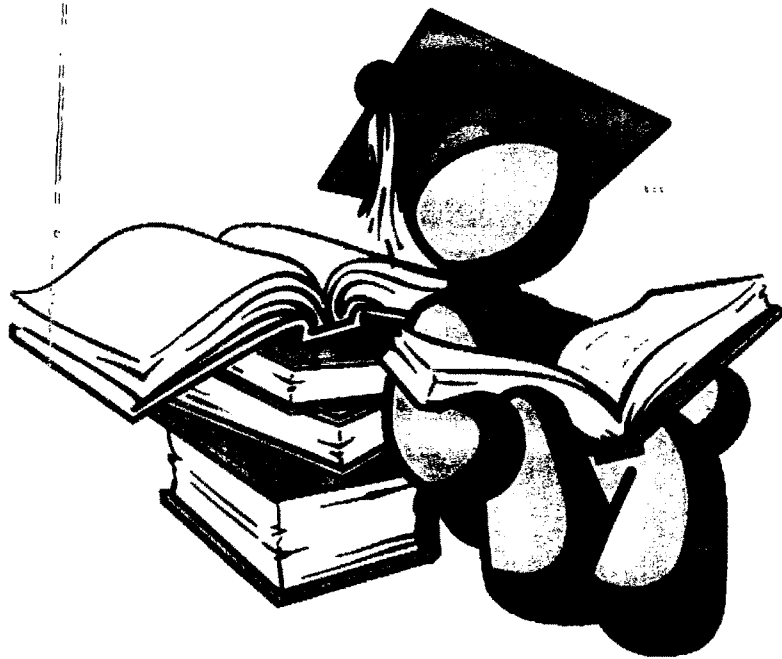


CHAPTER 2

LITERATURE REVIEW



Literature Review

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Considerable progress has been made, over the last 20 years, in understanding of the molecular pathophysiology of liver fibrosis. Liver fibrosis represents a wound healing response to a variety of chronic hepatic injuries including alcohol, autoimmune disease, viral hepatitis and toxic injury (Friedman, 2000). Common to wound healing in other tissues, the liver response to hepatocellular damage includes inflammation and tissue remodeling. Associated with this are changes in the quantity and quality of the extracellular matrix. There is accumulation of fibrillar collagen (mainly types I and III) in the space of Disse between the sinusoidal endothelial cells and the plates of hepatocytes. With chronic injury, the excess ECM in the liver causes architectural distortion and disruption of the normal pattern of blood flow through the liver. This eventually results in deterioration of hepatic performance with hepatic insufficiency and increased intrahepatic resistance to blood flow with portal hypertension.

Research into the pathogenesis of liver fibrosis has been facilitated by the observation that HSCs are the major mediators of the fibrotic process. Moreover, *in vitro* culture of extracted HSC and animal models has provided invaluable insights into hepatic fibrogenesis. Animal models include the carbon tetrachloride (CCl₄) model of liver fibrosis (Iredale et al., 1998) and the bile duct ligation model in the rat (Issa et al., 2002). In both models liver fibrosis may be induced in a reproducible manner. Furthermore, resolution of fibrosis may be observed to occur when the liver injury is stopped (cessation of injection or biliary jejunal anastomosis). These models have allowed identification of the activated HSCs as the key cell type responsible for production of the extracellular matrix (ECM) in chronic liver disease. Furthermore, they have highlighted that the stellate cell interacts with many other cell types within the liver, including hepatocytes, sinusoidal endothelial cells, lymphocytes, neutrophils and Kupffer cells (KCs) (liver macrophages). Many of these interactions play an important role in stellate cell biology and have been exploited to design and evaluate novel strategies and potential antifibrotic therapies.

2.1 MAJOR COMPONENTS OF THE LIVER

The liver is localized in the abdominal cavity between the digestive tract and the spleen. Functionally, it is in a strategic position between the gastro-intestinal tract and the general blood circulation. The organ has a double blood supply; most of the total blood influx is provided by the portal vein bringing nutrient-rich blood from the

digestive tract while the hepatic artery delivers blood supplemented with oxygen. Within the liver, the blood passes through a network of micro-vessels, called sinusoids, after which it is collected in the hepatic central veins and finally drained by the inferior vena cava.

The liver plays a central role in the metabolism of carbohydrates, proteins and fats, among other substances, and is thereby important for the maintenance of homeostasis in the body. The liver synthesizes most of the plasma proteins, such as albumin and globulins. Another function of the liver is detoxification, namely the biotransformation of xenobiotic compounds, pollutants and drugs into water-soluble compounds which then can be excreted either in bile or in urine. Importantly, the liver also eliminates particulate substances such as bacteria and viruses and different kinds of macromolecules from the blood stream.

At the microscopical level the liver is anatomically organised in functional units called lobules, built up from the three major liver cell types, the parenchymal cells or hepatocytes, the sinusoidal endothelial cells lining the sinusoids and a resident macrophage population, called KCs. The lobules have a hexagonal shape with the terminal hepatic vein in the centre and the portal triads at the corners of the hexagon. Each portal triad consists of a portal vein, a hepatic artery and a common hepatic bile duct. The parenchymal cells are structured in cords that are symmetrically distributed around the central vein and separated by liver sinusoids in which blood flows from portal triads towards the central vein.

The metabolic functions of the liver are maintained mainly by the parenchymal cells, which represent the major population of cells in the liver. In the narrow space between the endothelial cells and the hepatocytes, called the space of Disse, a fourth population of cells is localised, the HSCs. This population of non-parenchymal cells normally stores vitamin A and is involved in the production of ECM proteins. Other non-parenchymal cells that can be found in the liver are lymphocytes; including natural killer cells also known as pit cells, and biliary epithelial cells that line the bile ducts.

In the healthy liver, ECM constituents account for 0.5 % of the liver weight. In spite of its relatively small volume, the composition of the ECM in the space of Disse is of

great importance for the functioning of the liver cells. Hepatocytes, endothelial cells and HSCs require a proper ECM composition to express their phenotypes.

2.1.1 The Hepatocytes

Hepatocytes represent about 65 % of all liver cells in number and more than 90 % of liver volume and are the main functional unit of this organ. Although they have a polyhedral shape, functionally two major domains can be distinguished with regard to the outer plasma membranes of these cells. The bile canalicular (i.e. apical) surface is specialized in transport of bile salts, bilirubin, cholesterol, phospholipids as well as xenobiotics into the bile. The contact of hepatocytes with the blood occurs at the basolateral domain, bordering the space of Disse. Numerous microvilli on this membrane domain allow for a substantial increase in exchange surface. In addition, the plasma membrane of the basolateral domain contains many transmembrane transport proteins and also has endocytotic properties. Thus, this domain is rich in receptors such as the asialoglycoprotein receptor, the transferrin receptor and several lipoprotein receptors.

