direct correlation with the serum creatinine suggests that the kidney is a major excretory route (Hahn and Schuppan 1983).
Sakakibara et al. (1986) found, using monoclonal antibodies, that collagen III and procollagen III are localized not only in the extracellular matrix of hepatocytes and sinusoidal cells of cirrhotic liver, but also in the cytoplasm. Sato et al. (1986a) reported that in the liver some PIIIP molecules remain as constituents of mature collagen fibrils. In some liver diseases hepatic collagenase activity and extracellular collagen degradation may be altered, so the interval between secretion and extracellular cleavage may vary with the liver disease. The situation becomes even more complicated as one takes into consideration that a proportion of procollagen degradation occurs intracellularly. PIIIP was demonstrated intracellularly as well (Sakakibara et al. 1986).

LAMININ P1 FRAGMENT

Laminin is a glycoprotein and a major component of basement membranes (see chapter I.1). As laminin accumulates in the extracellular matrix in fibrosis attempts have been made to use assays of a product of laminin synthesis to get insight into the fibrotic process.
Two antigenic determinants of the laminin molecule have been identified: (1) A central heavily disulphide-linked domain, which can be isolated after treatment with proteolytic enzymes, known as fragment 1 (LP1) (Timpl et al. 1979) and (2) the globular parts located at the ends of the short arms of the laminin molecule, called fragment 2 (Rohde et al. 1979b). A sensitive radioimmunoassay for the measurement of LP1 in serum has been developed (Risteli and Timpl 1981). Elevated levels have been reported in alcoholic hepatitis with cirrhosis (Niemela et al. 1985), in alcoholic fatty liver and perivenular fibrosis (Nouchi et al. 1987) and in post-hepatitic and primary biliary cirrhosis (Kropf et al. 1988). In non-cirrhotic alcoholic liver disease the LP1 level correlated well with the hepatic fibrosis (Robert et al. 1989). LP1 concentrations were found to be normal in alcoholics with an intact liver architecture and pure steatosis (Sotaniemi et al. 1986). In severe chronic active hepatitis elevated LP1 levels improved but did not normalize during therapy; elevated LP1 values at biochemical and clinical remission correlated with the presence of cirrhosis and predicted relapse (McCullough et al. 1987). In patients with liver fibrosis and cirrhosis concentrations of LP1 were higher in the hepatic vein than in the renal vein and femoral artery, suggesting that LP1 was being produced by the fibrotic liver (Gressner et al. 1986).
In several studies a correlation between serum LP1 levels and portal hypertension was found. Increased concentrations were correlated with elevated portal venous pressure (Gressner and Tittor 1986, Mal et al. 1988). Serum LP1 level was suggested to be a potentially useful serum marker for portal hypertension (Babbs et al. 1987, Gressner et al. 1988). In normal pregnancy (before delivery) LP1 levels increase to nearly twice the levels found in non-pregnant women (Bieglmayer et al. 1986). LP1 concentrations were also elevated in serum of patients with diabetes mellitus (Hogemann et al. 1986) and malignant tumors (Brocks et al. 1986, Rochlitz et al. 1987).

N-ACETYL-β-D-GLUCOSAMINIDASE

N-acetyl-β-D-glucosaminidase (NAG) (E.C. 3.2.1.30) is a lysosomal enzyme involved in the breakdown of glycosaminoglycans from

extracellular matrix. The activity of NAG was found to be increased in liver biopsies and serum of patients with chronic liver disease (Pott et al. 1978, Pott et al. 1979a).
A correlation between activity of the enzyme and degree of hepatic fibrosis was suggested (Pott et al. 1979b). Elevated serum levels have also been reported in acute viral hepatitis (Calvo et al. 1982) and several non-hepatic disorders including acute myocardial infarction, breast cancer, hypertension and diabetes mellitus (Calvo et al. 1982, Nomura et al. 1985, Perdichizzi et al. 1985).

OTHER PROPOSED MARKERS OF HEPATIC FIBROSIS

Several other possible parameters of connective tissue metabolism have been studied. The most important ones will be briefly summarized.
The carboxyterminal peptide of procollagen type I is increased in the serum of patients with liver diseases, especially alcoholic hepatitis and cirrhosis, while in fatty liver moderately increased levels are found. The test may be useful in diagnosing alcoholic hepatitis but the overlap between different stages of alcoholic liver disease makes it impossible to distinguish between the early and late stages of fibrosis (Savolainen et al. 1984). Increased serum levels have also been reported in patients with Paget's disease of bone (Simon et al. 1984).

Specific radioimmunoassays have been developed for the aminoterminal and the carboxyterminal end of the collagen type IV molecule (the so called 7S and NC1 domains respectively). Serum levels of these peptides have been reported to be elevated in alcoholic liver disease (Niemela et al. 1985, Schuppan et al. 1986). In dimethylnitrosamine-induced hepatic fibrosis in rats increases in the two type IV collagen-related antigens were found to be early events (Savolainen et al. 1988). The clinical significance of these peptides as markers of hepatic fibrosis remains to be elucidated.
Prolyl hydroxylase is the enzyme required for the hydroxylation of prolyl residues in procollagen polypeptide chains into 4-hydroxyprolyl residues, which are essential for the stable triple helical conformation of collagen. Prolyl hydroxylase is thought to be the rate-limiting enzyme in the collagen biosynthesis (Fessler and Fessler 1978). A radioimmunoassay recognizes the β-subunit of this enzyme. Increased serum levels were reported in chronic active hepatitis and primary biliary cirrhosis (Kuutti-Savolainen et al. 1979a, Savolainen et al. 1983). The relationship between the β-subunit of prolyl hydroxylase in serum and the collagen synthesis in the liver has not been studied in detail.

I.7 PRIMARY BILIARY CIRRHOSIS

Primary biliary cirrhosis (PBC) is a nonsuppurative destructive cholangitis resulting in destruction of small and medium-sized intrahepatic bile ducts (Rubin et al. 1965). Clinical and histologic features, the possible etiology, diseases associated with PBC, prognosis and treatment have recently been reviewed (Kaplan 1987, Vierling 1989). Although not a common disease (it has been estimated to account for 0.6 to 2% of deaths from cirrhosis throughout the world) PBC is of major economic importance because it is one of the leading reasons for liver transplantation.
Middle-aged women are primarily affected by the disease.
The main problem is the cholestasis resulting from destruction of bile ducts. Most patients present with pruritus eventually accompanied by jaundice. Other clinical features comprise hepatosplenomegaly, cholelithiasis, the complications of portal hypertension like gastrointestinal bleeding and symptoms and signs of associated diseases including scleroderma, Sjogren's syndrome, arthropathy, thyroiditis and renal tubular acidosis. Because of the cholestasis resorption of fat-soluble vitamins may be impaired giving rise to deficiencies of these substances. Impaired dark adaptation (vitamin A), osteomalacia (vitamin D) and coagulation disturbances (vitamin K) may occur. The diagnosis of PBC is based on laboratory and histologic characteristics. Routine biochemical liver tests are abnormal in most patients with PBC. Alkaline phosphatase is almost always elevated and of hepatobiliary origin. Serum bilirubin levels depend on the clinical stage of the disease, being normal in most early cases and elevated in later stages. γ-glutamyltranspeptidase levels are elevated in most patients, while the levels of aminotransferases usually are normal. Fasting serum bile acids are elevated in 93 to 100% of patients with PBC. Many patients show a secondary hyperlipoproteinemia. Antibodies to mitochondrial antigens in serum can be detected in over 90% of patients with PBC. A positive test may help to discriminate between patients with PBC and patients with extrahepatic biliary obstruction or primary sclerosing cholangitis. Unfortunately positive tests may also be found in patients with chronic active hepatitis, cryptogenic cirrhosis and collagen vascular diseases.
In recent years more and more asymptomatic patients are being identified following routine biochemical tests.
The etiology of PBC is still unknown, although it is generally considered a immunological disorder. Several possible agents have been postulated including hepatotropic viruses, fungi, bacteria, abnormal or toxic bile acids and hepatotoxic medicaments. Recently it has been demonstrated that the rough form of intestinal Escherichia coli may be involved in the

pathogenesis of PBC (Hopf et al. 1989). According to Scheuer (1983) liver histology in PBC is subdivided into 4 overlapping stages in the pathologic evolution of the disease, from cholangitis with duct lesion in stage I to cirrhosis in stage IV. The prognosis of patients with PBC is variable. Prolonged survival may occur and asymptomatic subjects may have a normal life expectancy. However, in the typical case the disease follows a progressive course with death because of complications of portal hypertension and hepatocellular failure (James et al. 1981, Roll et al. 1983). In a recent study in 195 patients with PBC the mortality from liver disease was calculated to be 40% after 5 years and 60% after 10 years. Independent clinical risk factors correlating with reduced survival were serum bilirubin level, ascites, bleeding from esophageal varices and age (Goudie et al. 1989). Up till now there is no established curative treatment for PBC. Several medicaments including D-penicillamine, azathioprine and chlorambucil have been tested in controlled trials but have not been shown to have a beneficial effect on the progression of the disease (Kaplan 1990). In one controlled trial prednisolone therapy improved histology but accelerated osteopenia (Mitchison et al. 1986). After two years of treatment with ursodeoxycholic acid there was a significant improvement in clinical and biochemical parameters. The results of long-term treatment of patients with PBC with ursodeoxycholic acid should be awaited. Liver transplantation offers the only opportunity for prolonged survival for patients in the terminal phase of PBC. The results of transplantation in 76 patients with PBC were reported. Fifty-two of the 76 patients (68%) survived with a follow-up period ranging from 1 to 6.5 years. Retransplantation was necessary in 25% of patients, being performed as early as 3 days and as late as 3 years after the first transplantation. The 5-year survival was 66% (Esquivel et al. 1988).

I.8 ALCOHOLIC LIVER CIRRHOSIS

The liver is the main site where alcohol is metabolized and therefore it is prone to the toxic effects of this substance.
Several authors have reviewed the pathogenesis of alcohol-induced liver injury, the clinical and histologic features and treatment (Lieber 1984, Teschke and Gellert 1988, Zakim et al. 1989).
In the U.S.A. about 100.000 deaths a year can be attributed to alcohol. In Sweden a 6-year study amongst middle-aged men in the early 1980's showed that premature deaths caused by alcoholism were as frequent as deaths resulting from cancer or coronary artery disease. Nineteen percent of the deaths due to alcoholism were caused by liver cirrhosis (Trell et al.

1985). In a national survey conducted in the U.S. 18% of all men and 5% of all women were found to be frequent, heavy drinkers (i.e. they drink 5 or more drinks per occasion, at least weekly). Orrego et al. (1983) reported that 15% of alcoholics with liver damage without cirrhosis developed cirrhosis in a follow-up period of at least 10 years. Sorensen et al. (1984) found in a study of 258 alcoholics who did not have cirrhosis at the start, that 38 patients develop a cirrhosis in a follow-up of 10 to 13 years, which means 2% a year. In clinical studies the risk of developing alcoholic cirrhosis seems to increase with the quantity of alcohol consumed (Lelbach 1975, Pequignot and Tuyns 1980, Norton et al. 1987) and women run a higher risk than men.
In The Netherlands from the 1960's to the 1980's the use of alcohol increased from an average of 7 to about 25 g a day per person (persons > 14 year) (Gips 1982). Alcoholic liver diseases, mainly liver cirrhosis, belong to the most important sequelae of excessive drinking. The mortality from liver cirrhosis has multiplied by ten in the past 20 years. Apart from the mortality alcoholic cirrhosis is associated with a considerable morbidity, so that attempts should be made to detect patients who are prone to develop cirrhosis at an early phase and when possible prevent further progression of the disease. The metabolism of ethanol and its metabolic effects have been reviewed by Weiner et al. (1988). Pathophysiology and clinical aspects were recently reviewed by Zakim et al. (1989). The direct effect of acetaldehyde on hepatic fibrogenesis was described in chapter I.5.
The diagnosis of alcoholic liver cirrhosis is based on the patient's history combined with clinical, biochemical and histological findings (Leevy et al. 1976). In the determination whether liver disease is likely to be present the quantity of ethanol consumed by the patient should be assessed. As little as 20 g of ethanol consumed daily by women or 40 to 60 g of ethanol taken by men over a long period is associated with an increased incidence of serious liver disease. In screening a general population a standardized questionnaire has been proven useful to detect alcohol abuse (Feuerleyn et al. 1977, Ewing 1982, Van Limbeek and Walburg 1987). This procedure has not been investigated in hospitalized patients. The clinical picture of alcoholic cirrhosis comprises a spectrum from asymptomatic subjects to patients suffering from the complications of portal hypertension and hepatic insufficiency (e.g. gastrointestinal bleeding, ascites, encephalopathy). Laboratory tests frequently used in diagnosis of alcoholic liver disease, apart from serum ethanol levels, are γ-glutamyl transpeptidase (γ-GT) and mean corpuscular volume of red cells (MCV). Elevated serum levels of γ-GT and higher MCV's may be found in patients consuming more than 4 drinks daily. Serum uric acid is raised in

10% of alcoholics. Though none of these tests is specific the combination of the results with other observations may lead to accurate diagnosis of alcoholic liver disease and may be used in the follow-up (Keso and Salaspuro 1989, Pol et al. 1990).
The only way to establish the diagnosis alcoholic liver cirrhosis is by histologic examination of the liver. The pathological spectrum of alcoholic liver disease was discussed by Hall (1985). Fatty liver, alcoholic hepatitis and cirrhosis are the main features of alcoholic liver disease. Some characteristics, though not specific, are steatosis, formation of steatogranulomas, megamitochondria and Mallory bodies. Steatosis refers to accumulation of fat in the cytoplasm of the hepatocytes, usually in the form of droplets. Distended fat-laden hepatocytes may rupture with the resultant formation of steatogranulomas. By light microscopy Mallory bodies are seen as eosinophilic amorphous masses within the cytoplasm of hepatocytes. These Mallory bodies consist of randomly oriented filaments, often with parallel arrays at the periphery as can be shown by electron microscopy. The formation of Mallory bodies is thought to be a manifestation of liver cell injury, which has resulted in a disturbance of intermediate filament metabolism. It has been suggested that Mallory bodies may be involved in immune complex-mediated liver injury in alcoholic liver disease.

Abstinence is the cornerstone of therapy for all stages of liver disease. There is no established therapy for alcoholic liver disease for those who continue to drink. Corticosteroids, anabolic steroids and colchicine have been tested in controlled trials in patients with alcoholic hepatitis but none was found to have a beneficial effect on long-term prognosis. Testosterone given to patients with alcoholic cirrhosis did not prove to be better than placebo on liver tests, histology and mortality (Gluud et al. 1986). Recently a favourable effect of treatment with propylthiouracyl was reported. In this study however the patients who benefited most from treatment with propylthiouracyl were those with the lowest alcohol consumption (Orrego et al. 1987). Once alcoholic liver cirrhosis is present the life expectancy is shortened. Tygstrup et al. (1985) reported an one year mortality of 23% in a study of 191 patients. In another Danish study a mortality of 29% was found during a median follow-up of 31 months (Gluud et al. 1988). Tanaka et al. (1987) stated a 50% mortality at 5.8 years of follow-up. Pignon et al. (1986) followed 210 patients with alcoholic cirrhosis for two years and found a mortality of 33%.

Many studies have been performed to find clinical and laboratory variables correlating with prognosis in cirrhosis. The Child-Pugh (CP)

classification is by far the most widely used and reported prognostic index for cirrhosis. This index is based on serum bilirubin and albumin levels, prothrombin time and absence or presence of ascites and encephalopathy.
The CP classification was originally utilized to predict prognosis after portal systemic shunt surgery. Operative mortality and long-term survival proved to be related to the CP classification (Pugh et al. 1973). Apart from the shunt surgery the CP classification may be applied as a prognostic index for cirrhosis in general (Conn 1981).

In recent years several other methods to predict prognosis in liver disease have been described. The combined clinical and laboratory index (CCLI) uses 12 items including the 5 components of the CP classification together with collateral circulation, edema, weakness, anorexia and spider naevi as clinical abnormalities and hematocrite and alkaline phosphatase as laboratory tests (Orrego et al. 1983).
The Copenhagen Study Group for Liver Diseases (CSL) described a Cox's regression model for identifying prognostic factors. The prognostic index is calculated on the basis of 13 variables. The variables utilized ressemble those of the CCLI, but in the CSL index collateral circulation and anorexia are replaced by sex and age. The acetylcholinesterase level in serum is added (Schlichting et al. 1983). With a growing number of variables the methods are getting more difficult and less practicable.
Applied to the CSL material the CCLI was less effective, indicating that one should be cautious in using the methods in other patient groups (Tygstrup et al. 1985).
Other factors reported to be of prognostic significance in cirrhosis include variceal bleeding and large esophageal varices at esophagoscopy (Gluud et al. 1988), high alcohol consumption (Borowsky et al. 1981, Tanaka et al. 1987) and mean arterial pressure and plasma noradrenalin concentration in patients with ascites (Llach et al. 1988).

In cirrhosis the main causes of death are liver failure and gastrointestinal bleeding (Schlichting et al. 1983). Other causes may be hepatocellular carcinoma and non-liver related causes including infections and cardiovascular disease. Treatment of the main causes of death may influence the prognosis of cirrhosis. Endoscopic sclerotherapy of esophageal varices and medical or surgical treatment of portal hypertension may diminish gastrointestinal bleeding (Orloff and Bell 1986, Sarin et al. 1986, Pasta et al. 1989). Once liver insufficiency is present, liver transplantation is the only option for long-term survival (Kumar et al. 1990).