Hepatocytes are crucial for the glucose homeostasis in the body and can store as well as release glucose. Thus, their cytoplasm contains the storage form of glucose, the glycogen granules. Beside, hepatocytes harbour specific metabolic pathways such as the urea cycle, regulating the blood levels of amino acids and ammonia derived from the intestine, and the specific lipogenic and lipolytic enzyme systems involved in the synthesis and metabolism of a variety of serum and intestinal lipoproteins. Also the formation of bilirubin from heme and the excretion of cholesterol and its conversion into bile acids are specific hepatocytic processes.

2.1.2 Liver Sinusoidal Cells

KCs, endothelial cells and HSCs are major cells of hepatic sinusoid (Figure 2.1). Each of these cell populations has its own specific characteristics and functionality. Although these cells form a minority of the total liver cell population, they are essential for the proper functioning of the organ.

2.1.3 Liver Endothelial Cells

Liver endothelial cells (LEC) comprise approximately one fifth of all liver cells and represent about half of the non-parenchymal cells in number. They form the

endothelial lining of the hepatic sinusoids and as such possess unique features allowing them to provide a selective barrier between the blood stream on the one hand and the hepatocytes and HSCs on the other. LEC distinguish themselves from other vascular endothelial cells because they contain pores or fenestrations varying in diameter from 150–175 nm that are grouped in clusters (Wisse et al., 1985). Furthermore, a basal lamina underneath the all other endothelial cells in the body is lacking in the sinusoids of the liver. This architecture of the sinusoidal capillaries allows substances smaller than the diameter of the fenestrations to freely exchange between the blood and the space of Disse.

LEC also play an important functional role in a variety of liver-associated processes. High endocytotic activity of these cells enables them to clear the blood from different macromolecular waste products. Moreover, LEC are known to secrete cytokines, such as tumor necrosis factor - α (TNF- α) (Nagano et al., 1992) and interleukin (IL)-6 (Knolle et al., 1997) and express adhesion molecules such as intercellular adhesion molecule - 1 (ICAM-1) and vascular cell adhesion molecule - 1 (VCAM-1) (van Oosten et al., 1995) which are crucial in the interaction with leukocytes and neutrophils. In addition LEC express several scavenger receptors, which bind and take up negatively charged proteins and lipoproteins from the blood and are involved in the regulation of lipoprotein metabolism as well as in antigen presentation.

In the healthy liver, LEC play a role in the turn-over of ECM. They secrete collagen type IV and laminin (Maher and McGuire, 1990) and clear hyaluronan (McGary et al., 1993), (pro)collagen and fibronectin (Smedsrod et al., 1994), degradation products of ECM.

2.1.4 Kupffer Cells

KCs account for 15 % of the total liver cell population. Together with macrophages of the spleen they are a major part of the so-called mononuclear phagocyte system. KCs are predominantly located in the sinusoids, where they are directly exposed to the blood stream while being anchored to the endothelial cells. In addition to their strategic location, KCs are characterised by their high phagocytic activity and they are responsible for the removal of circulating microorganisms, immune complexes, dead cells and other debris from the blood stream as well as the detoxification of bacterial endotoxins. In addition to performing these phagocytic processes, KCs take up

different substances from the circulation via receptor mediated endocytosis. There are several receptors which facilitate this process, for example: mannose receptors, fucose receptors, Fc receptors, CD14 receptors (Smedsrod et al., 1994) and scavenger receptors class A I, A II and B I (Van Berkel et al., 2000).

As part of the innate immune system, KCs play a central role in the regulation of inflammatory processes and other immunological reactions in the liver. In response to stimuli, for example by bacterial endotoxin, KCs produce various cytokines, such as TNF- α , IL-1, IL-6 (Luster et al., 1994) eicosanoids and reactive oxygen species (ROS), which promote chemotaxis, phagocytosis and ROS production by other inflammatory cells, as well as stimulating different reactions of other liver cells. On the other hand, KCs are also capable to release factors which have an anti-inflammatory action, like IL-10 (Knolle et al., 1997).

2.1.5 Hepatic Stellate Cells

Hepatic stellate cells (HSC) represent 5 to 8 % of all liver cells in the healthy organ. A characteristic feature of these cells is the expression of two different phenotypes; quiescent in the normal liver, and activated in the diseased. As a consequence of this ability of transformation, HSCs also change their functions.

Quiescent HSC have a star-like shape and their cytoplasm contains vitamin A droplets, for which they were formerly also known as fat-storing cells (other former names are Ito cells, lipocytes). Storage and controlled release of retinoids is a major function of HSCs in the healthy liver. In the digestive tract esterified retinol is incorporated into chylomicrons which enter the blood stream after being secreted into the lymphatic system. After having been depleted from part of their triglyceride-load in the peripheral vasculature, the resulting chylomicron remnants are taken up from the blood by the hepatocytes and from there the retinol is transported to HSC with the help of retinol binding proteins (RBP), where it is stored as retinyl ester. From HSC, retinol bound to RBP can be secreted into the circulation or transported back to the hepatocytes.

The crucial process of ECM turnover in the space of Disse is also controlled by HSC. They secrete limited amounts of ECM proteins such as collagen type III, collagen type IV and laminin (Maher and McGuire, 1990). Furthermore, HSC express several matrix metalloproteinases (MMP), such as MMP-2, MMP-3, MMP-10, MMP-13 and

MMP-14, as well as their inhibitors (tissue inhibitors of matrix metalloproteinase -1 (TIMP-1) and TIMP-2) to control the matrix degradation processes (Knittel et al., 1999).

Because of their anatomical position, it is likely that quiescent HSCs also are involved in controlling the blood flow through the hepatic sinusoids. In fact, HSCs encircle the sinusoid with their long cellular processes in a cylindrical manner and can produce vasoactive proteins, including substance P, neuropeptide Y and somatostatin (Geerts, 2001).

Quiescent HSC are producers of hepatocyte growth factor (HGF) (Ramadori et al., 1992), which stimulates hepatocyte proliferation, and vascular endothelial growth factor (VEGF) (Ishikawa et al., 1999), a stimulus for growth of sinusoidal and vascular endothelial cells. In addition, molecules such as endothelin (ET)-1 (Shao et al., 1999), transforming growth factor- β (TGF- β) (De Bleser et al., 1997), neutrophins and erythropoietin (Maxwell et al., 1994) are secreted by HSC in the normal liver. All these mediators tightly control homeostasis within sinusoids and pathological processes within the liver.

2.2 LIVER FIBROSIS

Hepatic fibrosis, or scarring of the liver, is emerging as a treatable complication of advanced liver disease, following significant progress in understanding its underlying mechanisms. Efforts have focused on the HSCs, as these cells can undergo 'activation' into proliferative and fibrogenic myofibroblast-like cells during liver injury. Stimuli driving stellate cell activation include hepatocellular necrosis due to oxidant stress, apoptosis, and soluble growth factors. Specific lymphocyte subsets can also stimulate fibrogenesis. A cascade of signaling and transcriptional events in stellate cells underlies the fibrogenic response to liver injury, with each step in the cascade being a potential target for antifibrotic therapy. Disease specific fibrogenic mechanisms have also been uncovered: in hepatitis C, this may include direct stimulation of stellate cell activation by viral infection; in nonalcoholic steatohepatitis, elevated levels of leptin and increased leptin signaling by stellate cells increase fibrogenesis. Determinants of fibrosis progression include both environmental and genetic factors, with ongoing efforts to define specific polymorphisms correlating with fibrosis progression rates. Human studies now indicate that fibrosis and even cirrhosis could be reversible,

especially if the underlying disease is eradicated. A key challenge is to establish noninvasive means of assessing fibrosis stage and progression using either serum tests and/or imaging. In addition, endpoints of antifibrotic clinical trials need to be established so that reliable evidence of benefit can be identified. We are on the cusp of a new era in which antifibrotic therapies could become important in treating chronic fibrosing liver disease (Friedman, 2003; Friedman, 2008; Bataller and Brenner, 2005).

Hepatic fibrosis refers to the accumulation of interstitial or 'scar' ECM after either acute or chronic liver injury. Cirrhosis, the end-stage of progressive fibrosis, is characterized by septum formation and rings of scar that surround nodules of hepatocytes. The composition of ECM molecules in the fibrotic liver is similar to those of other fibrosing parenchyma, including lung and kidney, and is also similar among different etiologies of liver disease. Typically fibrosis requires years or decades to become clinically apparent, but notable exceptions in which cirrhosis develops over months may include pediatric liver disease (e.g. biliary atresia), drug-induced liver disease, and viral hepatitis associated with immunosuppression after liver transplantation.

Rapid progress in understanding the mechanisms of hepatic fibrosis exemplifies how basic research has begun to yield meaningful prospects for translation into new diagnostics and treatments for patients with liver disease. These advances include the isolation and characterization of fibrogenic cell types in liver, the clarification of general and disease-specific pathogenic mechanisms, and the broader appreciation of the natural history and reversibility of hepatic fibrosis. Recent reviews highlighting molecular mechanisms and historical aspects are recommended to the reader for more detail (Friedman, 2000; Schuppan et al., 2001; Geerts, 2001; Pinzani and Marra, 2001; Rocky, 2003).

2.2.1 The Cellular Basis of Hepatic Fibrosis

The HSC, a resident perisinusoidal cell that stores vitamin A, has been the focus of efforts to identify the source of extracellular matrix, because it can undergo 'activation' when the liver is injured. This activation is characterized by the acquisition of a proliferative, contractile, migratory, fibrogenic and inflammatory phenotype (Friedman, 2000) (Figure 2.1 and 2.2). Indeed, the model of sequential stellate cell

activation towards myofibroblasts has provided a robust conceptual framework, allowing new advances to be placed in a clear biological context. Recent studies have underscored the heterogeneity of mesenchymal populations in liver ranging from classic stellate cells to portal fibroblasts (Kinnman et al., 2003), with the variable expression of neural (Cassiman et al., 2002), angiogenic (Corpechot et al., 2002), contractile (Rocky, 2003) and even bone-marrow-derived (Forbes et al., 2004) markers. Moreover, experimental genetic 'marking' of stellate cells, by expressing fluorescent proteins downstream of either fibrogenic or contractile gene promoters, illustrates the plasticity of fibrogenic cell populations in vivo (Magness et al., 2004). In view of this capacity for 'transdifferentiation' between different mesenchymal cell lineages and possibly even epithelial cells (Kalluri and Neilson, 2003), the key issue is not necessarily where fibrogenic cells arise from, but rather whether they express target molecules such as receptors or cytokines (Figure 2.1) in sufficient concentrations in vivo to merit their targeting by diagnostic agents or antifibrotic compounds.

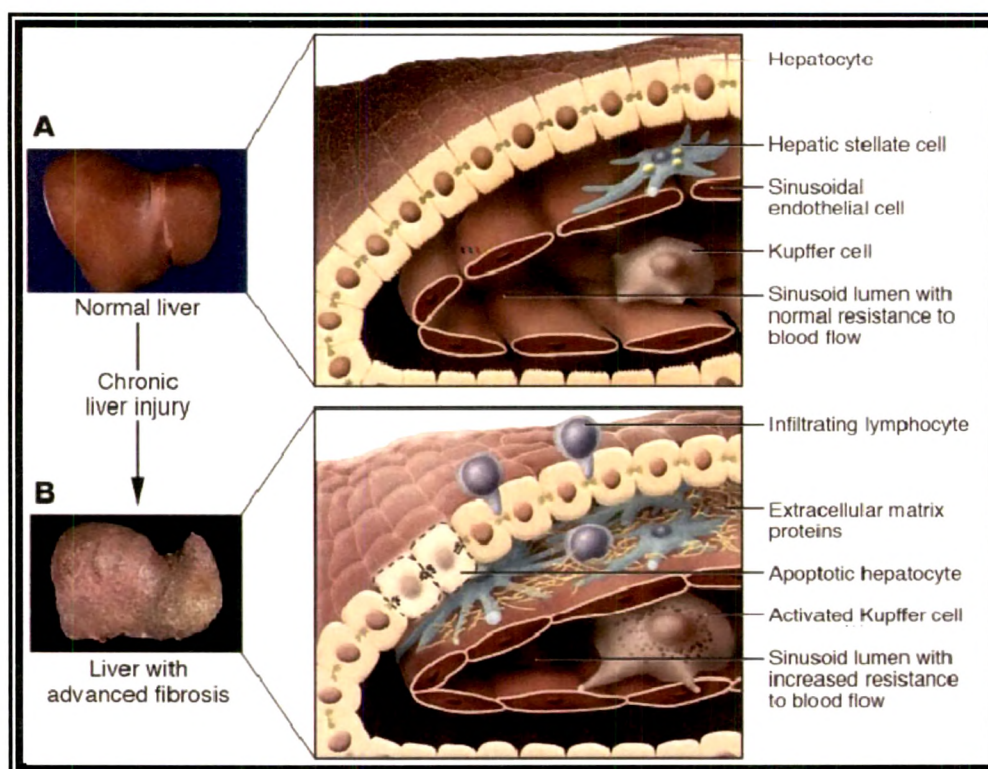


Figure 2.1 Sub-endothelial changes during stellate cell activation accompanying liver injury (Bataller and Brenner, 2005)