I.9 LIVER TRANSPLANTATION

Liver transplantation has been accepted as a treatment in various end-stage liver diseases. Good results after liver transplantation have been reported in PBC and alcoholic cirrhosis (Starzl et al. 1987, Markus et al. 1989, Kumar et al. 1990). However disease recurrence in the graft has been reported for several liver diseases including hepatitis B and D, PBC and hepatocellular carcinoma (Portmann et al. 1986, Demetris et al. 1986, Reynes et al. 1989, Dietze et al. 1990).

I.10 REVERSIBILITY AND TREATMENT OF LIVER FIBROSIS

Fibrosis of the liver may be reversible as documented in several published observations (Bianchi 1970, Popper and Udenfriend 1970, Baggenstoss 1975). It is generally taught that liver cirrhosis in humans is irreversible. However, in literature several cases, in which reversal of histologically documented cirrhosis to absent or minimal fibrosis with normal liver architecture is claimed, have been reported. Rojkind and Dunn (1979) summarized the data of 18 patients, collected from several publications. They also mention the most important problem: a normal-appearing needle biopsy can be obtained from patients with a documented cirrhosis due to sampling error.
Even taking this limitation into consideration, from the reported cases it seems possible that liver cirrhosis is reversible.
Assuming that liver fibrosis and cirrhosis may be reversible would suggest that it is possible to treat it. The first step in treatment involves, if possible, elimination of the agent that initiated the fibrosis e.g. alcohol abstinence. Various drugs have been proposed as anti-fibrotic agents. Kershenobich et al. (1988) reported the beneficial effect of colchicine on survival in cirrhotic patients. However, there is doubt whether this effect was achieved by influencing collagen synthesis. It has been suggested that any beneficial effect of colchicine in liver fibrosis was mediated by other mechanisms, including anti-inflammatory action and stimulation of collagenase secretion (Chojkier and Brenner 1988).
Many agents decrease collagen synthesis *in vitro*, including procollagen peptides (Wu et al. 1986), corticosteroids (Weiner et al. 1987, Kucharz 1988), malotilate (Ryle and Dumont 1987), γ-interferon (Jimenez et al. 1984) and prostaglandins (Baum et al. 1980). Malotilate is supposed to have an antifibrotic effect in liver disease because of inhibition of leucotriene synthesis (Zijlstra et al. 1989). *In vivo* corticosteroids might

also decrease collagen production by their anti-inflammatory action. Their side-effects however limit the application as anti-fibrotic drugs for chronic administration. γ-interferon treatment decreased collagen deposition in murine schistosomiasis (Czaja et al. 1989). Favourable effects were also reported for medroxyprogesterone acetate in rat liver cirrhosis (Stenback et al. 1989), a prolylhydroxylase inhibitor in fibrotic rat liver (Fujiwara et al. 1988) and 16, 16-dimethyl prostaglandin E2 on collagen formation in nutritional injury in rat liver (Ruwart et al. 1988). The usefulness of these drugs in human fibrotic liver disease remains to be established.

Chapter II PROGNOSIS AND PROGNOSTIC FACTORS IN ALCOHOLIC LIVER CIRRHOSIS

INTRODUCTION

Alcoholic liver cirrhosis has a considerable morbidity from ascites, bleeding from esophageal varices and hepatic encephalopathy and mortality due to variceal bleeding, infections, liver insufficiency and renal failure. The poor prognosis of patients with alcoholic cirrhosis has been described in several studies from some European countries (Tygstrup et al. 1985, Pignon et al. 1986, Gluud et al. 1988). A study by Van Toorn (1974), performed in the early 1970's, found a surprisingly good prognosis for a group of Dutch patients with cirrhosis of various etiology. As no systematic study of the prognosis of patients with alcoholic cirrhosis has been reported from the Netherlands, we decided to perform a follow-up study of patients admitted to hospital for the first time with alcoholic cirrhosis. In this study we examined clinical and biochemical factors which might be of prognostic importance. The clinical classification of the severity of the cirrhosis is generally made according to the Child-Pugh criteria, which are based on several clinical and biochemical parameters (Pugh et al. 1973). In recent years many attempts were made to provide a better scoring system for the severity of liver cirrhosis. Generally these have led to rather complicated methods, which were less practicable.
In this study we investigated the prognosis of patients with alcoholic cirrhosis to find out whether there are easily obtained factors that at the first admission to hospital can already give an impression about the prognosis.

MATERIAL AND METHODS

In the period from January 1st 1982 to December 31st 1987 99 patients were admitted in whom alcoholic cirrhosis was diagnosed for the first time. Sixty-four patients were men, 35 women. The mean age was 52 years, ranging from 28 to 77 years. The diagnosis was made on clinical, chemical and histological characteristics. For detection of esophageal varices, esophagoscopy or radiological examination was performed. A set of hematological (hemoglobin, hematocrit, mean corpuscular volume (MCV), leucocyte count, Normotest®, Thrombotest®), chemical (serum

creatinine, uric acid, alkaline phosphatase, γ-glutamyl transpeptidase (γ-GT), ASAT, ALAT, albumin, γ-globulins, hepatitis B surface antigen (HBsAg)) and clinical (presence of ascites, esophageal varices, encephalopathy, continued alcohol abuse) parameters was collected for every patient. The Child-Pugh classification was determined, modified in this way that the average of Normotest® and Thrombotest® replaced the APTT (Pugh et al. 1973). The information necessary for the follow-up, when it was not available in our hospital, was obtained via family doctors or civil registration. The duration of the follow-up was the period from the first day of admission to death or to the closing date of the follow-up (31 December 1989).
The number of haemorrhages from esophageal varices in this period was recorded. The causes of death were divided into causes related to the liver disease (liver insufficiency, variceal bleeding) and other causes.
Abstinence from alcohol was based on the patient's history and three consecutive blood samples that were negative for alcohol.
Survival was estimated and compared using Kaplan-Meier curves and logrank-tests (Peto et al. 1977, Pignon et al. 1986). Adjustment for confounding factors was done by stratification or use of multivariate Cox-regression. Correlation coefficients given are Pearson's. Other tests used are given in the text. The significance level used was 0.05 (two- sided).

RESULTS

Three patients were lost to follow-up, all directly after discharge (two sailors from abroad and another patient who could not be traced). Mean follow-up for the remaining 96 patients was 25 months and ranged from 1 day (died on the day of admission) to 69 months. During the study period 34 patients died. Causes of death were liver failure in 8 cases, variceal bleeding in 11 and other causes in 11; in 4 cases the cause of death was unknown. For the total group actuarial survival percentages were 69% (standard error (s.e.) 5%) and 50% (s.e. 8%) at 2 and 5 years respectively. Survival did not differ significantly between males and females. Figure II.1 shows survival according to the age of the patients. Patients with an age of 50 years or more had a significantly worse survival than younger patients ($p=0.01$). A finer subdivision of age did not show any further significant differences.
Survival according to the Child-Pugh (CP) classification is depicted in figure II.2. Prognosis in case of CP class A was best, in CP class C worst, while CP class B survival was intermediate (trend test: $p<0.001$). The decreasing survival with increasing CP class was present in both age

groups. The presence of varices was significantly associated with poor survival ($p<0.05$). For the groups with varices and without varices at diagnosis the survival percentages at two years were 62% and 82% respectively. However, this difference could be explained by the strong correlation between the presence of varices and the CP classification (see table II.1). When adjusted for CP class no significant difference in survival remained between the group with and the group without varices.
The hemoglobin-level also correlated significantly with survival when considered alone.
Two years survival for the groups hemoglobin less than 7.5 mmol/l, hemoglobin between 7.5 and 9.0 and hemoglobin greater than 9.0 was 54%, 73% and 100% (trend-test: $p=0.02$). After adjusting for CP class, however, the decreasing survival with decreasing hemoglobin was not present. This could be explained by the correlation of hemoglobin-level with CP class: with increasing CP class hemoglobin-level decreased.
Survival did not correlate significantly with hematocrit, MCV, leucocyte count, creatinine, uric acid, alkaline phosphatase, ASAT, ALAT, γ-GT, γ-globulins or HBsAg status.
Considering factors that contributed to CP class (bilirubin, albumin, ascites, encephalopathy and the average of Normotest® and Thrombotest®) it appeared that survival decreased significantly with increasing bilirubin, decreasing albumin, decreasing average of Normotest® and Thrombotest® and when ascites was present. Using multivariate analysis, bilirubin and albumin levels were the most important prognostic factors which were independently related to survival. A 10 g/l decrease in albumin level corresponds to an increase of the death rate by factor 1.8. A doubling of bilirubin level is associated with a 40% increase in death rate, although the latter finding is just above statistical significance ($p=0.06$) (see figure II.3). When both these factors are taken into account no further prognostic value of the other factors contributing to the CP class could be demonstrated. The average of Normotest® and Thrombotest® correlated significantly with serum bilirubin concentration ($r=-0.41$; $p<0.01$) and albumin in serum ($r=0.46$; $p<0.01$). Patients with ascites ($n=49$) had significantly higher bilirubin levels (mean: 43.0 ug/l) than patients without ascites (20.0 ug/l, Mann-Whitney test: $p<0.01$). The mean levels of albumin for patients with and patients without ascites were respectively 30.6 and 37.2 g/l (Mann-Whitney test: $p<0.01$).
Alcohol abuse after discharge could be reliable assessed in 40 patients only. Of the 15 patients who had abstained from alcohol none had died during the study period. Of those who continued their abuse ($n=25$) 12 died (Fisher exact test: $p=0.01$). Both groups were similar regarding age and CP class.

DISCUSSION

Several studies have shown that patients with alcoholic liver cirrhosis have a shortened life expectancy. Tygstrup et al. (1985) found a one year mortality of 23% in a study of 191 patients. In another Danish study a mortality of 29% was reported during a median follow-up of 31 months (Gluud et al. 1988). Tanaka et al. (1987) stated a 50% mortality at 5.8 years of follow-up in Japanese patients. Pignon et al. (1986) followed 210 patients with alcoholic cirrhosis for 2 years and found a mortality of 33%. These results are comparable with ours (one year mortality 20%, 2 year mortality 31% and 5 year mortality 50%). So despite possible differences in treatment between different countries the mortality of alcoholic cirrhosis appears to be similar. At least 19 of the 34 patients in our study died of a complication of the liver disease. Haemorrhages from esophageal varices were a main cause of death. In another Dutch study Van Toorn et al. (1974) reported that the finding of esophageal varices in patients with cirrhosis did not influence survival. This is in contrast with our findings and may be explained by the composition of their study group as only 25% of their patients had an alcoholic cirrhosis.

Sauerbruch et al. (1988) found a half-year mortality of 19% after bleeding from esophageal varices. Conn (1985) reported that 20 to 30% of the patients with cirrhosis die from variceal bleeding. Apart from mortality variceal bleeding also gave rise to considerable morbidity. For the treatment of ascites and hepatic encephalopathy frequent and mostly long hospital admissions were necessary.

In the determination of the chances of survival a number of clinical and chemical parameters proved to be of prognostic significance. The age, the presence of ascites and esophageal varices and the hemoglobin-level were all of prognostic importance with respect to death.

In recent years a number of factors that can predict the prognosis in alcoholic cirrhosis has been reported such as variceal bleeding and large esophageal varices at esophagoscopy (Gluud et al. 1988), high alcohol consumption (Tanaka et al. 1987, Borowsky et al. 1981) and age (Tanaka et al. 1987, Pignon et al. 1986, Christensen et al. 1986). Llach et al. (1988) found that the mean arterial pressure and plasma noradrenalin concentration in patients with ascites were the best indicators of prognosis. Orrego et al. (1983) proposed a scoring system based on 12 clinical and laboratory variables. This system was not equally effective in the material of the Copenhagen Study Group for Liver Diseases (Tygstryp et al. 1985). The Child-Pugh classification is by far the most widely used and reported prognostic index for cirrhosis. It is mainly utilized to predict prognosis after portosystemic shunt surgery. Operative

mortality and long-term survival proved to be related to the Child-Pugh classification (Conn 1981).
In multivariate analysis the main variables were the serum level of bilirubin and albumin, these were independently related to survival. In other studies abnormalities in these parameters were also associated with a higher mortality (Orrego et al. 1983, Sauerbruch et al. 1988, Christensen et al. 1986, Llach et al. 1988). The CP classification based on grade of hepatic encephalopathy, amount of ascites present, the bilirubin and albumin level in serum and an indicator of production of clot factors (in our study the average of Normo- and Thrombotest) was a good predictor of prognosis in our study. Considering only serum bilirubin and albumin however, nearly the same prognostic information was obtained. The hemoglobin-level and the presence of esophageal varices correlated significantly with survival when considered alone. Both factors were found to correlate significantly with the CP classification. Thus in a higher CP class varices were more frequently present, probably reflecting a higher portal tension in more severe cirrhosis. A lower hemoglobin-level in a high CP class may be explained by gastrointestinal bleeding or malnutrition.

The abstinence from alcohol has a favourable influence on prognosis in several studies (Borowsky et al. 1981, Powell and Klatskin, 1968, Galambos 1974, Capone et al. 1978). In our study only 40 patients met the criteria for determining ongoing alcohol consumption - a clear history and repeated blood alcohol measurements at out-patient visits. However of the 25 patients in this group who continued drinking 12 died patients in the follow-up period, of the 15 patients who abstained no one died. This difference was statistically significant. A favourable effect of abstinence is not found when a cirrhosis is associated with severe portal hypertension at the time of diagnosis (Soterakis et al. 1973). Because medicinal treatment of alcoholic liver disease is generally disappointing (Juhl and Christensen 1985, Morgan 1985, Orrego et al. 1987) the treatment should be directed to early detection of those patients who will develop liver cirrhosis and when possible prevention of development of cirrhosis via programs to stop alcohol abuse.

In conclusion alcoholic liver cirrhosis in the Netherlands has a considerable mortality. In our study the 5 year mortality was 50%. The main causes of death are variceal bleeding and liver insufficiency. In multivariate analysis of clinical and chemical parameters the Child-Pugh classification appeared to be the best predictor of prognosis. The combination of serum bilirubin and albumin was of nearly the same prognostic significance.

Table II.1
Relationship of esophageal varices with Child-Pugh classification

		A	B	C
Varices	present	21 (58%)	31 (78%)	18 (90%)
	absent	15	9	2
	total	36	40	20

Chi-Square test: p=0.03

Figure II.1
Survival according to age

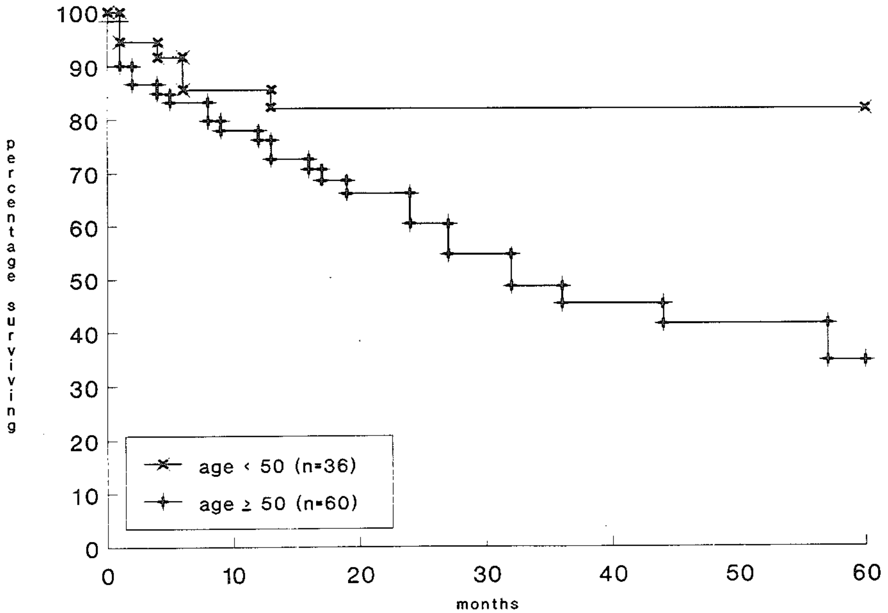


Figure II.2
Survival according to Child-Pugh classification

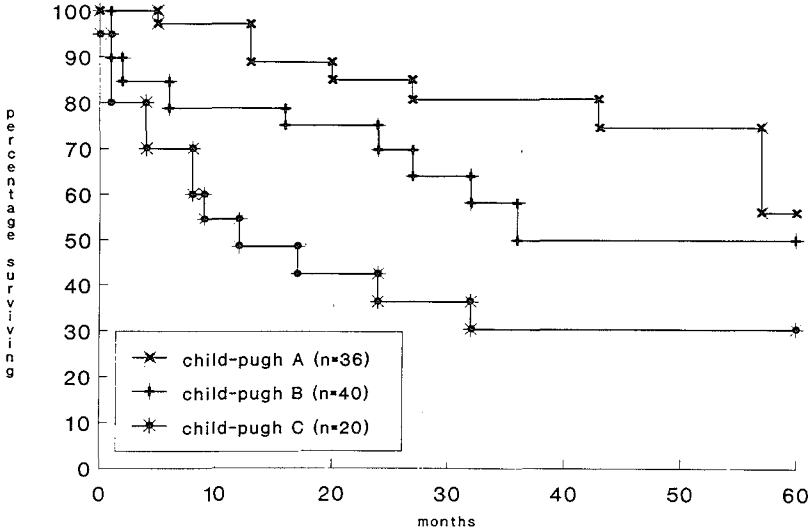
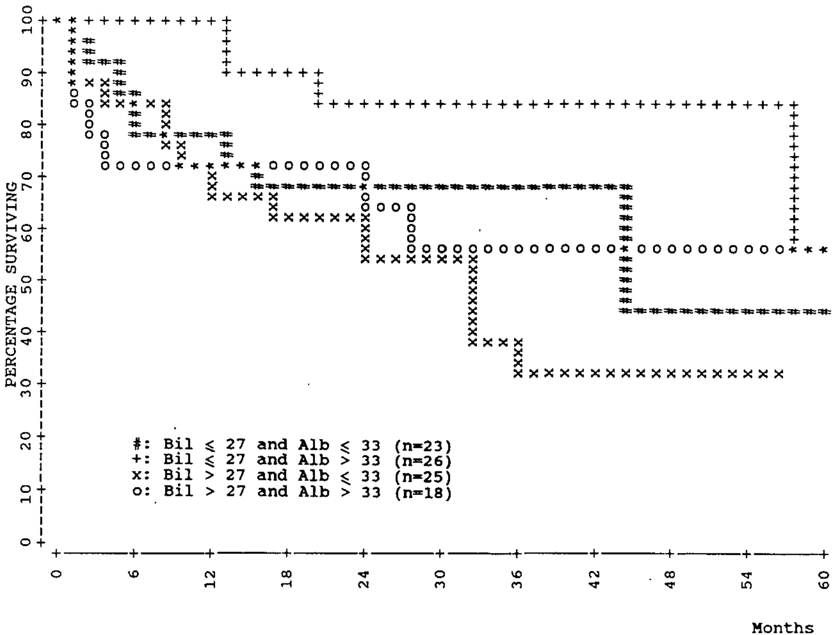


Figure II.3
Survival according to serum bilirubin and albumin levels



Chapter III PROCOLLAGEN III PEPTIDE LEVELS IN ALCOHOLIC LIVER DISEASE AND PRIMARY BILIARY CIRRHOSIS

INTRODUCTION

Fibrosis is a frequent finding in chronic liver diseases (Popper and Udenfriend 1970, Popper and Piez 1978, Chen and Leevy 1975). The most direct way to diagnose and quantitate fibrosis is by histological examination of liver biopsies. Liver biopsy, however, has some limitations. When liver tissue is obtained by means of a blind biopsy, the overall accuracy in diagnosis of a chronic liver disease is about 81%. When a directed biopsy is performed, the diagnostic accuracy can be increased to 95% (Pagliaro et al. 1983). Because of the inhomogeneous distribution of fibrosis and cirrhosis in the liver, there is a possibility of sampling error. In addition, liver biopsy is associated with a morbidity and slight mortality (Sherlock et al. 1984) and the frequency and number of biopsies that can be performed in an individual patient are limited.