2.2.2 General Mechanisms Regulating Hepatic Fibrosis

More importantly, HSCs and sinusoidal endothelial cells have emerged as inflammatory effectors. Sinusoidal endothelial cells, which are normally fenestrated to allow rapid bidirectional transport of solutes between sinusoidal blood and parenchymal cells, may rapidly lose their fenestrations on injury and express pro-inflammatory molecules including ICAM-1, Vascular Endothelial Cell Growth Factor (VEGF) and adhesion molecules (LeCouter et al., 2003; Olaso et al., 2003). Together with stellate cells, they activate angiogenic pathways in response to the hypoxia associated with local injury or malignancy (Olaso et al., 2003; Marra, 2002; Yoshiji et al., 2003; Ankoma-Sey et al., 2000).

HSCs are a nexus for converging inflammatory pathways leading to fibrosis. On activation, they release chemokines (Marra, 2002; Schwabe et al., 2003) and other leukocyte chemoattractants, and upregulate the expression of key inflammatory receptors including ICAM-1 (Bataller et al., 2004), chemokine receptors (Efsen et al., 2002), as well as those mediating lipopolysaccharides signaling, including Toll-like receptor 4 (Paik et al., 2003). Stellate cells might also contribute to the intrahepatic apoptosis of T lymphocytes (Kobayashi et al., 2003).

2.2.2.1 Stellate Cell Activation

Stellate cell activation is the central event in hepatic fibrosis. Activation consists of 2 major phases: (1) initiation (also called apreinflammatory stage) and (2) perpetuation (Figure 2.2) (Friedman, 2000). Initiation refers to early paracrine-mediated changes in gene expression and phenotype that render the cells responsive to other cytokines and stimuli. Perpetuation then results from the effects of these stimuli on maintaining the activated phenotype and generating fibrosis.

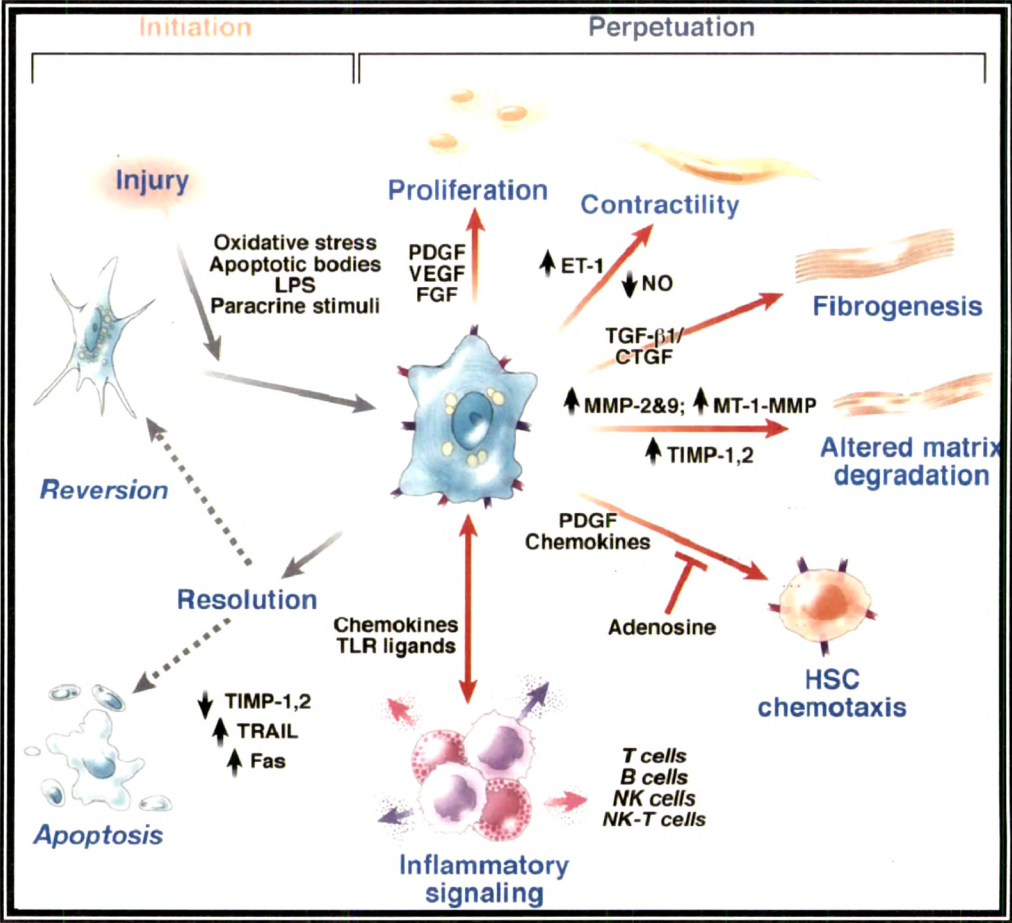


Figure 2.2 Pathways of stellate cell activation and resolution during liver injury
(Friedman, 2008)

2.2.2.1.1 Initiation of Stellate Cell Activation

The earliest changes in stellate cells are likely to result from paracrine stimulation by all neighboring cell types, including sinusoidal endothelium, KCs, hepatocytes, platelets, and leukocytes. Endothelial cells are also likely to participate in activation, both by production of cellular fibronectin and via conversion of TGF-β from the latent to active, profibrogenic form (Figure 2.3) (Bataller and Brenner, 2001).