During the past decade several serum parameters of collagen synthesis have been investigated. Serum concentrations of enzymes involved in synthesis of collagen have been measured. Some of these do not correlate with hepatic fibrosis; other enzymes appeared to, but their measurement proved too cumbersome for routine diagnostic use (Tuderman et al 1977, Kuutti-Savolainen et al. 1979a, Kuutti-Savolainen et al. 1979b, Stein et al. 1970). Potentially more valuable markers are peptides derived from collagen either during synthesis or breakdown. Rojkind et al. (1979) found the collagen content of cirrhotic livers to be elevated 4 to 7 times. This mainly involved collagen types I and III, independent of the etiology of the cirrhosis. In liver disease with increased connective tissue formation, synthesis of collagen type III preceeds that of collagen type I (Wick et al. 1978). In recent years, a sensitive radioimmunoassay for the N-terminal peptide of procollagen type III has been developed (Taubman et al. 1974, Nowack et al. 1976).
The procollagen peptide is enzymatically split off the procollagen molecule during the formation of collagen from procollagen (Fessler and Fessler 1978, Rojkind 1981) (Fig. III.1). Using radioimmunoassay techniques, this procollagen peptide has been demonstrated in serum, urine, ascites and bile (Rohde et al. 1979, Raedsch et al. 1982). Elevated serum concentrations of the procollagen peptide have been found in

patients with acute viral hepatitis, chronic active hepatitis, alcoholic liver disease and liver cirrhosis (Rohde et al. 1979).
To assess the diagnostic value of determining the procollagen type III peptide (PIIIP), we measured the levels of this peptide in serum of patients with alcohol abuse with and without cirrhosis and of patients with primary biliary cirrhosis.

PATIENTS AND METHODS

Serum PIIIP was measured in 15 healthy controls, in 18 patients with alcohol abuse without liver cirrhosis, in 23 patients with alcoholic cirrhosis and in 23 patients with primary biliary cirrhosis. In all individuals the diagnosis was based on clinical and biochemical findings. The diagnosis of cirrhosis was confirmed by histological examination in all instances. Primary biliary cirrhosis was graded histologically according to Scheuer (1983). All patients had normal serum urea and creatinine levels at the time blood samples were taken.

Serum Collection

Serum was obtained after clotting blood samples taken from fasting patients. The determination of PIIIP was carried out immediately or the serum was kept frozen at –70°C.

Radioimmunoassay

For the radioimmunological determination of PIIIP, a commercially available kit (Behringwerke, Frankfurt, Germany) containing ^{125}-I-labelled PIIIP peptide and the specific antibody from rabbit was used. The RIA procedure was performed as described by Rohde et al. (1979) with the modifications suggested by the manufacturer. Since antibody binding is not linear to the concentration of procollagen peptide, three dilution steps were carried out. All tests were performed in duplicate and a standard serum was run with all determinations for quality control (n=23:16.5 ng/ml vs manufacturer's value of 17.0 ng/ml; coefficient of variation 11%). Day-to-day variation has been reported to be 7.0% (n=6) (Gressner and Tittor 1986).

Statistical Analysis

Comparison of assay data between groups was performed with Student's t-test. Correlations were determined by linear regression analysis.

RESULTS

The groups were comparable with regard to age and sex, with the exception of the primary biliary cirrhosis group in which there was a preponderance of women (2 men and 21 women). The serum PIIIP concentrations were low in the control group (Table III.1). The patients with alcoholic cirrhosis had significantly higher concentrations ($p<0.0001$). There was also a significant difference in serum PIIIP concentrations between the controls and the patients with primary biliary cirrhosis ($p<0.01$). Serum PIIIP concentrations were also significantly higher in the patients with alcoholic cirrhosis than in the patients with alcohol abuse without cirrhosis ($p<0.0001$). There was no correlation between serum PIIIP and serum ASAT or serum alkaline phosphatase concentration. In the cirrhosis group there was a positive correlation between serum PIIIP levels and serum bilirubin concentration, and a correlation was found between fasting serum bile acids and PIIIP concentrations (Table III.2). Some of the patients have been followed-up quarterly for a longer period. PIIIP levels dropped in the few individuals with alcohol abuse who abstained from alcohol and became almost normal in several weeks to months. This has also been observed by other authors (Niemela et al. 1983, Niitsu et al. 1985, Nouchi et al. 1987). In the group as a whole there was no correlation between serum PIIIP and γ GT levels. There was no significant correlation between serum PIIIP levels and histological grading in primary biliary cirrhosis, although patients in stage IV tended to have higher levels.

DISCUSSION

Synthesis and deposition of collagen in excess to degradation is a major feature of chronic liver disease. This may give rise to fibrotic scars and even to irreversible cirrhosis. Fibrosis has conventionally been estimated by liver biopsy, and until recently there was no practical or reliable method available for gaining insight into the dynamic process of synthesis, deposition and degradation of specific collagens. Rojkind et al. (1979) showed that about 70% of liver collagens are type I or type III collagens. In normal liver type I and type III collagens are present in approximately equal amounts (Rojkind et al. 1979). In liver disease, the relative contribution of the two collagen types may vary, depending on the stage of the disease. Type III is the predominant collagen type in early fibrosis; in late cirrhosis type I is the main type (Rojkind et al. 1979), which may explain why patients with inactive cirrhosis sometimes have a normal

procollagen peptide level ((Niemela et al. 1983). Determination of PIIIP may therefore be particularly important in detecting relatively early stages of fibrosing liver disease.
The upper level of normal of serum PIIIP mentioned in the literature is 15 ng/ml (Rohde et al. 1979, Annoni et al. 1981, Pencev et al. 1981, Torres-Salinas et al. 1986, Colombo et al. 1985, Hahn 1984, Frei et al. 1984). In alcoholic steatosis, normal or slightly elevated levels are found (Savolainen et al. 1984, Torres-Salinas et al. 1986, Sato et al. 1986). The concentrations are significantly higher in alcoholic cirrhosis; only two of our patients with alcoholic cirrhosis had normal serum PIIIP levels (14.9 and 13 ng/ml) and this may represent an inactive phase in their cirrhosis (Niemela et al. 1983, Heredia et al. 1985). PIIIP levels probably do not give information about the amount of collagen already present in a liver. For the follow-up of alcoholic liver disease, with or without fibrosis, the serum PIIIP determination may be valuable for detecting ongoing fibrosis.
In patients with primary biliary cirrhosis, serum PIIIP levels were also significantly raised (Fig. III.2). Savolainen et al. (1983) and Weigand et al. (1984) also reported elevated concentrations in patients with primary biliary cirrhosis. There was no correlation between the histological grade of the disease and the peptide levels. This could be due to sampling error or to variable fibrotic activity in the different stages of the disease. We found that in our patients with cirrhosis there was a significant correlation between serum PIIIP and fasting serum bile acid levels. Elevation of serum bile acids is caused by portosystemic shunting (Ohkubo et al. 1983) and decreased hepatic clearance of cholic acids (Gilmore and Thompson 1980). Raedsch et al. (1983) studied biliary excretion of PIIIP in patients with alcoholic cirrhosis. The biliary excretion was found to be higher than in normal subjects, so it seems more likely that the elevated serum PIIIP levels are due to overproduction than to decreased biliary excretion.
Serum bile acid levels have been reported to be of prognostic value in cirrhosis (Mannes et al. 1986). It was also suggested that serum PIIIP levels are of prognostic value in primary biliary cirrhosis (Eriksson and Zettervall 1986). Whether this last suggestion is true needs to be elucidated in further studies. In former studies serum PIIIP has been reported to correlate with fibrosis (Rohde et al. 1979, Torres-Salinas et al. 1986, Savolainen et al. 1984, Sato et al. 1986, Tanaka et al. 1986) but also with inflammation, necrosis and degeneration (Colombo et al. 1985, Savolainen et al. 1984, Surrenti et al. 1987). In our opinion, these processes should not be separated as increased fibroplastic activity may be triggered by inflammation and necrosis. The correlations reported between serum PIIIP and enzymes involved in collagen synthesis and serum proline levels may favour this view (Tanaka et al. 1986, Bolarin et

al. 1984). Further studies using histomorphological methods and specific collagen stainings, which are now underway, may clarify this problem. We conclude that serum PIIIP levels are markedly elevated in alcoholic and primary biliary cirrhosis and only slightly in alcohol abuse without cirrhosis. PIIIP assays are potentially valuable for detection of hepatic fibrosis and for studies on the effects of treatment of liver cirrhosis. The discrepancy between histological grading and serum PIIIP levels in primary biliary cirrhosis could be due either to biopsy sampling error or to different rates of fibrogenesis at different stages of the disease.

Fig. III.1
Schematic representation of the production of procollagen type III peptide

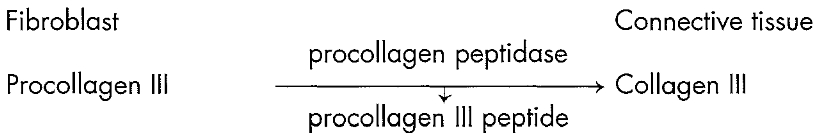


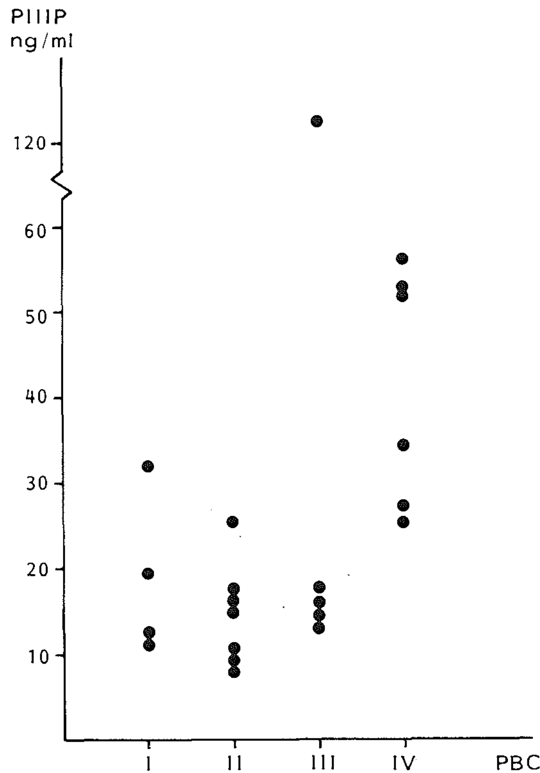
Table III.1
Serum procollagen type III peptide levels in healthy controls, patients with alcohol abuse with and without cirrhosis and patients with primary biliary cirrhosis (PBC).

	PIIIP (ng/ml)	
Controls (n=16)	9.2 +/- 1.8	
		p<0.01
PBC (n=23)	27.5 +/- 25.0	p<0.0001
Alcoholic cirrhosis (n=23)	37.8 +/- 21.2	
		p<0.0001
Alcoholic abuse (n=18)	12.5 +/- 2.6	

Table III.2
Correlation between serum procollagen type III peptide and other liver tests (n=29).

parameter	r	p
Bilirubin	0.55	< 0.01
γ-GT	0.30	> 0.1
ASAT	0.32	> 0.1
Alkaline phosphatase	0.16	> 0.1
Fasting bile acids	0.73	< 0.01

Fig. III.2
Serum procollagen type III peptide (PIIIP) levels in various stages of primary biliary cirrhosis (PBC).



Chapter IV SERUM PROCOLLAGEN TYPE III N-TERMINAL PEPTIDE AND SERUM LAMININ LEVELS IN ALCOHOLIC LIVER DISEASE AND PRIMARY BILIARY CIRRHOSIS

INTRODUCTION

Hepatic fibrosis may develop as a consequence of chronic inflammation in the liver. The most prominent feature of this fibrosis is excessive intercellular deposition of biomatrix, which is normally present at rather low concentrations (Popper and Udenfriend 1970, Rauterberg et al. 1981, Rojkind and Ponce-Noyola 1982) The mechanism of the accumulation of the main fractions of the biomatrix (collagens, noncollagenous high molecular weight glycoproteins (laminin and fibronectin) and some proteoglycans and glycosaminoglycans) involves their activated *de novo* synthesis in non-parenchymal and probably also parenchymal cells of the liver (Gressner 1986). During this process a fraction of the newly synthesized biomatrix escapes into the systemic circulation and becomes measurable in elevated levels in the serum (Hahn 1984).

Rojkind et al. (1979) found the collagen content of cirrhotic livers to be elevated 4 to 7 times. This was mainly collagen types I and III and this was independent of the etiology of the cirrhosis. In liver diseases with increased connective tissue formation synthesis of collagen type III preceeds that of collagen type I (Wick et al. 1978). A sensitive radioimmunoassay for the N-terminal peptide of procollagen type III (PIIIP) is now available. The procollagen peptide is split from the procollagen molecule by a specific peptidase during the formation of collagen from procollagen (Fessler and Fessler 1978, Rojkind 1981). Raised concentrations of PIIIP have been reported in sera from patients with alcoholic hepatitis and cirrhosis (Rohde et al. 1979a, Niemela et al. 1983, Frei et al. 1984).
Laminin is a major noncollagenous glycoprotein of basement mem-branes. A sensitive radioimmunoassay for the stable laminin P1 fragment has been developed (Risteli et al. 1981). This laminin P1 fragment (LP1) in serum probably reflects the metabolism of basement membranes. Raised serum LP1 levels have been reported in patients with alcoholic cirrhosis with hepatitis (Niemela et al. 1983, Sato et al. 1986).
In this study we evaluated the diagnostic application of serum PIIIP and LP1 levels in alcoholic liver disease and primary biliary cirrhosis (PBC).

PATIENTS AND METHODS

PATIENTS

Serum PIIIP and LP1 levels were measured in 14 healthy controls, in 27 patients consuming 60 gram of alcohol a day or more without cirrhosis, in 30 patients with alcoholic cirrhosis and 25 patients with PBC. Patients were classified on the basis of clinical, biochemical and ultrasound findings. The diagnosis of cirrhosis was confirmed by histological examinations in all cases. Primary biliary cirrhosis was graded histologically according to Scheuer (1983). Twelve of the 27 alcoholic patients without cirrhosis had steatosis. All patients had normal serum urea nitrogen and creatinine levels at the time blood samples were taken.

ASSAYS

Serum was stored at –70°C until PIIIP and LP1 were analyzed with radioimmunoassays (Behringwerke AG, Frankfurt, Germany). The concentration of PIIIP was calculated using a 50% intercept method (Niemela et al. 1982). The concentrations of LP1 were read from the corresponding standard curve. All tests were carried out in duplicate and a test serum was run with all determinations for quality control. For the PIIIP testserum the mean value of 23 determinations was 16.5 ng/ml (manufacturer's value 17.0 ng/ml; coefficient of variation 11%). With 4 batches of LP1 these values were 1,36 U/ml (manufacturer's value 1,35 U/ml; variation coefficient 2,2%).
Blood taken from a healthy individual on 6 different days showed a day-to-day variation of 7.1% and 4.9% respectively.

STATISTICAL ANALYSIS

Comparison of assay data between groups was performed with the Student's t-test. Correlations were determined by linear regression analysis.

RESULTS

In healthy subjects serum LP1 ranged from 1.09-1.44 U/ml (mean 1.28 U/ml) (figure IV.1). In patients with alcohol abuse without cirrhosis LP1 levels were significantly higher (mean 1.57 U/ml, $p<0.03$). Higher levels were found in patients with primary biliary cirrhosis (mean 2.14 U/ml, $p<0.001$) and in patients with alcoholic cirrhosis (mean 2.94 U/ml, $p<0.0001$). In alcoholic cirrhosis levels were significantly higher than in alcoholic abuse without cirrhosis ($p<0.0001$).

There were only weak correlations between serum LP1 and conventional liver tests such as bilirubin, ASAT and γ-GT (table IV.1). Only 3 patients with alcohol abuse without cirrhosis had serum LP1 levels above 2.0 U/ml. Only one patient with alcoholic cirrhosis had a normal value (14.0 U/ml). Three patients with primary biliary cirrhosis had normal values (1.37, 1.22 and 1.35 U/ml respectively). Patients with alcohol abuse without cirrhosis had normal or slightly elevated PIIIP levels (figure IV.2). Concentrations were significantly higher in PBC (26.8 +/- 23.8 ng/ml; $p<0.02$) and in alcoholic cirrhosis (35.9 +/- 29.6 ng/ml; $p<0.001$). In alcoholic cirrhosis serum PIIIP concentrations were significantly higher than in alcohol abuse without cirrhosis ($p<0.001$). Serum PIIIP levels showed weak correlations with biochemical liver parameters (table IV.1). There was a high correlation between the levels of PIIIP and LP1 in serum ($r=0.82$; $p<0.001$). In PBC there was no correlation between serum PIIIP or LP1 and the histological grading.

DISCUSSION

In the present study we evaluated the diagnostic application of radioimmunoassays for fragments of a basement membrane component and a collagen component in liver cirrhosis. Two different forms of cirrhosis were investigated: alcoholic cirrhosis with presumably intralobular fibrosis and primary biliary cirrhosis with periportal fibrosis. The concentrations of laminin-related antigens in serum has been reported to be elevated in patients with alcoholic liver disease, presumably alcoholic cirrhosis and hepatitis (Niemela et al. 1985, Sato et al. 1986). An association between serum LP1 levels and degree of hepatic fibrosis has been reported (Sato et al. 1986). In our study we found significantly elevated serum LP1 levels in patients with alcohol abuse without cirrhosis, some of whom had a fatty liver, and in patients with alcoholic cirrhosis. Only one of the patients with alcoholic cirrhosis had a normal serum LP1 level (1.40 U/ml) (3%). This patient may have inactive cirrhosis (Nouchi et al. 1987). Patients with PBC also had levels significantly higher than the controls. Patients with histological grade III and IV tended to have higher levels than patients with histological grade I and II, but there was no evident correlation. Twenty-two out of 25 patients with primary biliary cirrhosis had levels above 1.40 U/ml (89%).
Serum PIIIP concentrations were normal or slightly elevated in patients with alcohol abuse without cirrhosis. In alcoholic and primary biliary cirrhosis levels were significantly higher, as was reported by other authors (Rohde et al. 1979a, Niemela et al. 1983, Frei et al. 1984, Weigand et al.

1984, Niemela et al. 1985, Colombo et al. 1985). There was a strong correlation between serum LP1 and procollagen type III N-peptide. This correlation has been reported by other groups as well (Niemela et al. 1985, Nouchi et al. 1987). The observation that in alcohol abuse without cirrhosis serum LP1 is significantly elevated, but serum PIIIP is not, may be explained by assuming that the process of basement membrane production starts in an earlier phase than the formation of collagen.
In other studies serum PIIIP has been reported to correlate with fibrosis (Rohde et al. 1979a, Sato et al. 1986, Tanaka et al. 1986, Torres-Salinas et al. 1986) but also with inflammation and necrosis (Colombo et al. 1985, Surrenti et al. 1987). In our opinion these processes should not be separated as increased fibroplastic activity may be triggered by inflammation and necrosis.
Further study by means of histomorphological techniques and specific collagen stainings may clarify this problem.
In conclusion serum PIIIP and LP1 levels are raised in alcoholic and primary biliary cirrhosis. Serum LP1 levels are also elevated in patients with alcohol abuse without cirrhosis, whereas serum PIIIP levels are normal or slightly raised in these patients. There is a strong correlation between serum PIIIP and LP1 levels. The combination of these serum markers may be helpful in diagnosis of alcoholic liver disease and primary biliary cirrhosis, and is probably useful for monitoring fibroplasia in these diseases.

Table IV.1

Correlations between serum laminin P1 fragment and procollagen type III N-peptide levels and other liver tests (n=63).

	bili	alk.p.	alat	asat	γ-gt	PIIIP
LP1	0.56#	0,34**	0.41**	0.01*	0.37**	0.82#
PIIIP	0.56#	0.21*	0.35**	0.01*	0.30**	–

* not significant
** p<0.01
# p<0.001

FIGURE IV.1

Serum laminin P1 fragment levels in alcoholic liver disease and primary biliary cirrhosis.

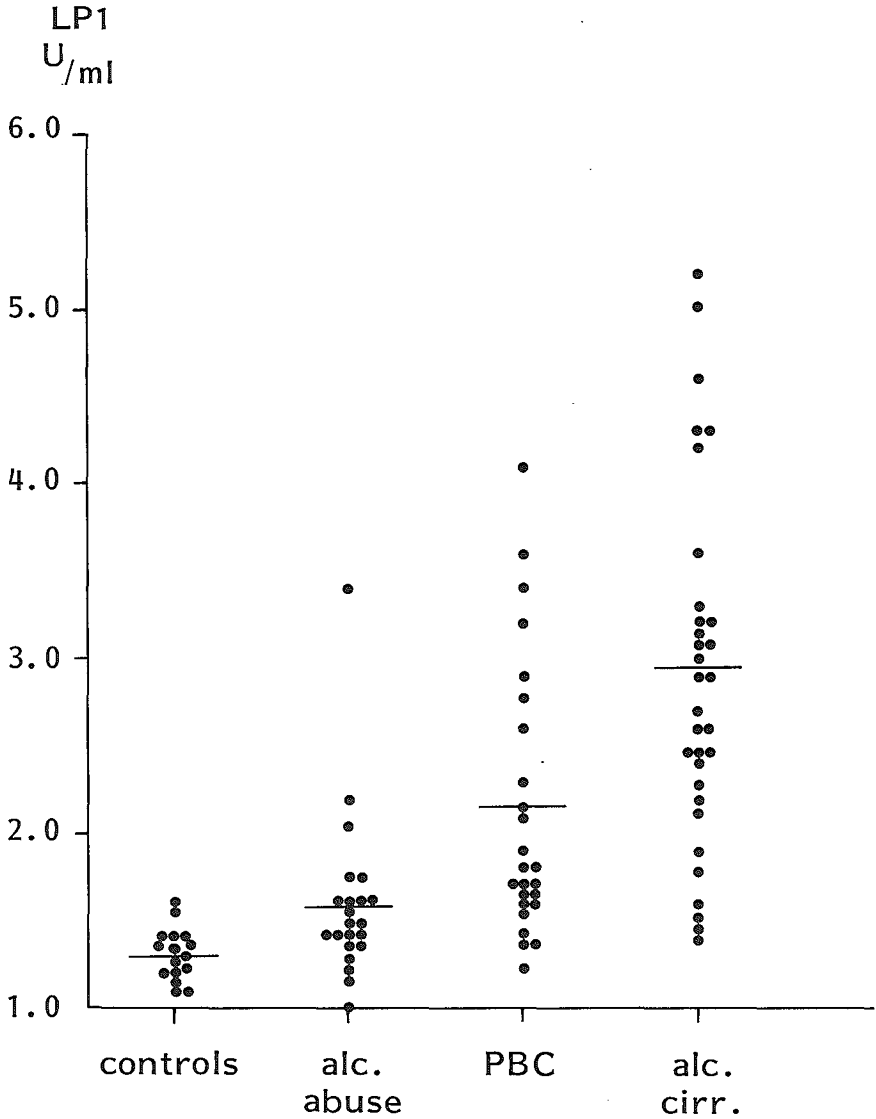
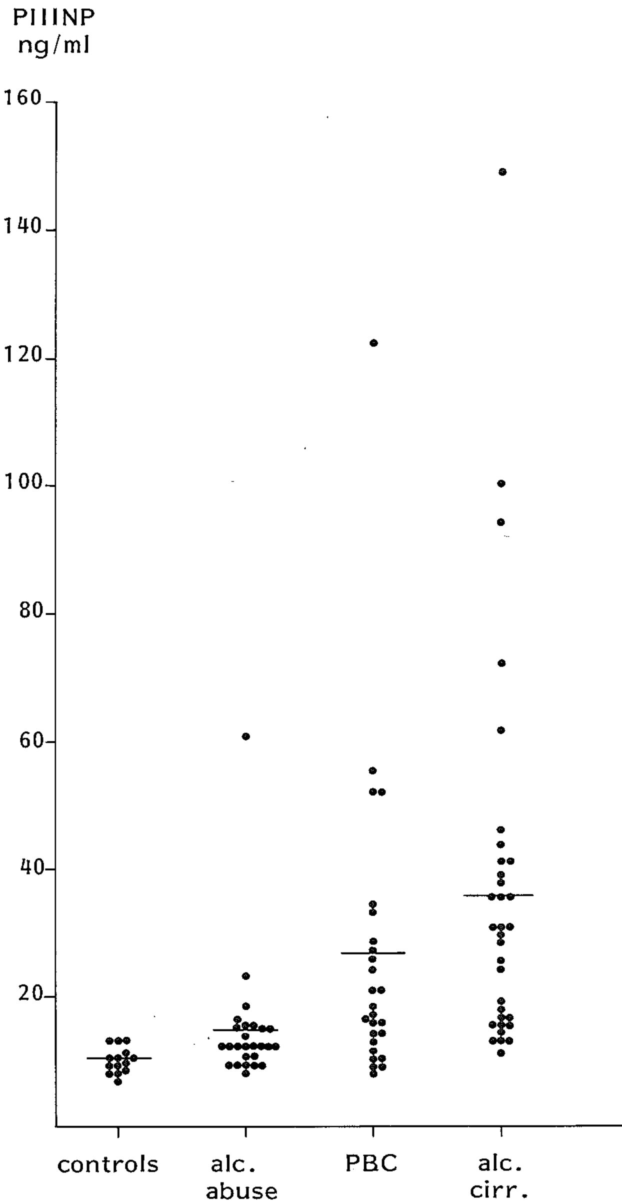


FIGURE IV.2

Serum procollagen type III N-terminal peptide in alcoholic liver disease and primary biliary cirrhosis.



Chapter V SERUM LAMININ LEVELS, INDOCYANINE GREEN AND ANTIPYRINE CLEARANCE AND ESOPHAGEAL VARICES IN CHRONIC LIVER DISEASE

INTRODUCTION

In chronic liver disease fibrosis is a frequent finding (Popper and Udenfriend 1970, Popper and Piez 1978). Progressive fibrosis may lead to cirrhosis, one of the most serious complications of chronic liver disease. The connective tissue laid down in the liver during fibrosis consists of the extracellular matrix components collagen, proteoglycans, elastin, laminin, fibronectin and other structural proteins. In attempts to develop non-invasive methods to assess fibrosis, several fragments which are split off during formation of collagen in liver fibrosis have been studied. One of the components that has attracted attention is the P1 fragment of laminin, which is split off during the formation of the laminin molecule. A sensitive radioimmunoassay for the laminin P1 fragment has been developed (Risteli et al. 1981). Raised levels of this laminin P1 fragment (LP1) in serum have been reported in patients with alcoholic cirrhosis (with hepatitis) and primary biliary cirrhosis (Niemela et al. 1983, Sato et al. 1988, van Zanten et al. 1988). Gressner and Tittor (1986) suggested a correlation between serum LP1 levels and portal hypertension.
In this study we evaluated the usefulness of serum LP1 as a marker of presence of esophageal varices. We also studied the relationship between serum LP1 and liver blood flow (using indocyanine green clearance as an indicator) and between serum LP1 and functional hepatic mass (using antipyrine clearance as a marker).

PATIENTS AND METHODS

In the first study serum LP1 levels were related to presence or absence of esophageal varices. Thirty-six patients with liver cirrhosis were evaluated. Cirrhosis was due to alcohol abuse (18 patients) or primary biliary cirrhosis. The diagnosis was based on clinical, biochemical and histological findings. Serum LP1 levels were measured by radioimmunoassay (Behringwerke, Frankfurt, Germany). Esophageal varices were detected by proximal endoscopy or by radiological examination. Patients who had esophageal bleeding or who were treated by endoscopic sclerotherapy were excluded. In a second study 34

patients with chronic liver disease were evaluated. Nine patients had cirrhosis due to chronic active hepatitis, 12 due to alcohol abuse, 3 due to sclerosing cholangitis and 3 had primary biliary cirrhosis. The remaining had chronic hepatitis without cirrhosis (2), fatty liver (3), portal vein thrombosis (1) and hepatocellular carcinoma (1). Indocyanine green (ICG) levels in serum were measured at 4, 6, 8, 10, 12, 15 and 20 minutes after intravenous bolus injection of 0.5 mg ICG/kg, while patients were in fasting state.
Antipyrine (AP) was measured in saliva samples collected 2, 6, 12, 18, 24 and 30 hours after intravenous injection of 250 mg AP. In both tests blanc samples were taken just before injection. Five patients underwent the tests on two occasions.
Correlations between tests were calculated by the geometric means method (Haeckel 1981). Differences between patients with and without esophageal varices were tested by the Student t-test.

RESULTS

In the first study 21 patients were found to have esophageal varices. Serum LP1 levels of these patients were significantly higher (3.17 +/–1.36 U/ml; mean +/– standard deviation) than in patients without varices (2.12 +/– 0.81 U/ml; $p<0.01$). Six patients with varices had serum LP1 levels lower than 2.93 U/ml and in only 2 patients without varices the levels were higher than 2.93 U/ml (3.2 and 5.0 U/ml respectively).
In the second study 36 fractional ICG clearances and 35 AP clearances were evaluable. Serum LP1 levels in this group varied from 1.13 to 5.2 U/ml (mean 2.58 U/ml). The fractional ICG clearance ranged from 0.5 to 21.3% per minute (mean 9.4%/min.). There was a significant negative correlation between serum LP1 levels and fractional ICG clearance of 0.68 ($p<0.001$). Fractional antipyrine clearances ranged from 0.7 to 9.5% per hour (mean 3.9%/hr) and correlated significantly with LP1 levels ($r=-0.65$; $p<0.001$). There was also a significant correlation between fractional ICG and AP clearances ($r=0.68$).

DISCUSSION

The standard method to diagnose and quantitate fibrosis is histological examination of a liver biopsy. Because sampling error may occur and a biopsy only gives a static impression of fibrosis, other methods to quantitate fibrosis have been studied (Hahn 1984).

Sato et al. (1986) reported an association between serum LP1 concentrations and degree of hepatic fibrosis. In a recent article Gressner et al. (1988) reported a significant positive correlation between the serum laminin and the portal vein pressure and suggested that serum LP1 could be used to predict portal hypertension in chronic liver diseases. In another study they found that the concentration of laminin in serum was related to the degree of esophageal varices in fibrotic liver disease (Gressner and Kropf 1988). In our study patients with esophageal varices had significantly higher serum LP1 levels than patients without varices. There was however an overlap in serum LP1 values between patients with and without varices. One possible explanation is that not all patients with portal hypertension have esophageal varices (Garcia-Tsao et al. 1985, Manzione et al. 1987, Lebrec et al. 1980).

In a preliminary study we found a relationship between low ICG clearance and the presence of esophageal varices (de Rave, unpublished data). In this study ICG clearance was used as an indicator of hepatic blood flow (Caesar et al. 1961, Grainger et al. 1983, Henriksen and Winkler 1987). As ICG is a high clearance drug its clearance depends mostly on the liver blood flow. There was a significant correlation between ICG clearance and serum LP1 levels. This is in accordance with the assumption that higher serum LP1 levels reflect a greater degree of fibrosis which causes decreased flow through the portal vein vascular bed. Serum LP1 may be therefore a non-invasive indicator of portal hypertension.

Gluud et al. (1988) reported that the ICG clearance contained significant prognostic information about the development of variceal hemorrhage. Because the LP1 levels are correlated with ICG clearance, LP1 may be of prognostic significance regarding variceal bleeding.

Serum LP1 levels were also significantly correlated with antipyrine clearance. Antipyrine is a low clearance drug and its hepatic clearance is largely dependent on functional liver mass and nearly independent of liver blood flow. Progressive fibrosis disturbes the normal function of the hepatocytes and reduces the functional liver mass.

In conclusion raised serum LP1 levels are correlated with decreased ICG and AP clearance and with the presence of esophageal varices. Serum LP1 may be a non-invasive marker of portal hypertension in liver disease.

Chapter VI SERIAL DETERMINATION OF TYPE III PROCOLLAGEN AMINO PROPEPTIDE SERUM LEVELS IN PATIENTS WITH HISTOLOGICALLY PROGRESSIVE AND NON-PROGRESSIVE PRIMARY BILIARY CIRRHOSIS

INTRODUCTION

Hepatic fibrosis, a common and important feature of chronic liver disease, is characterized by an increased synthesis and deposition of collagen, mainly types I and III, in the intercellular spaces (Rojkind et al. 1979). In liver disease with enhanced fibrogenic activity an increased synthesis of type III collagen preceeds that of type I (Wick et al. 1978). During the extracellular formation of type III collagen from type III procollagen, the aminoterminal peptide of the procollagen molecule is split off enzymatically and released into the circulation. In recent years a sensitive radioimmunoassay (RIA) for this type III procollagen aminopropeptide (PIIIP) has been developed (Rohde et al. 1979a). Increased PIIIP levels have been reported in acute and chronic hepatitis (Rohde et al. 1979a, Chang et al. 1989), alcoholic liver disease (Rohde et al. 1979a, Frei et al. 1984, Torres-Salinas et al. 1986), chronic active hepatitis (Frei et al. 1984, McCullough et al. 1987) and primary biliary cirrhosis (Savolainen et al. 1983, Weigand et al. 1984, Frei et al. 1984, Eriksson and Zettervall 1986, Niemela et al. 1988, Babbs et al. 1988, Mutimer et al. 1989). Serum PIIIP levels in liver disease probably reflect active fibrogenesis rather than the extent of fibrous tissue in the liver (Heredia et al. 1985, Torres-Salinas et al. 1986).