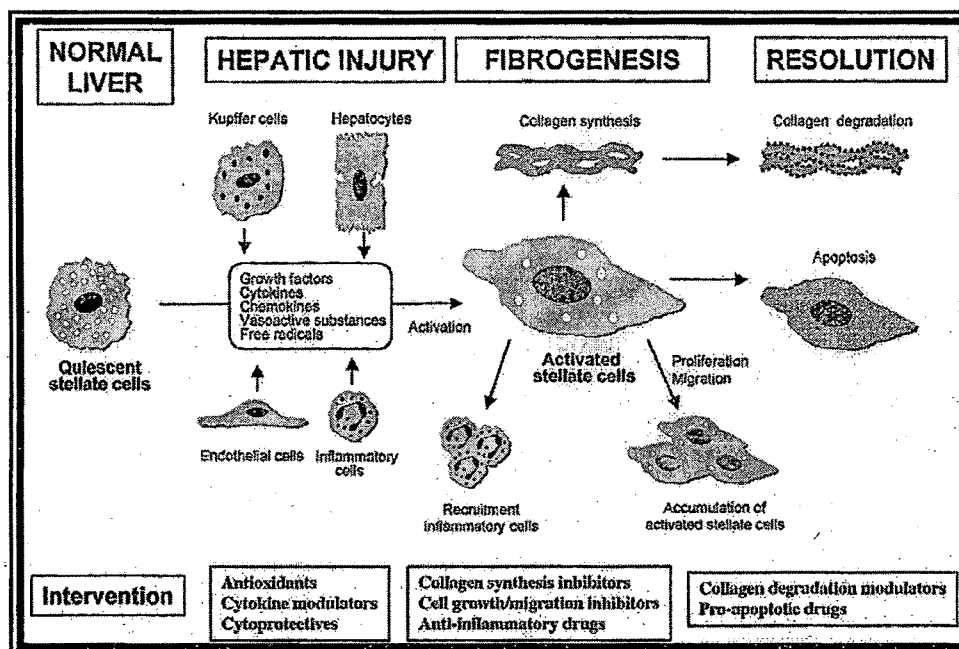


Figure 2.3 Biological actions of hepatic stellate cells (HSCs) during liver fibrogenesis and potential sites for therapeutic intervention (Bataller and Brenner, 2001)

Kupffer cell infiltration and activation also play a prominent role in the process. The influx of KCs coincides with the appearance of stellate cell activation markers. KCs can stimulate matrix synthesis, cell proliferation, and release of retinoids by stellate cells through the actions of cytokines (especially, TGF- β_1) and reactive oxygen intermediates/lipid peroxides. Proliferation has been attributed to Kupffer cell-derived TGF- α (Gressner, 1995; Friedman and Arthur, 1989). TGF- β derived from KCs markedly stimulates stellate cell ECM synthesis (Gressner, 1995; Gressner et al., 1993). KCs produce anti-inflammatory as well as proinflammatory cytokines, including IL-10 (Nieto et al., 2000). Of interest, IL-10 downregulates fibrogenesis in cultured stellate cells by decreasing collagen synthesis and increasing production of collagenase (Wang et al., 1998(b)).

Another means by which KCs can influence stellate cells is through secretion of MMP-9 (gelatinase B) (Winwood et al., 1995). MMP-9 can activate latent TGF- β , which in turn can stimulate stellate cell collagen synthesis (Yu and Stamenkovic, 2000). Lastly, KCs are an important source of ROS in the liver. ROS, whether produced internally within stellate cells (Nieto et al., 1999; Svegliati-Baroni et al.,

2001) or released into the extracellular environment (Lee et al., 1995), are capable of enhancing stellate cell activation and collagen synthesis. KCs also produce nitric oxide (NO), which can counterbalance the stimulatory effects of ROS by reducing stellate cell proliferation and contractility. Hepatocytes, as the most abundant cell type in liver, are a potent source of fibrogenic lipid peroxides in inflammatory liver diseases.

Platelets are a potent source of growth factors, and are present in the injured liver (Bachem et al., 1989). Potentially important platelet mediators include platelet derived growth factor (PDGF), TGF- β_1 , and epidermal growth factor.

Leukocytes that are recruited to the liver during injury join with KCs in producing compounds that modulate stellate cell behavior. Neutrophils are an important source of ROS, which have a direct stimulatory effect on stellate cell collagen synthesis. The specific role of neutrophils as a stimulus to stellate cells was demonstrated in a coculture experiment in which cells activated by N-formyl-methionyl-leucyl-phenylalanine were plated in direct contact with stellate cells (Casini et al., 1997). Activated neutrophils increased stellate cell collagen synthesis 3-fold over control levels. Superoxide was identified as the principal mediator of the neutrophil effect. Activated neutrophils also produced NO, which dampened the effect of superoxide on collagen expression but did not abrogate it completely. Lymphocytes, including CD4+ T-helper (Th) cells, reside in the liver (Winnock et al., 1995; Tiegs et al., 1992) and represent a further potential source of cytokines. Th lymphocytes help orchestrate the host-response via cytokine production and can differentiate into Th1 and Th2 subsets, a classification that is based on the pattern of cytokines produced. In general, Th1 cells produce cytokines that promote cell-mediated immunity and include interferon (IFN)- γ , TNF, and IL-2. Th2 cells produce IL-4, IL-5, IL-6, and IL-13, and promote humoral immunity. Th1 cytokines inhibit the development of Th2 cells and Th2 cytokines inhibit the development of Th1 cells. Thus, the host response to infection or injury frequently polarizes to either a Th1 or Th2 response, but not both.

Several experimental models offer evidence implicating Th cell-derived cytokines in determining the immune response. The polarization of the immune response is enhanced when chronic exposure to an agent occurs, such as with persistent infections or exposure to environmental toxins. Furthermore, polarization of the immune

response to Th1 or Th2 cytokines is under genetic control, as demonstrated by divergent responses of different inbred strains of mice to experimental murine leishmaniasis (Guler et al., 1996). Genetically resistant mice - such as C57BL/6 mice - exhibit an expansion of IFN- γ -producing Th1 cells and control the infection, whereas susceptible strains (BALB/c mice) develop an IL-4-mediated Th2 response (Heinzel et al., 1989; Wang et al., 1994).

More intriguing data have been obtained from studies comparing the effects of T lymphocytes on liver fibrosis. Overall, data suggest that Th2 lymphocytes favor fibrogenesis in liver injury over Th1 lymphocytes. The effects of CCl₄ have been examined in mice with several different lymphocyte profiles, including T-cell depletion (severe combined immunodeficiency, SCID), Th1 predominance (C57/BL6), and Th2 predominance (BALB/c) (Shi et al., 1997). SCID mice from both C57/BL6 and BALB/c backgrounds develop liver fibrosis after treatment with CCl₄ for 4 weeks. The degree of fibrosis is modified significantly, however, in immunocompetent mice from both strains. Immunocompetent C57/BL6 mice, whose lymphocyte cytokine profile includes IFN-gamma, actually exhibit less fibrosis than SCID mice from the same background. Indeed, when C57/BL6 mice with targeted disruption of IFN- γ are treated with CCl₄, fibrosis returns to the level seen in C57/BL6 SCID mice. However, immunocompetent BALB/c mice, whose lymphocyte cytokine profile includes the fibrogenic compounds IL-4 and TGF- β , exhibit more fibrosis than BALB/c SCID mice. Among all of the studies examining immunoregulation of fibrosis, those demonstrating modulation by T-lymphocytes appear the most convincing.