Chronic non-suppurative cholangiolytic hepatitis, usually called primary biliary cirrhosis (PBC), even in stages without characteristics of cirrhosis, runs a variable course. The disease may progress to liver cirrhosis with complications of portal hypertension and death from hepatic failure, but it also may run a benign course with a normal life expectancy for asymptomatic patients (Kaplan 1987). Apart from liver transplantation in end-stage disease, effective treatment for PBC is still not available (Wiesner et al. 1988). Theoretically patients with active and progressive disease could benefit the most from therapeutic intervention. Parameters to identify such patients, however, are lacking. Serum bilirubin has proven to be an important prognostic factor (Shapiro et al. 1979, Christensen et al. 1985), but rising serum bilirubin levels and jaundice develop only late in the course of the disease. Histologically the later stages of the disease are characterized by increasing fibrosis (stage III) and eventually cirrhosis

(stage IV) (Scheuer et al. 1967). Several authors have suggested that serum PIIIP concentrations provide independent prognostic information (Eriksson and Zettervall 1986, Niemela et al. 1988, Babbs et al. 1988), although this has been disputed (Nutimer et al. 1989). In the former studies, however, PIIIP levels were only determined at a single point in time. Data on serial PIIIP measurements for individual patients are scarce (Weigand et al. 1984, Mutimer et al. 1989). Patients with histologically progressive PBC may be expected to have increased hepatic fibrogenic activity. Therefore, during follow-up these patients may show elevated serum PIIIP levels compared with patients with histologically stable disease. To assess the value of serum PIIIP measurements for predicting histological progression of PBC, we studied serial PIIIP levels in patients with histologically progressive disease compared with those with histologically stable early PBC, all of whom were followed for up to 13 years. In addition the effects of 3 different therapeutic regiments on serum PIIIP concentrations were investigated in several small series of patients.

MATERIAL AND METHODS

PATIENTS

The patients in this study comprise 9 patients selected for histologically progressive PBC, i.e. progression from early stage (I and II) to late stage (III and IV) disease, and 9 patients with early stage disease without histological progression as assessed by repeated liver biopsies. They will be referred to as the progressive group and non-progressive group, respectively. The diagnosis of PBC was established according to clinical, biochemical and histological criteria (Kaplan 1987) in all cases. Entry and follow-up liver biopsies were taken blindly or under laparoscopic control with a Tru-Cut needle and classified according to Scheuer (1967) by one experienced hepatic pathologist. The timing and results for the liver biopsies for individual patients are shown in table VI.1. For each patient of the progressive group, except patient 5, at least 2 liver biopsies were available for assessment of the phase of early stage disease, thereby for the most part circumventing the problem of sampling error. During follow-up a total of 52 and 39 liver specimens were obtained from the patients of the progressive and non-progressive group, respectively. Entry data, including liver histology, were obtained at the time of diagnosis or -in referred cases- at the time of the first examination in our hospital. The patient data and characteristics at entry are given in table VI.2. The 2 groups did not differ significantly. All patients of the progressive group and 8 patients with non-progressive disease had symptoms attributable to

PBC, including fatigue, pruritus or jaundice or symptoms of portal hypertension. During follow-up 3 patients in the non-progressive group (patient 10, 15 and 17; table VI.1) and all of the progressive group received one or more treatment courses with colchicine, azathioprine, penicillamine, cyclosporin A or combinations of these drugs.

The patients were evaluated clinically, biochemically and histologically at regular intervals. Additional serum samples were stored at –20°C for future analysis.

Clinical progression, defined as a definite rise in serum bilirubin (i.e. above 34 umol/l, confirmed on at least 2 occasions), progressive jaundice or the development of signs and symptoms of portal hypertension, was observed in 4 patients from the progressive group (patients 2, 5, 7 and 9; table VI.1). Patient 2 died of liver failure 7 years after diagnosis, patient 7 also developed progressive systemic sclerosis and patient 9 underwent liver transplantation 7 years after diagnosis but died 4 months later.
The non-progressive patients were followed by means of histology and/or serum PIIIP determinations for 4-9.6 years (table VI.2), but clinically for 8.5-11.5 years (mean: 10 years). None of these patients showed clinical progression as defined above. At the end of the clinical follow-up all non-progressive patients had normal serum bilirubin levels (mean: 8 umol/l; range: 5-14). Furthermore, mean serum levels of aspartate amino-transferase (30 U/l; range: 17-49), immunoglobulin G (13.5 g/l; range: 8.0-17.4) and albumin (43 g/l; range: 40-45) were normal, but mean serum immunoglobulin M levels were moderately elevated (3.5 g/l; range: 1.6-5.6). As to be expected, alkaline phosphatase levels were elevated in all cases (mean 108 U/l; range: 62-200), but the mean level was not increased compared with that at entry. Seven of the 9 non-progressive patients were examined during the last 2 years of clinical follow-up for signs of portal hypertension by means of endoscopy, radiology or ultrasonography, with negative results in all cases.

The effect of anti-inflammatory and immunosuppressive drug therapy on PIIIP levels was studied in 7 patients of the progressive group, one non-progressive patient and 4 additional patients (2 with early and 2 with late stage disease). In all of these cases serum samples and liver biopsies were taken before and after treatment. The following drugs or combinations of drugs were studied, each in 5 patients: penicillamine (250 mg) plus prednisone (10 mg) for 12 months, cyclosporin A (2-5 mg/kg) for 6 months and cyclosporin A plus prednisone for 16-24 months.

LABORATORY INVESTIGATIONS

Bilirubin, alkaline phosphatase and aspartate aminotransferase (ASAT) levels in the serum were assessed by standard techniques. Serum PIIIP concentrations were measured by radioimmunoassay, using a commercially available kit (Behringwerke, Frankfurt, Germany). The RIA was performed as described by Rohde et al. (1979) with modifications suggested by the manufacturer. The concentration of PIIIP was calculated using a 50% intercept method (Hahn 1984). All tests were carried out in duplicate and a standard serum was run together with all determinations for quality control. For the standard serum the mean value of 23 determinations was 16.5 ng/ml (manufacturer's value 17.0 ng/ml). The coefficient of variation was 11%. Determinations of PIIIP were done in sera frozen at –20°C for up to 12 years. Storage at –20°C for ut to 2 years (Rohde et al. 1979a, Trivedi et al. 1985) or at 70°C (Niemela et al. 1988) does not influence PIIIP concentrations. The mean serum PIIIP level plus and minus 2 standard deviations for 16 normal controls was used as normal reference range (mean +/- SD: 9.2 +/- 1.8 ng/ml).

STATISTICAL METHODS

Differences between groups were tested with the Wilcoxon rank test for unpaired data and within groups with the Wilcoxon rank test for paired data. The association between serum PIIIP concentrations and serum levels of ASAT, alkaline phosphatase, bilirubin and albumin as well as histological stage was evaluated by means of the Spearman correlation coefficient (r).

RESULTS

The follow-up data on serum PIIIP levels for patients from both groups are depicted in figure VI.1. For the progressive group the same data, rearranged according to an artificial point in time indicating the transition from histologically early to late stage disease, are shown in figure VI.2. The time of the histological transition is given by the median time between the last early stage and the first late stage liver specimen for each patient. No consistent pattern in the course of PIIIP levels could be recognized in either the progressive group or the non-progressive group (figure VI.1). The same holds for the PIIIP levels corresponding to early stage disease compared with those corresponding to late stage disease for patients of the progressive group (figure VI.2). For each patient the mean of all serum PIIIP levels obtained during follow-up was calculated. No significant difference in these mean PIIIP levels between the progressive group and

non-progressive group was observed (table VI.3). Two patients of the non-progressive group and 5 of the progressive group had serum PIIIP levels above the upper limit of normal (12.8 ng/ml). Elevated PIIIP levels occurred at least once during follow-up for 4 patients of the non-progressive group and all those with histologically progressive disease. Furthermore, in the progressive group the means of the PIIIP levels during early stage disease were compared with the means during late stage disease. For one patient no PIIIP levels were obtained during early stage disease. In the other 8 patients the mean PIIIP levels did not change significantly with histological progression (figure VI.3).

To investigate whether the serum PIIIP levels at a given time reflect the histological stage of PBC, we compared the peptide levels corresponding to the different stages of the disease. Mean values were used in case a patient had more than one PIIIP value corresponding to a given histological stage. PIIIP levels corresponding to stage IV disease were available for only 2 patients. Therefore, only stage I, II and III were compared. We found no significant difference between mean PIIIP levels corresponding to stage I (11.7 ng/ml), stage II (15.0 ng/ml) and stage III (13.2 ng/ml) disease (Wilcoxon test for paired data).

For each patient the correlations between PIIIP levels and corresponding bilirubin, alkaline phosphatase, ASAT and albumin concentrations as well as histological stage were calculated. The median number of observations available for correlation was 6 (range: 3-11) for each variable tested. The correlation coefficients appeared to be widely distributed without a consistent trend towards a positive or negative correlation for either of the variables (figure VI.4).

The effects on PIIIP levels of 3 different treatment regiments are shown in figure VI.5. In these small series of patients only penicillamine combined with prednisone resulted in a significant decrease in PIIIP concentrations.

DISCUSSION

Serum PIIIP levels have been found to be elevated in acute and chronic liver diseases of various etiologies (Rohde et al. 1979a, Savolainen et al. 1983, Weigand et al. 1984, Frei et al. 1984, Eriksson and Zettervall 1986, Torres-Salinas et al. 1986, McCullough et al. 1987, Niemela et al. 1988, Babbs et al. 1988, Mutimer et al. 1989, Chang et al. 1989). Some investigators have reported a good correlation between serum PIIIP

and established fibrosis (Frei et al. 1984, Babbs et al. 1989), while others concluded that the PIIIP levels primarily reflect active fibrogenesis (Heredia et al. 1985, Galambos et al. 1985, Torres-Salinas et al. 1986) or necrosis and inflammation (Rohde et al. 1979a, Surrenti et al. 1987). Furthermore, serum PIIIP has been proposed as an useful marker of disease activity in autoimmune chronic active hepatitis (McCullough et al. 1987) and as a prognostic factor in PBC (Eriksson and Zettervall 1986, Niemela et al. 1988, Babbs et al. 1988). Several investigators have reported on serum PIIIP levels in patients with PBC (Savolainen et al. 1983, Weigand et al. 1984, Frei et al. 1984, Eriksson and Zettervall 1986, Niemela et al. 1988, Babbs et al. 1988). Savolainen et al. (1983) found elevated PIIIP concentrations in the majority of their 21 patients. The peptide levels exceeded the upper limit of normal in three-quarters of the 24 patients studied by Eriksson and Zettervall (1986). Mean serum PIIIP levels, however, were significantly elevated in symptomatic patients only. Niemela et al. (1986) reported elevated peptide levels in all 11 patients with histologically late stage PBC, whereas 6 of the 11 patients with early stage disease in their series had normal PIIIP levels. In the largest series reported sofar, Babbs et al. (1988) found that the serum PIIIP levels were elevated in 73% of the 63 patients studied. In all of these series PIIIP measurements were carried out at a single point in time. Few data are available on follow-up determinations of serum PIIIP for the same patients. Weigand et al. (1984) followed PIIIP levels in 4 patients with PBC for up to 7 years; the initially increased peptide levels remained elevated in all cases. In contrast, during a mean follow-up of 42 months PIIIP concentrations dropped from the initial value in 10 out of 18 patients in the series reported by Mutimer et al. (1989). In addition, PIIIP levels decreased prior to death in 5 of the 7 patients with a fatal outcome of their disease.
In the present series, to our knowledge the largest report on serial PIIIP measurements in PBC, mean peptide levels during follow-up exceeded the upper limit of normal in 40% of the patients, whereas serum PIIIP was elevated at least once during follow-up in 70%. As stated earlier, in PBC patients with histologically progressive disease and therefore active fibrogenesis, one would expect serum PIIIP levels to be elevated. Although the peptide levels of patients of the progressive group were elevated more often during follow-up than those of the non-progressive group, the means of the individual values did not differ between the groups. Moreover, progression from histologically early to late stage disease was not reflected in serum PIIIP levels. We selected our patients on the basis of histological criteria, i.e. histological progression versus stable disease. All patients included in the progressive group had been treated with immuno-

suppressive or anti-inflammatory drugs. In contrast, only 3 of the non-progressive patients had been treated medically. Therefore, incorrect patient selection due to sampling error as well as drug treatment could have influenced our results. The hazard of sampling error applies especially to the inclusion of patients in the progressive group. We believe that we have overcome this problem by establishing the phase of early stage disease in these patients on the basis of at least 2 liver biopsies in all but one case. The correctness of our selection appears to be supported by the clinical progression of 4 patients, 2 of whom died, in the progressive group, versus none in the non-progressive group. Although tested in small series of patients, penicillamine (Savolainen et al. 1983, Weigand et al. 1984) and azathioprine and prednisolone (Weigand et al. 1984) have been reported to have no influence on serum PIIIP levels. In agreement with others (Minuk et al. 1988) we observed no effect on serum PIIIP due to treatment with cyclosporin A. The same applies for cyclosporin A combined with prednisone. Only treatment with the combination of penicillamine and prednisone for one year resulted in a significant decrease in PIIIP levels. Five out of 9 patients of the progressive group, but non of the non-progressive group had been treated in the past with this combination of drugs; apparently this did not prevent histological progression in the former group. Only in 2 cases was a serum sample for PIIIP determination obtained during treatment. Therefore we believe it to be unlikely that our results have been influenced by medical treatment.

We found no significant difference between PIIIP levels corresponding to the different histological stages. In contrast, other investigators did find a positive correlation between PIIIP levels and histology with significantly lower peptide levels in early disease. In these studies, however, the overlap between groups was considerable (Babbs et al. 1988, Mutimer et al. 1989). Therefore, we believe that PIIIP is not a reliable marker of the histological stage of PBC.

Serum PIIIP has been reported to correlate with parameters of cholestasis (alkaline phosphatase, bilirubin), hepatic necrosis and inflammation (ASAT) and protein synthesis (albumin) in PBC (Niemela et al. 1988, Babbs et al. 1988, Mutimer et al. 1989) as well as other liver diseases (Rohde et al. 1979a, Frei et al. 1984, Chang et al. 1989). Although statistically significant, the correlations in all these studies were weak. Because serial data were available for all patients in our series, we were able to calculate individual correlation coefficients. Of course, our results may have been influenced by the relatively small number of observations per patient. The wide distribution of the correlation coefficients without an

obvious trend towards positive or negative for either of the variables tested, however, throws doubt upon the relationship of PIIIP with cholestasis and hepatic necrosis, inflammation or protein synthesis.

Babbs et al. (1988), using the Cox proportional hazard model, concluded that serum PIIIP is an independent prognostic variable in PBC. Although this seems to be supported by data from other investigators (Eriksson and Zettervall 1986, Niemela et al. 1988), the conclusions of these studies were criticized in a recent report by Mutimer et al. (1989). Our data do not allow firm conclusions on the value of serum PIIIP for predicting the prognosis of PBC. Nevertheless, there are arguments to assume that, despite the lack of difference in PIIIP levels, prognosis is worse for patients included in the progressive group compared with those in the non-progressive group. Firstly, there is the unfavourable clinical outcome for some patients in the former group, in contrast to the absence of clinical deterioration in the latter. Secondly, several authors have reported histologically late stage disease, i.e. cirrhosis, to be correlated with shortened survival (Roll et al. 1983, Portmann et al. 1985, Christensen et al. 1985).
We conclude that serum PIIIP level determinations are of no value for predicting histological progression of PBC. This could be due to the complexity of collagen metabolism and variations in PIIIP clearance (Mutimer et al. 1989). Another explanation could be that during the slow process of progression to the fibrotic stages of the disease, active hepatic fibrogenesis results in increments in serum PIIIP concentrations which are too small to be detected with the present methods. Serum PIIIP measurements may however be of some use in evaluating response to treatment.

Fig. VI.1: Follow-up serum PIIIP concentrations in patients with (A) histologically non-progressive and (B) progressive PBC. Each dot at the bottom of the graphs represents a liver biopsy. In this and following figures the hatched bar indicates the normal range.

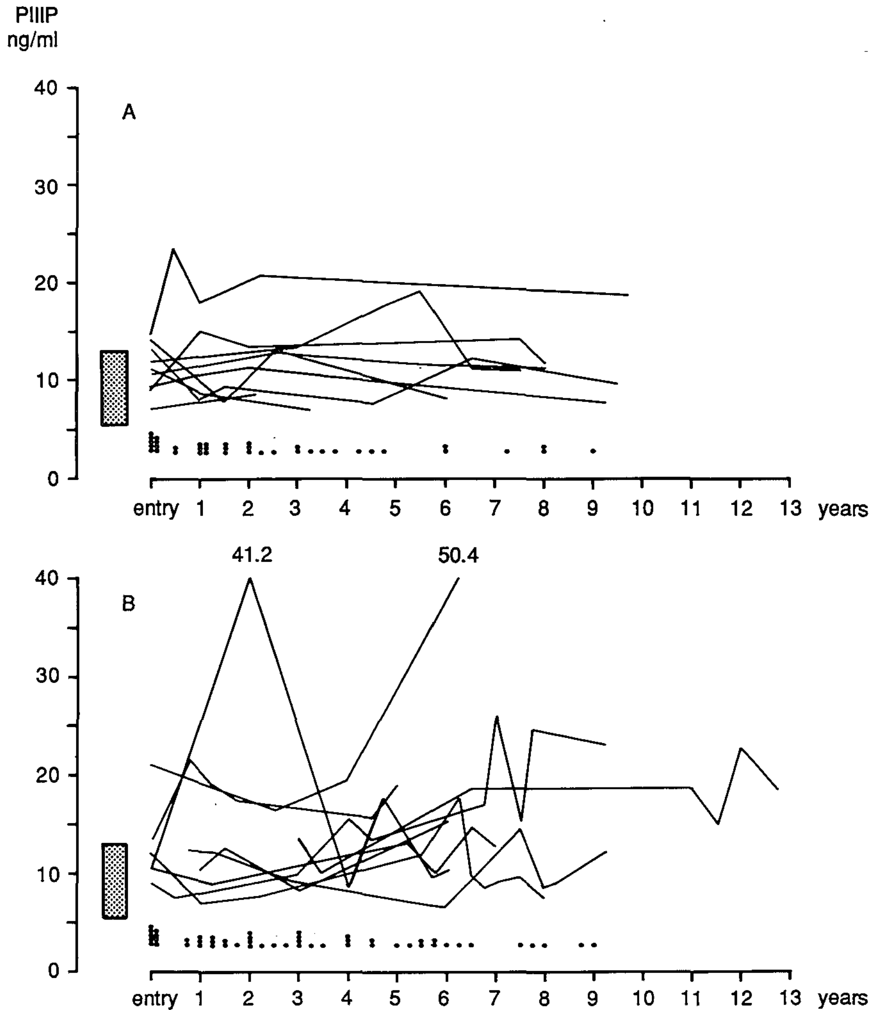


Fig. VI.2:Follow-up serum PIIIP concentrations in patients with histologically progressive PBC. The data are arranged according to an artificial histological turning point (time zero), separating early from late stage disease. Each dot at the bottom of the graph represents a liver biopsy.

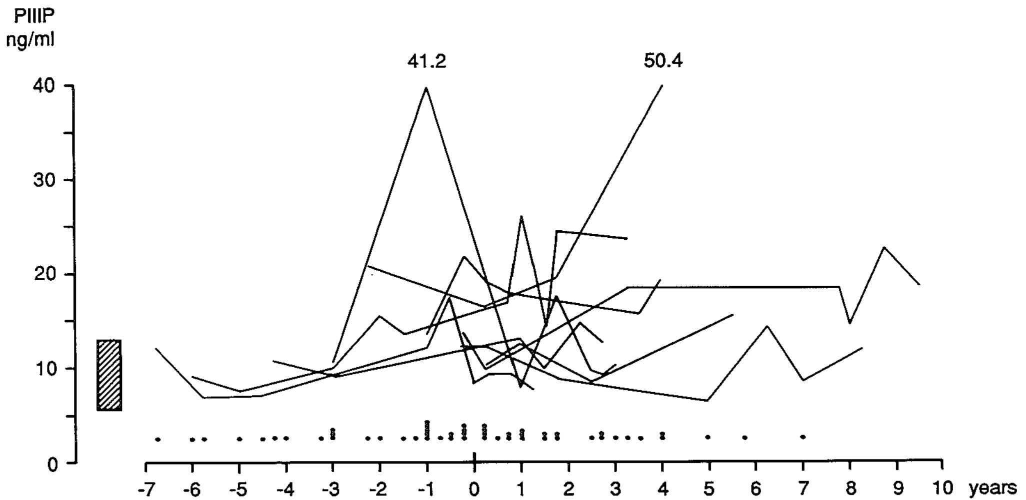


Fig. VI.3: Mean serum PIIIP concentrations found for early stage disease compared with those found for late stage disease in 8 patients with histologically progressive PBC.

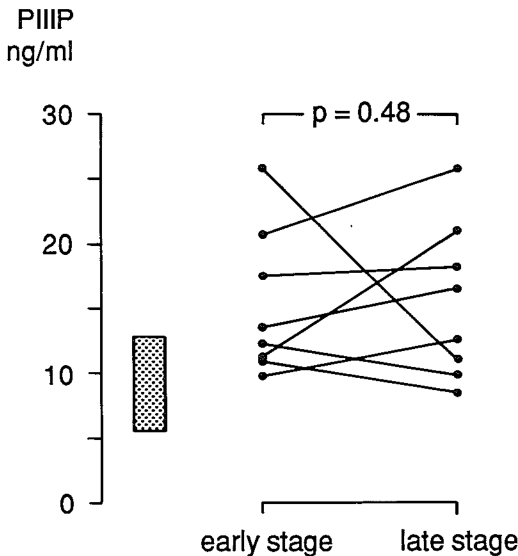


Fig. VI.4: Distribution of the correlation coefficients found for 18 patients with PBC indicating the correlation between serum PIIIP levels and corresponding biochemical values and histology.

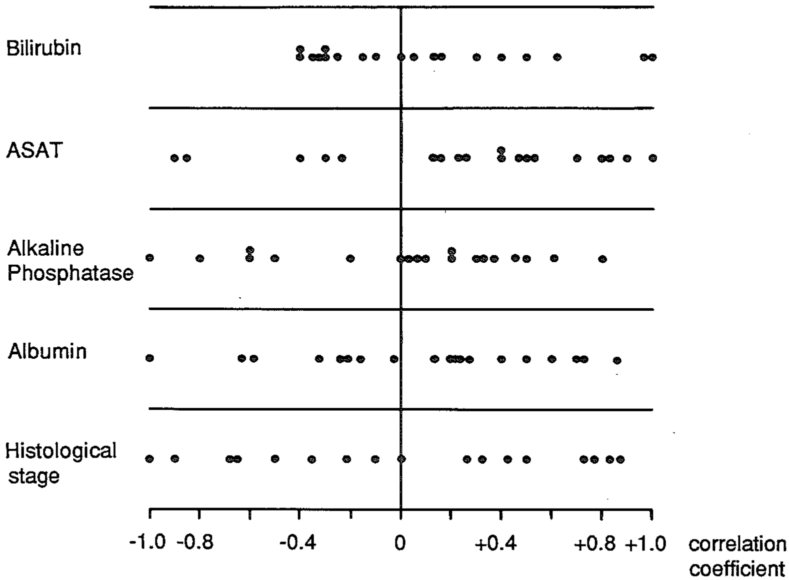


Fig. VI.5: Effect on serum PIIIP concentrations of 3 immunosuppressive and anti-inflammatory treatment regimens in patients with PBC.
NS = statistically not significant.

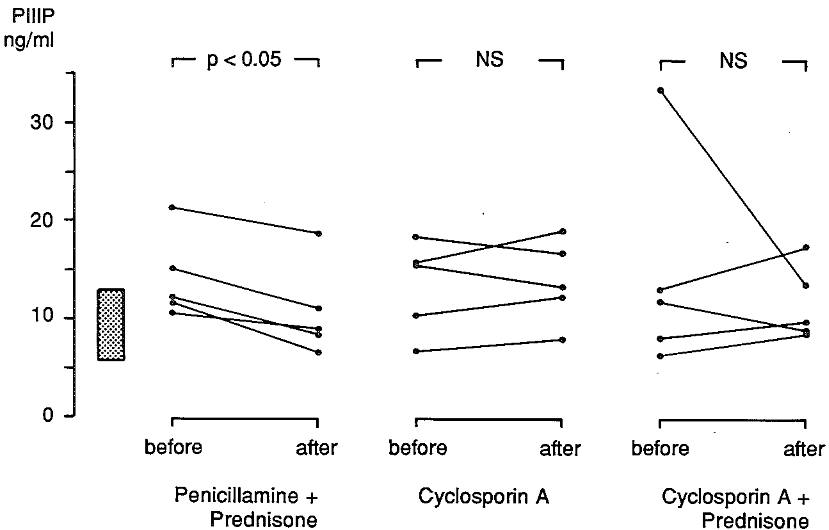


Table VI.2: Patients characteristics

variable	normal values	progressive group (n=9)	non-progressive group (n=9)
Entry data*:			
sex (F/M)		8/1	9/0
age (yr)		45 (31-63)	52 (43-61)
bilirubin (umol/l)	<17	23 (5-90)	12 (5-26)
alkaline phoshpatase (U/L)	18-45	200 (70-470)	135 (51-418)
ASAT (U/l)	5-30	84 (22-148)	63 (25-156)
PIIIP (ng/ml)	5.6-12.8	12.8 (8.9-20.8)	11.2 (7.1-14.8)
Follow-up data:			
duration (yr)**		8 (6-13)	8 (4-9.5)
medical treatment (n)		9	3
clinical progression (n)		4	0
mortality (n)		2	0

Age, duration and biochemical variables are expressed as mean values (range)

ASAT: aspartate aminotransferse.

* Entry data were not significantly different.

** Duration of follow-up by means of histology and/or PIIIP determinations.

Table VI.1: Timing and results of sequential liver biopsies in patients with histologically progressive and non-progressive primary biliary cirrhosis.

	Patient no.	Sex	Age (yr)*	Entry	Histological stage at:																	
						1		2		3		4		5		6		7		8		9 years
Progressive Group	1	M	58	1				2				3			3							
	2	F	39	1			2	2	3	3		3		3			3					
	3	F	31	1		1		1							2				3			
	4	F	63	1		2				1					3							
	5	F	55	2		3	2			4												
	6	F	38	2		2	3			3						3				3		
	7	F	38	1		1		1		1		2	2		2				3		3	
	8	F	38	1				1		1	3						3					2
	9	F	45	2	1	3	3						4	4								
Non-progressive Group	10	F	54	1			2		1							2				2		
	11	F	52	2		1		1			2											
	12	F	60	1		1		1		1		1										
	13	F	50	1		2		1												2		
	14	F	42	1	2	2		2			2											
	15	F	49	1						2				2								
	16	F	49	1		1				1								1				
	17	F	57	2	2	1	1						2									1
	18	F	53	2			1									1						

For practical purposes the histological stages of PBC are presented in this table in Arabic numerals.
* Age et entry.

Table VI.3: Means of follow-up serum PIIIP concentrations in patients with histologically progressive and non-progressive PBC

Patient	PIIP concentrations (N: 5, 6-12, 8 ng/ml)		
	number of values	mean values*	SEM
Progressive group			
1	9	14.5	3.5
2	5	24.8	6.5
3	11	9.7	0.9
4	6	11.7	0.9
5	5	11.6	1.4
6	9	10.2	0.8
7	11	16.5	1.9
8	9	16.2	1.2
9	6	17.7	1.2
Non-progressive group			
10	4	11.6	0.4
11	5	9.7	0.6
12	3	7.8	0.5
13	5	12.6	1.1
14	5	19.0	1.6
15	6	14.0	1.4
16	3	8.8	1.3
17	7	9.9	0.8
18	4	10.8	1.6

* Mean values for the two groups were not significantly different (p=0.10; Wilcoxon test).

SEM: standard error of the mean.

Chapter VII SERUM AMINOTERMINAL PEPTIDE OF PROCOLLAGEN III CORRELATED TO LIVER BIOPSIES AFTER LIVER TRANSPLANTATION

INTRODUCTION

In recent years liver tranplantation has become an accepted treatment for end-stage liver disease, including chronic hepatitis and primary biliary cirrhosis (Starzl et al. 1987).
However, recurrence of disease in the liver graft has been reported (Portmann et al. 1986, Demetris et al. 1986, Dietze et al. 1990). When this happens liver fibrosis may result. Serum procollagen type III aminoterminal peptide (PIIIP) has been suggested as a marker of hepatic fibrogenesis (Rohde et al. 1979a, Torres-Salinas et al. 1986, Gabrielli et al. 1989). The N-terminal peptide is split off the procollagen III molecule by a specific peptidase in the formation of collagen III (Schuppan et al. 1988).
After liver transplantation patients undergo serial liver biopsies for the evaluation of rejection of the graft; this offers us an excellent model to study fibrogenesis in the graft as well. To our knowledge no studies concerning the serum PIIIP levels after liver transplantation have been published.
We evaluated the possible application of serum PIIIP in the follow-up of patients who underwent liver transplantation.

PATIENTS AND METHODS

In this retrospective study 18 patients who underwent liver transplantation were included (13 men, 5 women). Liver transplantation was performed because of liver insufficiency due to chronic active hepatitis with cirrhosis (8), primary biliary cirrhosis (2), alcoholic cirrhosis (1), primary sclerosing cholangitis (3), acute hepatitis (3) and hepatocellular carcinoma (1). In 13 patients auxiliary partial liver transplantation was performed (Terpstra et al. 1988). The remaining 5 patients underwent orthotopic liver transplantation; one of these patients was retransplanted 5 months after the original procedure.
Biopsies of the graft were taken 6 weeks, 3 months and one year after transplantation. On the same occasion blood samples were taken for routine laboratory tests and determination of serum PIIIP. Liver biopsies

were taken with a Tru-cut needle under ultrasound control and classified by a single pathologist. In the liver biopsies the grade of fibrosis was scored as follows: grade 0: no fibrosis; grade 1: minimal periportal fibrosis; grade 2: periportal fibrosis with some septum formation between portal tracts; grade 3: periportal fibrosis with extensive septum formation; grade 4: cirrhosis.
The serum PIIIP levels were measured using a commercially available radioimmunoassay (RIAgnost® PIIIP coated tube, Behringwerke, Germany). All tests were carried out in duplicate and a standard serum was run with all determinations for quality control. The first sample was taken 6 weeks after the operation to avoid elevations of serum PIIIP levels due to wound healing (Haukipuro et al. 1987).
Statistical analysis of correlations was performed.

RESULTS

In the study period one patient died because of cholangitis and septicaemia in the 4th month after transplantation. One other patient had to be retransplanted 5 months after the original procedure because of dysfunction of the graft. In most patients the serum PIIIP levels were highest 6 weeks after transplantation and then lowered remaining under this 6th week's level. The serial biopsies of the liver graft of 12 patients showed no or minimal fibrosis. Six patients had more evident fibrosis (grade 2) to cirrhosis (grade 4). Five patients who underwent auxiliary partial liver transplantation had a recurrence of hepatitis B in the graft, one patient of non A- non B- hepatitis. In one patient who underwent orthotopic liver transplantation, hepatitis B recurred in the graft. Five of the 6 patients with hepatitis B of the graft developed fibrosis and one of them cirrhosis. Though serum PIIIP levels decreased in 11 of 12 patients without fibrosis or with minimal fibrosis in the period from 6 weeks to 3 months after transplantation, in 4 of the 6 patients in whom fibrosis developed the levels raised or remained stable (see table VII.1). The difference failed to reach statistical significance, though there was a trend towards significance. The only patient with an elevation of PIIIP levels without fibrosis was the patient who had a severe cholestasis and who died of cholangitis. We found only weak correlations between serum PIIIP levels and the conventional liver tests (see table VII.2).

DISCUSSION

The main problems that threaten the liver graft after a surgically succesful implantation are rejection and recurrence of the original disease. The use of cyclosporin A, an immunosuppressive drug, has considerably improved the overall results, as rejection could be treated in a better way (Starzl et al. 1982). Recurrence of disease in the liver graft has been described for several hepatic diseases. Development of hepatitis B has been reported frequently, sometimes leading to cirrhosis (Starzl et al. 1987, Portmann et al. 1986, Demetris et al. 1986). In our study group in 6 patients hepatitis B developed in the transplant in the first year after transplantation. This led to fibrotic changes in the graft in 5 patients. The 6th patient who showed fibrosis of the graft had recurrence of non A/non B- hepatitis. As cirrhosis was present in the graft of a patient one year after transplantation rapidly progressive fibrogenesis appears to be possible and may be the cause of dysfunction of the graft.
The value of serum PIIIP determinations in liver diseases is still a matter of debate. A good correlation between serum PIIIP and established fibrosis has been reported by some investigators (Frei et al. 1984, Babbs et al. 1988) while others concluded that the peptide levels primarily reflect active fibrogenesis (Heredia et al. 1985, Galambos et al. 1985, Torres-Salinas et al. 1986, McCullough et al. 1987) or necrosis and inflammation (Rohde et al. 1979a, Surrenti et al. 1987).
A study starting with livers without fibrosis (the liver grafts in our study) during which serial PIIIP levels are correlated to liver biopsy findings however has not been published. The fact that we did not find a significant correlation between serum PIIIP levels in the first months after transplantation and the occurrence of fibrosis in the liver graft in the first year, but only a trend towards significance is probably due to the small number of patients included in the study. The correlation would become better if one takes into account that cholestasis per se can give rise to elevated serum PIIIP levels (Raedsch et al. 1985). In 2 patients a certain grade of fibrosis was present after one year, though there was a drop in PIIIP levels between 6 weeks and 3 months after transplantation. One possible explanation is that in these patients fibrogenesis took place in the period between 3 months and one year post-transplantation. Our results, though the number of patients is small, can support the assumption that serum PIIIP levels may reflect active fibrogenesis. In one study with patients with acute viral hepatitis a similar result was reported (Bentsen et al. 1987).
We conclude that serial measurements of serum PIIIP may be useful in the detection of hepatic fibrogenesis after liver transplantation.

Table VII.1
Grade of fibrosis in liver biopsies and difference in PIIIP levels between 6 weeks and 3 months after liver transplantation.

Patient	Grade of fibrosis	PIIIP
A	0/1	–0.35
B	0/1	0
C	0	–0.54
D	0	–0.36
E	0	+0.47
F	0	–0.33
G	1/2	+0.48
H	0	–1.81
I	0/1	–0.38
J	1	+1.92
K	1/2	–2.12
L	0	–0.24
M	0	–0.32
N	3	+0.11
O	0	–0.67
P	4	+0.09
Q	2	–0.08
R	0/1	–0.15

Table VII.2
Correlations between serum PIIIP levels and conventional liver tests.

parameter	PIIIP
Bili	r=0.25
ASAT	r=0.26
ALAT	r=0.13
Alk. p.	r=0.29

Chapter VIII MEASUREMENT OF PROCOLLAGEN TYPE III AMINOTERMINAL PEPTIDE IN SERUM.