2.2.2.1.2 Perpetuation of Stellate Cell Activation

Perpetuation of stellate cell activation involves several discrete changes in cell behavior, as discussed below.

2.2.2.1.2.1 Proliferation

PDGF is the most potent stellate cell mitogen identified. Induction of PDGF receptors early in stellate cell activation increases responsiveness to this potent mitogen (Wong et al., 1996). Downstream pathways of PDGF signaling have been carefully characterized in stellate cells.

2.2.2.1.2.2 Chemotaxis

Stellate cells can migrate towards cytokine chemoattractants (Maher, 2001; Marra et al., 1999), an action that is characteristic of woundinfiltrating mesenchymal cells in other tissues as well. Chemotaxis of stellate cells explains in part why stellate cells align within inflammatory septa in vivo.

2.2.2.1.2.3 Fibrogenesis

Increased matrix production is the most direct way that stellate cell activation generates hepatic fibrosis. The most potent stimulus to collagen I production is TGF- β , which is derived from both paracrine and autocrine sources.

Lipid peroxidation products are emerging as important stimuli to ECM production; their effects may be amplified by loss of antioxidant capacity of stellate cells as they activate (Whalen et al., 1999). These important insights have provoked efforts to use antioxidants as therapy for hepatic fibrosis.

2.2.2.1.2.4 Contractility

Contractility of stellate cells may be a major determinant of early and late increases in portal resistance during liver fibrosis. Activated stellate cells impede portal blood flow by both constricting individual sinusoids and contracting the cirrhotic liver, because the collagenous bands typical of end-stage cirrhosis contain large numbers of activated stellate cells (Rocky, 2001). The major contractile stimulus towards stellate cells is ET-1. Receptors for the latter are expressed on both quiescent and activated stellate cells, but its subunit composition may vary (Housset et al., 1993). Contractility of stellate cells in response to ET-1 has also been observed in vivo (Zhang et al., 1994). Other, less potent contractile stimuli have also been identified (Rocky, 2001). For example, KCs produce eicosanoids, including prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), and thromboxanes (Peters et al., 1990; Kawada et al., 1992(b)). Eicosanoids modulate stellate cell contractility, with thromboxanes typically promoting contraction and PGE2 mediating relaxation (Kawada et al., 1992(a)).

Locally produced vasodilator substances may counteract the constrictive effects of ET-1 (Rocky, 2001). NO, which is also produced by stellate cells, is a well-characterized endogenous antagonist to ET.

2.2.2.1.2.5 Matrix Degradation

Quantitative and qualitative changes in matrix protease activity play an important role in ECM remodeling accompanying fibrosing liver injury. Stellate cells express virtually all of the key components required for pathologic matrix degradation and therefore play a key role not only in matrix production, but also in matrix degradation. An enlarging family of matrix-metalloproteinases has been identified that are calcium-dependent enzymes which specifically degrade collagens and noncollagenous substrates (Benyon and Arthur, 2001). Broadly, these enzymes fall into 5 categories based on substrate specificity: (1) interstitial collagenases (MMP-1, -8, -13); (2) gelatinases (MMP-2,-9) and fibroblast activation protein (Levy et al., 1999); (3) stromelysins (MMP-3, -7, -10, 11); (4) membrane-type (MMP-14, 15, -16, -17, -24, -25); and (5) a metalloelastase (MMP-12). Inactive metalloproteinases can be activated through proteolytic cleavage by either membrane-type matrix metalloproteinase 1 (MTMMP-1) or plasmin, and inhibited by binding to specific inhibitors known as TIMPs. Thus, net collagenase activity reflects the relative amounts of activated metalloproteinases and their inhibitors, especially TIMPs.

In liver, "pathologic" matrix degradation refers to the early disruption of the normal subendothelial matrix that occurs through the actions of at least 4 enzymes: MMP2 and MMP-9 degrade type IV collagen; MTMMP-1 or -2 activates latent MMP-2; and stromelysin-1 degrades proteoglycans and glycoproteins, and also activates latent collagenases. Stellate cells are a key source of MMP-2 (Arthur et al., 1992; Milani et al., 1994) as well as increases in the specific MMP inhibitor molecules, TIMP-1 (Murawaki et al., 1997; Iredale et al., 1998) and TIMP-2, (Herbst et al., 1997) leading to a net decrease in protease activity, and therefore, more unopposed matrix accumulation. Moreover, an emerging role for TIMPs in regulating apoptosis suggests that their influence on liver homeostasis extends beyond that of direct effects on ECM turnover.

2.2.2.1.2.6 Retinoid Loss

As stellate cells activate, they lose their characteristic perinuclear retinoid (vitamin A) droplets and acquire a more fibroblastic appearance. In culture, retinoid is stored as retinyl esters, whereas as stellate cells activate, the retinoid released outside the cell is retinol, suggesting that there is intracellular hydrolysis of esters prior to export

(Friedman, 1996). However, it is generally unknown whether retinoid loss is required for stellate cells to activate and which retinoids may accelerate or prevent activation *in vivo*. Recently, peroxisome proliferator-activated receptors (PPAR), in particular PPAR γ , have also been identified in stellate cells, and their expression increases with activation (Miyahara et al., 2000; Marra et al., 2000). Ligands for this newly identified nuclear receptor family downregulate stellate cell activation (Marra et al., 2000).

2.2.2.1.2.7 WBC Chemoattractant and Cytokine Release

Increased production and/or activity of cytokines may be critical for both autocrine and paracrine perpetuation of stellate cell activation. Direct effects on stellate cell matrix production and contractility have been attributed to autocrine TGF- β and ET-1, respectively. Stellate cells can amplify the inflammatory response by inducing infiltration of mono- and polymorphonuclear leukocytes.

2.2.2.2 Necrosis

Hepatic injury, whether subclinical or overt, indicates that there is a perturbation of normal liver homeostasis, with the extracellular release of either free radicals (i.e. 'oxidant stress'), intracellular constituents, and/or cytokines and signaling molecules. Sources of these mediators may be circulating (i.e. endocrine), transferred between cells (i.e. paracrine) or act within the same cell (i.e. autocrine). In particular, oxidant-stress-mediated necrosis leading to stellate cell activation may underlie various liver diseases, including hemochromatosis, alcoholic liver disease, viral hepatitis and nonalcoholic steatohepatitis (NASH) (Tsukamoto, 2002; Bataller et al., 2003(b); Tuma, 2002). Liver injury is typically associated with the infiltration of inflammatory cells, but even in their absence the liver contains sufficient resident macrophages (KCs) and natural killer cells (pit cells) to initiate local inflammation before the arrival of extrahepatic cells.