A comparison between the conventional and a new coated tube radioimmunoassay.

INTRODUCTION

In recent years the measurement of procollagen type III aminoterminal peptide (PIIIP) in serum has attracted much attention. Many reports have been published on its potential clinical usefulness in fields like liver diseases (Niemela et al. 1988, McCullough et al. 1987, Annoni et al. 1989) rheumatic diseases Horslev-Petersen et al. 1988) endocrine disorders (Tapanainen et al. 1988, Triolo et al. 1989) and gynaecology (Risteli et al. 1987, Kauppila et al. 1989).
The conventional radioimmunoassay for PIIIP (RIA-gnost® PIIIP, tachysorb) was based on polyclonal antibodies raised in rabbits.
The non-linear behaviour of this conventional radioimmunoassay requires at least 3 different dilutions of the sample to be analysed. From these 3 values a line is constructed plotting the analyte dilution versus the percentage of ligand binding. Using this line one can can usually make a reading at the point representing 50% ligand binding. With this procedure one circumvents non-linearity because each unknown concentration is in fact (artificially) read under identical antibody-ligand conditions (i.e. the 50% binding point).

Although this is a valid procedure to overcome non-linearity of an assay, it is tedous and inefficient, non-suitable for routine laboratory use. Recently a new radioimmunoassay procedure was introduced for measuring PIIIP based on a straight forward coated tube method: RIA-gnost® PIIIP coated tube. The new method uses a monoclonal mouse antibody and shows good linearity upon dilution of samples and a wide measuring range. The whole procedure is based on antibody-coated tubes, eliminating chemical separation of free and bound fractions, which increases speed and improves precision.
This new method has been greatly improved and appears convenient for routine use in clinical laboratories. In this study we present a comparison of the results obtained with both methods on samples from patients with a variety of liver diseases and from pregnant women.

PATIENTS AND METHODS

We measured the PIIIP in the sera of 129 patients. Thirty-one patients had an alcoholic liver cirrhosis, 27 had an alcohol abuse without liver cirrhosis, 30 had a chronic hepatitis, 32 had a primary biliary cirrhosis and 9 non-diseased women were pregnant. Serum PIIIP was measured by the conventional RIA-gnost® PIIIP and the new coated tube method (RIA-gnost® PIIIP coated tube), both manufactured by Behringwerke, D3550 Marburg, Germany (Instruction Booklet RIA-gnost® Procollagen-III-Peptid 1986). Results were compared by means of orthogonal regression analysis according to Passing and Bablok (1983).

RESULTS AND DISCUSSION

In the "RIA-gnost® tachysorb method" results are expressed as ng PIIIP/ml of serum. With the new coated tube method the standards are stated as units PIIIP/ml of serum. A factor relating ng and units had to be established. The results of the comparative measurements are given in table VIII.1. Considering all samples there was a high correlation between the conventional and the new method (r=0.96). When we make a division in groups according to diagnosis we see that the correlation is highest in alcoholic cirrhosis (r=0.97) and lowest in the pregnant women (r=0.77). This difference may be explained by the fact that the last group is rather small. In addition pregnant women all have low values of PIIIP so the assay variation has to be taken into account. In other groups we found high correlations between the data of the two assays. When all samples are taken into account we find the following correlation equation: coated tube method, in U/ml = 0.05 x (PIIIP tachysorb, in ng/ml) + 0.08.
The interassay coefficient of variation was 11% for the conventional radioimmunoassay and 5.4% for the coated tube method (4 samples measured in 6 different RIA kits).
An advantage for the routine use of the coated tube method is that this assay can be performed relatively rapidly, in 5 to 6 hours. As serial dilution of the serum samples is not necessary, the laboratory work is faster and simpler. The interassay variation is very low which is favourable when several samples are measured during follow up of a patient. The fact that the results are not influenced by inaccuracy of repeated pipetting is at least partly responsible for the low interassay variation.
As the results of the coated tube method correlate well with those of the conventional RIA-gnost® PIIIP we think this coated tube assay may be introduced as an easy and rapidly performable test for measuring PIIIP.

We conclude that using this coated tube method it will be possible to use routinely PIIIP as a marker for monitoring collagen forming activities during follow-up of various diseases.

Table VIII.1

Comparison of RIA-gnost® PIIIP (old method) with RIA-gnost® PIIIP coated tube (new method).

Group	N	A	B	r
1. All	129	0.05	0.08	0.963
2. Alc. steat	27	0.06	0.11	0.958
3. Chron. hep.	30	0.04	0.37	0.955
4. PBC	32	0.05	0.21	0.851
5. Alc. Cirr.	31	0.06	–0.06	0.972
6. Pregnant	9	0.08	–0.48	0.771

Values obtained by the two kids were compared by orthogonal regression analysis: result new method = A x result old method + B

Chapter IX SERUM N-ACETYL-β-D-GLUCOSAMINIDASE ACTIVITY IN CHRONIC LIVER DISEASE

INTRODUCTION

Fibrosis of the liver is encountered frequently in chronic liver disease (Chen and Leevy 1975, Popper and Udenfriend 1970, Popper and Piez 1978). Fibrosis occurs when collagen is formed and deposited in excess to degradation. Active liver fibrosis goes along with an increase of the synthesis as well as an increase of the catabolism of the ground substance. N-acetyl-β-D-glucosaminidase (NAG) (E.C.3.2.1.30) is a lysosomal glycosidase involved in the breakdown of glycosaminoglycans from connective tissue.
Activity of NAG was found to be raised in liver biopsies and serum of patients with chronic liver disease (Pott et al. 1979a, Pott et al. 1979b). A correlation between activity of the enzyme and degree of liver fibrosis was suggested (Pott et al. 1979b).
In this study we measured serum concentrations of NAG in patients with chronic liver disease with and without fibrosis to investigate the value in the differential diagnosis of chronic liver disease.

PATIENTS AND METHODS

Serum NAG activity was studied in 16 healthy controls, in 27 patients with alcohol abuse with steatosis, but without fibrosis or cirrhosis, in 11 patients with chronic active hepatitis without cirrhosis, in 14 patients with primary biliary cirrhosis, in 26 patients with alcoholic cirrhosis and in 18 patients with cirrhosis due to chronic active hepatitis. At last NAG was measured in serum of 15 patients with other liver diseases (lymphoma, sclerosing cholangitis, adenoma). In all individuals the diagnosis was made on clinical and biochemical findings. In all patients with cirrhosis and most of the other patients the diagnosis was confirmed by histological examination. Patients with renal disease or diabetes mellitus were excluded.

DETERMINATION OF NAG

The enzyme was measured by a colorimetric method (Pugh et al. 1957) using p-nitrophenyl-N-acetyl-glucosaminide as a substrate.

STATISTICAL ANALYSIS

Comparison of assay data between groups was performed with Students' t-test. Correlations were determined by linear regression analysis.

RESULTS AND DISCUSSION

The serum activity of NAG in the different groups is shown in table IX.1. Significant differences were found between all disease-groups and the controls. Activities found in the controls were similar to those reported by other groups (Koslinen et al. 1984, Ackermann et al. 1981). In the patients with cirrhosis serum concentrations of NAG were higher and comparable to the values in active fibrosis (Pott et al. 1979b). Alcoholics with steatosis but without cirrhosis also showed significantly raised activities, as did patients with chronic active hepatitis without cirrhosis. No significant difference could be demonstrated between alcoholics with and without cirrhosis ($p = 0.13$) or between patients with chronic active hepatitis with and without cirrhosis ($p > 0.43$). This could be partly due to sampling error or the presence of patients with inactive cirrhosis. On the other hand increased serum NAG activities have been reported in acute viral hepatitis and several other non-liver related disorders, such as acute myocardial infarction, breast cancer, hypertension and silicosis (Koslinen et al. 1984, Calvo et al. 1982, Nomura et al. 1985, Perdichizzi et al. 1985). Casola et al. (1979) suggested that the increased serum glycosidase levels found in some diseases may be associated with structural changes in enzymes, which make the liver cell unable to recognize them, thereby preventing their clearance from blood. From the fact that in our study raised levels are also found in steatosis and chronic active hepatitis without evident fibrosis one can assume that the structural changes in the enzyme may also occur in these conditions apart from formation of fibrous tissue. Maybe one measures defective clearing as an epiphenomenon. The correlations (table IX.2) we found between serum NAG activity and biochemical parameters of liver tissue damage and bile duct obstruction could give some support to this theory.
We conclude that measurement of serum NAG is of no value in the differential diagnosis of chronic liver disease.

Table IX.1

Serum N-acetyl-β-D-glucosaminidase activity in controls and patients with liver disease.

patients groups		β-NAG (U/l))	p(t-test)
controls	(n=16)	19.6 +/- 4.2	–
alcohol abuse	(n=27)	30.4 +/- 16.4	p<0.01
alcoholic cirrhosis	(n=26)	37.9 +/- 18.7	p<0.006
primary biliary cirrhosis	(n=14)	37.7 +/- 15.1	p<0.0001
cirrhosis due to chronic active hepatitis	(n=18)	35.0 +/- 8.3	p<0.0001
chronic active hepatitis	(n=11)	32.6 +/- 7.5	p<0.0001
other liver diseases	(n=15)	33.8 +/- 13.3	p<0.0008

inter-assay variation coefficient (n=17)
19.8 +/- 1.6 U/l (x +/- S.D.) (7.9%)

intra-assay variation coefficient (n = 7)
19.8 +/- 1.5 U/l (x +/- S.D.) (7.7%)

Table IX.2

Correlations between serum N-acetyl-β-glucosaminidase and other liver tests (n = 102).

parameter	coefficient of correlation	p value
bilirubine	r=0.20	p<0.10
ASAT	r=0.73	p<0.001
ALAT	r=0.41	p<0.001
γ-GT	r=0.63	p<0.001
alk. p.	r=0.32	p<0.001

Chapter X GENERAL DISCUSSION AND CONCLUSIONS

The mortality of alcoholic cirrhosis is high. As reported in chapter II we found a 5 year mortality of 50%. In the Netherlands the number of death due to alcoholic cirrhosis has multiplied by ten in the past 20 years. Though the per capita consumption of alcohol is stabilizing or declining a little, alcohol abuse still remains a great social and medical problem. Up till now an effective medical treatment for the prevention of alcoholic cirrhosis has not been found. As about 15% of alcoholics will develop cirrhosis, efforts should be made to identify this group via a procedure that could be used as a screening. Once these patients have been identified, one can try harder to make them stop drinking as this is the best and for the moment the only treatment we have to prevent the development of liver cirrhosis.

In our study of patients hospitalized for the first time with alcoholic cirrhosis the CP classification was a good indicator of prognosis as was the combination of serum bilirubin and albumin.

Because cirrhosis is preceeded by fibrosis a marker of fibrosis or fibrogenesis would be very useful in the screening of alcoholics. When a reliable marker of liver fibrosis would be available this could also be used for the assessment of effectiveness of antifibrotic drugs. In this way such a test could replace serial liver biopsies with all their limitations.

We studied PIIIP, a product of collagen synthesis and LP1, a peptide split off in the production of a glycoprotein, in alcoholic liver disease and PBC. Both peptides were elevated in the sera of patients with alcoholic cirrhosis and PBC. In alcoholic steatosis the PIIIP levels were normal, while the LP1 levels were significantly higher than in controls. Because of this last finding it was suggested that glycoprotein production preceeds synthesis of collagen in the process of fibrogenesis and that LP1 might be an early marker of fibrosis in alcoholic liver disease. In this way serial measurements of LP1 in the sera of alcoholics could be helpful in identifying patients at risk of developing fibrosis.

A correlation between serum LP1 levels and presence of esophageal varices in cirrhotic patients was found. It has been suggested that LP1 may also be a non-invasive serum marker of portal hypertension, though further study in this field is necessary.

Patients with alcoholic steatosis had normal PIIIP values. This could be due to absence of excessive collagen production or to a slightly elevated

synthesis, too small to detect with the used radioimmunoassay. This last problem could also be the cause of the failure of PIIIP levels to differentiate between PBC patients with and without histological progression.
The newly developed RIAgnost PIIIP coated tube is easier to perform, takes less time and has a lower coefficient of variation. Therefore this test is superior for routine laboratory use and may also detect low grade fibrogenesis.
In patients who underwent liver transplantation serum PIIIP concentrations at 6 weeks and 3 months after the operation were measured with this coated tube method. The absence of a decrease in serum PIIIP levels in this period appeared to correlate with occurence of fibrosis in the graft. It was concluded that serial measurements of PIIIP levels might be useful in the detection of hepatic fibrogenesis after liver transplantation.
From a theoretical point of view a collagen III molecule should give rise to a release of a PIIIP to the serum. From this assumption it results that elevation of collagen III production causes elevation of serum PIIIP levels. However, as described in the introduction the situation is more complicated. Intracellular degradation of procollagen, variation in procollagen secretion, PIIIP molecules remaining as constituents of mature collagen and renal function are some factors that trouble the interpretation of the PIIIP level as a representative of the collagen production in a liver. Moreover at this moment it is not possible for the pathologist to differentiate between recently deposited and formerly deposited collagen. This is the reason why in fact a correlation between a serum PIIIP level, which is the result of recently formed collagen, and the collagen content of a liver, which may be built up in the course of years can not exist.
Taking this into consideration one can conclude that one measurement of PIIIP is useless to get insight into the total collagen content of a liver and that PIIIP is not a marker of liver fibrosis. It is however possible that the process of fibrogenesis, thus the new formation of collagen, may be monitored using serial PIIIP determinations; then the integrated value of PIIIP levels over time may give the best prediction of newly formed hepatic collagen.
The information whether new collagen has been formed in a certain period is probably more important than the knowledge of total collagen content at a certain moment, as it offers us insight into the course of a hepatic disease and, when medical therapy is prescribed, into the effect of this intervention.
Because the eventual development of fibrosis is the result of synthesis and degradation of biomatrix a marker of degradation would be helpful in the evaluation of collagen metabolism. We tested the enzyme N-acetyl-β-glucosaminidase, involved in the breakdown of glycosaminoglycans from

connective tissue, as a possible serum marker of degradation. However, this test failed to discriminate between different forms of liver disease with and without fibrosis. It was concluded that determination of serum N-acetyl-β-glucosaminidase is of no value for differentiation between chronic liver diseases with and without fibrosis.
Further study should aim at finding a reliable serum marker of collagen degradation. The combination of serial measurements of serum PIIIP levels and the levels of a degradation marker then may give a good insight into the eventual occurrence of collagen deposition in the course of a liver disease.

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SUMMARY

This thesis is concerned with an investigation into serum parameters of liver fibrosis. Together with this investigation a study concerning the prognosis and prognostic factors in alcoholic liver cirrhosis was performed.
In **chapter I** a review of the extracellular matrix in normal and fibrotic liver is given. Fibrosis is defined as an excessive accumulation of connective tissue or extracellular matrix (mainly collagen, glycoproteins and glycosaminoglycans) within the organ. The cells involved in the production of the extracellular matrix are described, and the metabolism of collagen is discussed. The mechanism of liver fibrosis, some etiologic varieties and factors that may influence the fibrotic process are dealt with. The evaluation of fibrosis is discussed, including the limitations of the liver biopsy and the poor association between conventional liver tests and liver fibrosis.
In cirrhotic liver the collagen content may be elevated 4 to 7 times. This is mainly collagen types I and III and is independent of the etiology of the cirrhosis. In liver diseases with increased connective tissue formation synthesis of collagen type III preceeds that of collagen type I. During the process of fibrogenesis a fraction of the newly synthesized matrix escapes into the systemic circulation and becomes measurable in elevated levels in the serum.
The aminoterminal peptide of procollagen type III (PIIIP) is split from the procollagen molecule by a specific peptidase during the formation of collagen from procollagen. PIIIP concentrations in serum can be measured by radioimmunoassay. In **chapter III** the results of measurements of serum PIIIP levels in patients with alcoholic liver disease and primary biliary cirrhosis (PBC) are given. Concentrations were significantly raised in both alcoholic cirrhosis and PBC and were slightly increased in patients with alcohol abuse, some of whom had a fatty liver. In patients with PBC serum levels did not correlate with the histological grade of the disease.
Laminin is a major non-collagenous glycoprotein of basement membranes. In addition to PIIIP laminin P1 fragment (LP1) has also been proposed as a marker of liver fibrosis. In **chapter IV** a study, in which the diagnostic application of both peptides in alcoholic liver disease and PBC is evaluated, is described. Serum PIIIP and LP1 levels appeared to be significantly raised in patients with alcoholic cirrhosis and PBC.