2.2.2.3 Apoptosis

Although necrosis is considered a classical inflammatory and fibrogenic stimulus, recent findings also implicate apoptosis, or programmed cell death, in the fibrogenic response. Apoptotic fragments released from hepatocytes are fibrogenic for cultured stellate cells (Canbay et al., 2003), and FAS((TNF receptor superfamily, member 6)) -

mediated hepatocyte apoptosis in vivo in experimental animals is also fibrogenic (Canbay et al., 2002).

2.2.2.4 Lymphocytes

Remarkably, little attention has focused on the contribution of different lymphocyte subsets to hepatic fibrogenesis even though lymphocyte infiltration is a major feature of many forms of chronic liver disease. Interest has increased recently, in part because of the observation that patients infected with hepatitis C virus (HCV) who are co-infected with Human immunodeficiency virus (HIV), as well as those who are immunosuppressed after liver transplantation, have accelerated fibrosis rates, which implicates the immune system as a determinant of fibrogenesis. These observations are supported by animal studies demonstrating that the immune phenotype regulates fibrogenesis by means other than simply increasing the extent of hepatocyte injury (Shi et al., 1997). These findings led in turn to efforts to map the genetic loci accounting for these differences (Hillebrandt et al., 2002). Most recently, CD8⁺ lymphocytes have emerged as potential profibrogenic cells based on their ability to induce early fibrogenesis after adoptive transfer from animals with liver injury to naive SCID mice (Safadi et al., 2004).

2.2.2.5 Soluble Growth Factors

The range of soluble growth factors regulating stellate cell activation continues to broaden, although the key proliferative, fibrogenic, and contractile stimuli that were identified previously (Friedman, 2000) (including PDGF, TGF- β , and ET-1, respectively) remain the dominant stimuli in current models of hepatic fibrosis. In addition, CCN2 (previously known as connective tissue growth factor, or CTGF), is a downstream target of TGF- β 1 that has been recognized as an additional fibrogenic signal (Rachfal and Brigstock, 2003). Pathways regulating the synthesis, secretion and activation of cytokines during fibrosis are well characterized, and offer many potential therapeutic targets. In addition, the proteolytic release by activated stellate cells of bound growth factors from ECM reservoirs may regulate local fibrogenic activity within the pericellular milieu (Schuppan et al., 2001).

2.2.3 Disease-Specific Mechanisms Regulating Hepatic Fibrosis

In addition to generic mechanisms of fibrogenesis that are common to all experimental and human liver disease, there has been progress in elucidating disease-specific mechanisms, in particular in hepatitis C and NASH.

2.2.3.1 Hepatitis C

A recent study has raised the possibility that stellate cells could be infected by HCV, by identifying the expression of putative HCV receptors in activated stellate cells, including CD80, Low-density lipoprotein (LDL) receptor and Clq. Moreover, adenoviral transduction of HCV nonstructural and core proteins induces stellate cell proliferation and the release of inflammatory signals (Bataller et al., 2004). In HCV infected liver, chemokines and their receptors are upregulated, stimulating lymphocyte recruitment (Bonacchi et al., 2003). HCV proteins could also interact directly with sinusoidal endothelium (Pohlmann et al., 2003).

2.2.3.2 Non-Alcoholic Steatohepatitis

The rising prevalence of obesity in the US and Western Europe is associated with an alarming increase in NASH (Angulo, 2002) leading to advanced fibrosis and cirrhosis. Leptin, a circulating adipogenic hormone whose serum levels are proportionate to adipose mass in circulating blood, has been clearly tied to stellate cell fibrogenesis (Ikejima et al., 2002; Leclercq et al., 2002; Saxena et al., 2003). Sources are likely to be both endocrine and autocrine, and its activity is amplified because of enhanced signaling through the leptin receptor, the expression of which is upregulated during stellate cell activation (Ikejima et al., 2002). Concurrently, downregulation of adiponectin in obesity, a counter-regulatory hormone, might amplify the fibrogenic activity of leptin. This possibility is supported by findings in mice lacking adiponectin, which have enhanced fibrosis after toxic liver injury (Kamada et al., 2003).

2.2.4 Transcription Factors and Signaling Pathways

There have been too many advances in dissecting the pathways of membrane and intracellular signaling and transcriptional gene regulation in activated HSCs to detail here (Mann and Smart, 2002). A growing number of transcription factors could regulate stellate cell behavior, including PPAR- β (Hellemans et al., 2003; Marra et al.,

2000; Miyahara et al., 2000) and retinoid receptors (Hellemans et al., 2004), nuclear factor B (NFB) (Paik et al., 2003), Jun D (Mann and Smart, 2002), Kruppel like factor 6, Foxf1 (Kalinichenko et al., 2003), among others. Similarly, both general and cell-type-specific membrane receptors and signaling pathways have also been thoroughly characterized, including Receptor Tyrosine Kinases (Pinzani and Marra, 2001), chemokine receptors (Marra, 2002), and Integrins (Yang et al., 2003(a)).

2.2.5 Different Types of Liver Fibrosis

To date, therapies that interfere with the underlying cause of the disease represent the best pharmacological treatment options for liver fibrosis. The following paragraphs will therefore give a brief outline of the generally applied pharmacological interventions in patients with this disease. Pharmacological treatment strategies are discussed in relation to the different injurious events leading to liver scarring.

2.2.5.1 Hepatitis B Virus-Induced Fibrosis

Viral hepatitis is an important cause of chronic liver disease and fibrosis. World wide, approximately 350 million people are chronically infected with hepatitis B virus (HBV), and it is estimated that approximately 25% of these patients will develop liver cirrhosis. The virus infects hepatocytes by binding to certain cell surface receptors, subsequently followed by uncoating of the virus particle and translocation of the HBV genome to the nucleus. Viral deoxyribonucleic acid (DNA) resides in hepatocytes very persistently and its clearance appears to be dependent on the clearance of infected hepatocytes by CD8⁺ T-lymphocytes. This immune response is mainly responsible for the liver injury that follows from HBV infection, although also direct cytopathic effects of HBV have been described in patients with very high viral loads (Pinzani et al., 2005; Colombo and Sangiovanni, 2003; Fung and Lok, 2005; Lok, 2000).