Patients with alcohol abuse without cirrhosis had normal or slightly elevated PIIIP levels, but significantly raised LP1 levels. There was a clear correlation between PIIIP and LP1 concentrations. On the basis of this study it was not possible to conclude that both parameters should be measured or that use of one parameter only was sufficient. There were some indications that the two assays provide slightly different information; raised LP1 levels were seen early in alcoholic steatosis and also appeared to correlate with signs of portal hypertension.
Basement membrane is laid down in the sinusoids in cirrhosis and this could be a factor in the development of portal hypertension. Serum laminin levels in cirrhosis have been reported to correlate with portal hypertension. To determine the possible role of serum laminin assays as a non-invasive test for portal circulation changes in cirrhosis we measured serum LP1 levels in patients with cirrhosis and related these to the presence or absence of esophageal varices (**chapter V**). In a second group of patients with chronic liver disease we also measured serum LP1 levels. Indocyanine green (ICG) clearance was used as an indicator of hepatic blood flow and antipyrine (AP) clearance as a measure of functional liver mass. Patients with esophageal varices had significantly higher LP1 levels than patients without. There were significant negative correlations between serum LP1 levels and ICG and AP clearances. Raised serum LP1 levels in chronic liver disease are thus correlated with a reduced ICG clearance (and possibly reduced hepatic blood flow) and reduced AP clearance. As serum LP1 levels are higher if varices are present, serum LP1 is a candidate non-invasive marker of portal hypertension in chronic liver disease.
In **chapter VI** we describe a study concerning the value of serum PIIIP for predicting histological progression of PBC. Serial PIIIP measurements were obtained for patients with histologically progressive PBC and patients with histologically stable early disease, assessed by repeated liver biopsies and followed for up to 13 years. The means of the follow-up PIIIP concentrations were elevated in 40% of the cases; moreover, PIIIP levels were elevated at least once during follow-up in 70% of the cases. Mean follow-up PIIIP concentrations did not differ significantly between patients with progressive and non-progressive disease. In addition, in the progressive group, histological progression was not reflected by PIIIP levels. No difference was found between the serum PIIIP levels corresponding to the histological stages I, II and III. The individual coefficients of the correlation between serum PIIIP and biochemical variables (bilirubin, alkaline phosphatase, ASAT, albumin) and histology showed a wide distribution without a consistent trend towards positive or negative. Treatment with cyclosporin A or cyclosporin A combined with

prednisone did not influence serum PIIIP levels. Treatment with penicillamine combined with prednisone, however, resulted in a significant decrease in PIIIP concentrations.
We concluded that serum PIIIP measurements were of no value for predicting histological progression of PBC. Serum PIIIP measurements may however be of some use in evaluating response to treatment.
Liver transplantation is now an accepted treatment for end-stage liver disease. Disease recurrence in the graft, which may lead to fibrosis, has however been reported. We studied the clinical value of serum PIIIP in patients who underwent liver transplantation, as reported in **chapter VII**. Blood samples for measurement of serum PIIIP levels were taken at regular intervals. In 4 of 6 patients who showed fibrosis in the biopsy one year after transplantation serum PIIIP levels did not drop between 6 weeks and 3 months, while in 11 of 12 patients without fibrosis the 3 months' levels were lower than those after 6 weeks.
It was concluded that serial measurements of serum PIIIP may be useful in the detection of hepatic fibrogenesis after liver transplantation.
Measurement of serum PIIIP levels was originally performed using a conventional radioimmunoassay kit (RIAgnost® PIIIP tachysorb). A major problem of this assay is the non-linear behaviour. Recently a new radioimmunological procedure based on a "coated tube" method was developed (RIAgnost® PIIIP coated tube), which has not the problem of non-linearity, is simpler, needs less laboratory work and is faster.
As described in **chapter VIII**, we compared the results obtained by both methods in the sera of 129 patients (120 patients with different liver diseases and 9 pregnant women).
There was a high correlation between the conventional and the new method (r=0.96). We conclude that with the coated tube method it will be possible to use routinely PIIIP as a marker for monitoring collagen forming activities during follow-up of various diseases.
Serum concentrations of the enzyme N-acetyl-β-D-glucosaminidase (NAG), involved in the breakdown of glycosaminoglycans from connective tissue, were measured in healthy controls and patients with different liver diseases in order to evaluate its possible role in the differential diagnosis of liver diseases, mainly the detection of fibrosis (**chapter IX**).
Serum NAG was determined in patients with alcohol abuse without fibrosis, chronic active hepatitis without cirrhosis, PBC, alcoholic cirrhosis and cirrhosis due to chronic active hepatitis. Finally serum NAG was measured in patients with other liver diseases.
Serum NAG levels were significantly elevated in all patient groups.
No difference could be demonstrated between patients with and without fibrosis.

It is concluded that serum NAG levels are of no value in the differential diagnosis in chronic liver disease.
Alcoholic liver cirrhosis is associated with a considerable morbidity and mortality. We performed a follow-up study of patients admitted to hospital for the first time with alcoholic cirrhosis to derive factors of prognostic importance (**chapter II**). A set of 20 clinical, hematological and chemical parameters was collected for every case. The actuarial survival percentage was 50% at 5 years. Patients aged 50 years or more had a significantly poorer survival. Prognosis was best for Child-Pugh (CP) class A and worst for CP class C. The presence of esophageal varices was associated with a significantly lower 2 years survival. Survival correlated with the individual factors of the CP classification and the hemoglobin-level. In multivariate analysis bilirubin- and albumin-levels were the most important independent prognostic factors. The main causes of death were variceal bleeding and liver insufficiency. Alcohol consumption after discharge could be reliably assessed in 40 patients. None of the 15 patients who abstained died during follow-up as opposed to 12 deaths amongst the 25 who continued drinking.
In **chapter X** the results of the studies described in this thesis are evaluated in the light of the aims of the thesis.

SAMENVATTING

In dit proefschrift wordt een onderzoek beschreven naar serumparameters bij leverfibrose. Gelijktijdig met dit onderzoek werd een studie verricht naar de prognose en prognostische factoren bij alcoholische levercirrose.
Hoofdstuk I geeft een overzicht van de extracellulaire matrix bij een normale en een fibrotische lever. Fibrose wordt gedefinieerd als een overmatige ophoping van bindweefsel of extracellulaire matrix (voornamelijk collageen, glycoproteïnen en glycosaminoglycanen) in een orgaan. De cellen betrokken bij de productie van extracellulaire matrix en het metabolisme van collageen worden beschreven. Het mechanisme van leverfibrose, enkele etiologische varianten en factoren die het proces van fibrose kunnen beïnvloeden worden besproken.
De evaluatie van fibrose wordt belicht inclusief de beperkingen van de leverbiopsie en de slechte correlatie tussen conventionele levertesten en leverfibrose.
Bij een cirrotische lever kan het collageengehalte 4 tot 7 maal verhoogd zijn. Dit betreft met name de collageentypes I en III en is onafhankelijk van de etiologie van de cirrose. Bij leverziekten met verhoogde bindweefselvorming gaat de synthese van collageen type III vooraf aan die van collageen type I. Tijdens het proces van fibrogenese ontsnapt een fractie van de nieuwgevormde matrix naar de systemische circulatie en wordt meetbaar in het serum in verhoogde concentraties. Het aminoterminale peptide van procollageen type III (PIIIP) wordt door een specifiek peptidase van het procollageen-molecuul afgesplitst tijdens de vorming van collageen uit procollageen. PIIIP-concentraties in het serum kunnen bepaald worden middels een radioimmunologische methode.
In **hoofdstuk III** worden de resultaten van metingen van serum PIIIP-concentraties bij patiënten met alcoholische leverziekte en primair biliaire cirrose (PBC) gegeven. De concentraties waren significant verhoogd bij zowel alcoholische cirrose als PBC en waren licht verhoogd bij patiënten met alcoholabusus, van wie enkelen een vetlever hadden. Bij patiënten met PBC correleerden de serum PIIIP-spiegels niet met het histologisch stadium van de ziekte.
Laminine is een belangrijk niet-collageen glycoproteïne in basaalmembranen. Naast PIIIP is het laminine P1 fragment (LP1) ook voorgesteld als een merkstof voor leverfibrose. In **hoofdstuk IV** wordt een studie beschreven, waarin de diagnostische toepassing van beide

peptiden bij alcoholische leverziekte en PBC geëvalueerd wordt. Serum PIIIP- en LP1-spiegels bleken significant verhoogd te zijn bij patiënten met alcoholische cirrose en PBC.
Patiënten met alcoholabusus zonder cirrose hadden normale of licht verhoogde PIIIP-concentraties, maar significant verhoogde LP1-concentraties. Er was een duidelijke correlatie tussen PIIIP- en LP1-spiegels. Op basis van deze studie was het niet mogelijk om te concluderen dat beide parameters gemeten zouden moeten worden of dat het gebruik van slechts een parameter voldoende zou zijn. Er waren enige aanwijzingen dat de 2 bepalingen enigszins verschillende informatie geven; verhoogde LP1-concentraties werden gezien in een vroeg stadium van alcoholische steatose en leken ook te correleren met tekenen van portale hypertensie. Basaal membraan wordt afgezet in de sinusoïden bij cirrose en dit zou een factor kunnen zijn bij de ontwikkeling van portale hypertensie. Er zijn studies verschenen, die beschrijven dat serum laminine-spiegels correleren met portale hypertensie. Om de mogelijke rol van serum laminine-bepalingen als een niet-invasieve test op portale circulatie-veranderingen te onderzoeken werden serum LP1-concentraties bij patiënten met cirrose gemeten en gerelateerd aan aan- of afwezigheid van slokdarmvarices (**hoofdstuk V**). Bij een tweede groep patiënten met chronische leverziekte bepaalden wij eveneens de serum LP1-spiegels. De Indocyanine Groen (ICG)-klaring werd gebruikt als een indicator van de bloedstroom door de lever en de Antipyrine (AP)-klaring als een maat voor functionele levermassa.
Patiënten met slokdarmvarices hadden significant hogere LP1-spiegels dan patiënten zonder. Er waren significante negatieve correlaties tussen serum LP1-concentraties en ICG- en AP-klaringen. Verhoogde serum LP1-waarden zijn derhalve gecorreleerd met een verminderde ICG-klaring (en mogelijk verminderde bloedstroom door de lever) en verminderde AP-klaring. Omdat de serum LP1-concentraties hoger zijn als varices aanwezig zijn, is serum LP1 mogelijk een niet-invasieve parameter voor portale hypertensie bij chronische leverziekte.
In **hoofdstuk VI** beschrijven wij een studie naar de waarde van serum PIIIP bij het voorspellen van histologische progressie van PBC. Seriële PIIIP-bepalingen werden verricht bij patiënten met histologisch progressieve PBC en bij patiënten met een histologisch stationair vroeg stadium van de ziekte, aangetoond middels herhaalde leverbiopsieën en gevolgd gedurende maximaal 13 jaar. De gemiddelden van de PIIIP-concentraties tijdens de onderzoeksperiode waren in 40% van de gevallen verhoogd; bovendien waren de spiegels tenminste eenmaal verhoogd bij 70% van de patiënten. De gemiddelde PIIIP-concentraties verschilden niet significant bij patiënten met progressieve en niet-progressieve ziekte. Daarnaast

werd, in de progressieve groep, histologische progressie niet weerspiegeld in PIIIP-concentraties. Er werd geen verschil gevonden in de serum PIIIP-waarden corresponderend met de histologische stadia I, II en III. De individuele coëfficiënten van de correlatie tussen serum PIIIP en biochemische variabelen (bilirubine, alkalische fosfatase, ASAT, albumine) en histologie vertoonden een grote spreiding zonder een duidelijke neiging naar positief of negatief. Behandeling met cyclosporine A of cyclosporine A gecombineerd met prednison beïnvloedde de serum PIIIP-spiegels niet. Behandeling met penicillamine gecombineerd met prednison resulteerde echter in een significante vermindering van de PIIIP-concentraties.
Wij concludeerden dat in deze studie serum PIIIP-bepalingen geen waarde hadden bij het voorspellen van histologische progressie van PBC. Serum PIIIP-bepalingen zouden echter bruikbaar kunnen zijn bij het evalueren van de reactie op behandeling.
Levertransplantatie is nu geaccepteerd als behandeling voor terminale leverziekte. Het opnieuw optreden van de ziekte in het transplantaat, wat kan leiden tot fibrose, is echter mogelijk. Wij bestudeerden de klinische waarde van serum PIIIP bij patiënten die levertransplantatie ondergingen (**hoofdstuk VII**). Met regelmatige intervallen werden bloedmonsters voor PIIIP-bepaling en leverbiopten genomen. Bij 4 van de 6 patiënten, bij wie fibrose in het biopt aanwezig was een jaar na transplantatie, daalden de serum PIIIP-concentraties niet tussen 6 weken en 3 maanden, terwijl bij 11 van de 12 patiënten zonder fibrose de spiegels na 3 maanden lager waren dan die na 6 weken. Er werd geconcludeerd dat regelmatige bepalingen van serum PIIIP nuttig kunnen zijn bij het opsporen van fibrogenese in de lever na levertransplantatie.
Bepaling van serum PIIIP-spiegels werd aanvankelijk met gebruikmaking van een conventionele radioimmunologische methode (RIAgnost® PIIIP tachysorb) verricht. Een belangrijk probleem van deze methode is het niet-lineaire gedrag. Recent werd een nieuwe radioimmunologische procedure, waarbij de antistof op de testbuis is aangebracht (RIAgnost® PIIIP coated tube), ontwikkeld, die niet het probleem van het niet-lineair zijn heeft, makkelijker is, minder laboratoriumwerk vergt en sneller is. Zoals beschreven in **hoofdstuk VIII** vergeleken wij de resultaten verkregen middels beide methoden in de sera van 129 patiënten (120 patiënten met verschillende leverziekten en 9 zwangere vrouwen). Er was een hoge correlatie tussen de conventionele en de nieuwe methode ($r=0.96$). Wij concluderen dat het met de "coated tube"-methode mogelijk zal zijn PIIIP routinematig te gebruiken als een merkstof om collageen-vormende activiteiten te vervolgen in het verloop van verschillende ziekten.

Serum concentraties van het enzym N-acetyl-β-D-glucosaminidase (NAG), betrokken bij de afbraak van glycosaminoglycanen uit bindweefsel, werden bepaald bij gezonde controle personen en patiënten met verschillende leverziekten om de mogelijke rol van dit enzym bij de differentiële diagnostiek van leverziekten, voornamelijk het opsporen van fibrose, te onderzoeken (**hoofdstuk IX**). Serum NAG werd bepaald bij patiënten met alcoholabusus zonder fibrose, chronische actieve hepatitis zonder cirrose, PBC, alcoholische cirrose en cirrose ten gevolge van chronisch actieve hepatitis. Tenslotte werd serum NAG bepaald bij patiënten met andere leverziekten.
Serum NAG-spiegels waren significant verhoogd bij alle patiëntengroepen. Er kon geen verschil worden aangetoond tussen patiënten met en zonder fibrose. Concluderend hebben serum NAG-spiegels geen waarde bij de differentiële diagnostiek van chronische leverziekten.
Alcoholische levercirrose gaat gepaard met aanzienlijke morbiditeit en mortaliteit. Wij verrichtten een vervolgonderzoek bij patiënten die voor de eerste maal met alcoholische cirrose in het ziekenhuis werden opgenomen om voor de prognose belangrijke factoren vast te stellen (**hoofdstuk II**). Voor iedere patient werden 20 klinische, chemische en hematologische parameters verzameld. Het actuariële overlevingspercentage was 50% na 5 jaar. Patiënten ouder dan 50 jaar hadden een significant slechtere overleving. De prognose was het best voor Child-Pugh (CP) klasse A en het slechtst voor CP klasse C. De aanwezigheid van slokdarmvarices ging gepaard met een significant lagere tweejaars overleving. De overleving correleerde met de individuele factoren van de CP classificatie en het hemoglobine-gehalte. Bij multivariate analyse waren de bilirubine- en albumine-concentraties de belangrijkste onafhankelijke prognostische factoren. De belangrijkste doodsoorzaken waren varixbloedingen en leverinsufficiëntie. Het gebruik van alcohol na ontslag kon betrouwbaar worden vastgesteld bij 40 patiënten. Geen van de 15 patiënten die zich onthielden van alcoholgebruik overleed tijdens de vervolgperiode tegenover 12 doden onder de 25 die niet stopten.
In **hoofdstuk X** worden de in het begin van het proefschrift genoemde doelstellingen geëvalueerd in het licht van de gegevens van het onderzoek beschreven in de hoofdstukken II tot en met IX.

This thesis is based on the following publications:

R.A.A. van Zanten, R.E.W. van Leeuwen, R. Beukers, J.H.P. Wilson.
Procollagen III peptide levels in alcoholic liver disease and primary biliary cirrhosis. Neth. J. Med. 1988; 32:278-284.

R.A.A. van Zanten, R.E.W. van Leeuwen, J.H.P. Wilson.
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Serum laminin levels, indocyanine green and antipyrine clearance and esophageal varices in chronic liver disease. Gastroenterology 1989; 96:A670.

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Prognosis and prognostic factors in alcoholic liver disease. Submitted for publication.

R. Beukers, R.A.A. van Zanten, S.W. Schalm.
Serial determination of type III procollagen amino propeptide serum levels in patients with histologically progressive and non-progressive primary biliary cirrhosis. Submitted for publication.

R.A.A. van Zanten, J.H.P. Wilson.
Serum aminoterminal peptide of procollagen III correlated to liver biopsies after liver transplantation. Submitted for publication.

R.A.A. van Zanten, J.W.O. van den Berg, A. Edixhoven-Bosdijk, T. Rietveld.
Measurement of procollagen type III aminoterminal peptide in serum. A comparison between the conventional and a new coated tube radioimmunoassay. Submitted for publication.

R.A.A. van Zanten, J.H.P. Wilson.
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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 9 april 1958 te Rotterdam. Aldaar werd in 1976 het eindexamen Gymnasium-β aan het Sint Franciscuscollege behaald. In datzelfde jaar werd de studie in de geneeskunde aan de Erasmus Universiteit Rotterdam aangevangen. Tijdens de studie was hij aanvankelijk in het kader van een keuzepracticum en later als student-assistent betrokken bij onderzoeksprojecten van de afdeling Interne Geneeskunde II (hoofd destijds Prof. Dr. M. Frenkel).
In mei 1983 begon hij de opleiding tot internist in het Diakonessenhuis Refaja te Dordrecht (opleider destijds C. Verdoorn†). In juli 1985 werd de opleiding voortgezet in het Academisch Ziekenhuis Rotterdam-Dijkzigt (opleider Prof. J.H.P. Wilson).
Op 1 juli 1988 volgde inschrijving in het specialistenregister.
Vanaf 1 januari 1989 was hij als chef de clinique verbonden aan de afdeling Interne Geneeskunde III (hoofd Prof. Dr. J.C. Birkenhäger).
Sinds 1 juni 1990 is hij als internist werkzaam in het Twenteborg Ziekenhuis te Almelo.