Until recently, only (pegylated) IFN- α was available for the treatment of patients with chronic hepatitis B infection. IFN- α is an immunomodulating drug that improves the cytotoxic T-lymphocyte response against infected hepatocytes. Unfortunately, its use is contra-indicated in patients with clinical cirrhosis. Because IFN- α acts by increasing the clearance of HBV infected hepatocytes, in cirrhotic patients with decompensated liver function, this can result in the loss of a relative large fraction of

the remaining functional liver parenchyma and thus in an acute deterioration of liver function. Moreover, an increased incidence of bacterial infections has been associated with the use of IFN- α in cirrhotic patients, which is related to myelosuppressive effects of the drug (Fung and Lok, 2005; Lok, 2000).

However, with the advent of the HBV DNA polymerase inhibitors lamivudine and adefovir, also the treatment of HBV-infected patients with clinical cirrhosis has become an option. In contrast to IFN- α , lamivudine and adefovir have an excellent tolerability and side effect profile. Lamivudine is metabolized intracellularly to its active triphosphate, which acts as a nucleoside analog that inhibits HBV DNA polymerase and causes DNA chain termination. Adefovir is a nucleotide analog to adenosine monophosphate and acts via a similar mechanism (Hardman et al., 2001). Long term monotherapy with either drug has been shown to exert beneficial effects on fibrosis and inflammation. In HBV-infected patients with cirrhosis and decompensated liver function, treatment with lamivudine extended the time to liver transplantation (Hadziyannis et al., 2003; Marcellin et al., 2003; Dienstag et al., 2003; Lau et al., 2005; Lai et al., 1998).

For all anti-HBV drugs it is necessary to maintain viral suppression for long periods of time, because these drugs only inhibit active viral replication and leave the intracellular reservoir of viral DNA in hepatocytes largely intact. Indeed, relapse occurs in a large percentage of treated patients after cessation of therapy. Furthermore, development of lamivudine-resistant mutants presents a serious problem. Resistance occurs in 28% of treated patients after 1 year of treatment and increases to 68% after 4 years (Leung et al., 2001; Liaw et al., 2000). Interestingly, no viral resistance has been reported in response to adefovir therapy yet, and therefore adefovir is usually added to lamivudine monotherapy at the first signs of development of drug-resistant mutants (Fung and Lok, 2005). New anti-HBV drugs, such as entecavir, clevudine and emtricitabine, are expected to find their way to the clinic soon and it is very well possible that a combination therapy with mechanistically different acting drugs can improve the response rate to therapy in the near future (Hardman et al., 2001).

2.2.5.2 Hepatitis C Virus-Induced Fibrosis

Worldwide, approximately 150 million people are infected with HCV. After HCV infection approximately 85% of patients will develop a chronic hepatitis. Similar to HBV, HCV itself is not directly cytotoxic to hepatocytes, but by continuous stimulation of the immune system, cytotoxic T lymphocyte-mediated clearance of infected hepatocytes forms the main mechanism of hepatic injury. After the initial infection, it may take up to 20 years for these patients to develop advanced fibrosis/cirrhosis and approximately 25-30 years for the development of hepatocellular carcinoma (Boyer and Marcellin, 2000). This long asymptomatic period of time often prevents an early onset of therapy.

The number of drugs available for the pharmacological treatment of chronic HCV infection is even smaller than that for HBV-infected patients. Combination therapy of pegylated IFN- α with ribavirin has been proven to be effective in suppressing HCV activity (Torriani et al., 2004). Similar to HBV-infected patients, the treatment aims at reducing viral replication to induce seroconversion and reduce the risk of hepatocellular carcinoma and progression of fibrosis and cirrhosis into end-stage liver disease. Ribavirin is a purine nucleoside analog that inhibits the replication of a wide variety of ribonucleic acid (RNA) and DNA viruses (Hardman et al., 2001). Dose-limiting side effects of the drug are anemia and leucopenia, whereas IFN- α therapy is associated with depression, flu-like symptoms, fever and myelosuppression. Especially in cirrhotic patients a dose reduction is required to avoid drug related morbidity, which generally results in low sustained viral response rates in those patients. Although adjuvant therapy with erythropoietin and granulocyte colony stimulating factor has been proposed in order to reduce anemia and leucopenia in cirrhotic patients, limited clinical evidence for its efficacy is available and treatment of patients with advanced HCV-induced cirrhosis therefore remains very difficult (Boyer and Marcellin, 2000; Everson, 2005).

The overall response rate to IFN- α /ribavirin in HCV-infected patients is approximately 50%, and as pointed out in the above paragraphs, for nonresponders there is no alternative treatment available. Particularly difficult to treat are the HCV/HIV co-infected and patients with an HCV re-infection of the liver after liver

transplantation. These patients often develop fulminant fibrosis, which progresses to end stage liver disease within 5 years (Pinzani et al., 2005).

2.2.5.3 Liver Fibrosis Due to Genetically Inheritable Factors

Wilson's disease and hemochromatosis are genetically inheritable disorders that can lead to liver fibrosis. Wilson's disease, or copper overload disease, is a result of a mutation in the ATP7B gene which codes for a protein that is essential for copper excretion into the bile. The resulting decrease in copper excretion into the bile leads to its accumulation in the body, which is primarily deposited within the liver, and in a later stage of the disease, also in the brain (Brewer and Askari, 2005). Hemochromatosis, or iron overload disease, is the result of an increased iron uptake from the small intestine due to a mutation in the HFE (High Iron Fe) gene. It is believed that the increased levels of metal ions in hepatocytes induce necrosis via facilitation of the generation of reactive oxygen intermediates within hepatocytes, which leads to hepatocellular injury and, ultimately, hepatic fibrosis (Pietrangelo, 2004; Bacon et al., 2003).

The treatment of cirrhotic patients with Wilson's disease is mainly based on procedures aimed at the reduction of tissue copper levels. A well known anticopper agent is penicillamine, which acts as a reductive chelator that mobilizes intracellular copper deposits in the liver and brain, and facilitates its urinary excretion. However, the use of penicillamine has become obsolete with the arrival of much safer alternatives. One option is treatment with zinc, which inhibits the copper uptake from the small intestine via the induction of metallothionein in the intestinal wall. Another possibility is treatment with tetrathiomolybdate, which complexes copper in the intestine and in the circulation, thus preventing cellular uptake and favoring its urinary excretion either via faeces or urine. Treatment of patients with decompensated cirrhosis with these drugs has been shown to improve liver function, often successfully postponing a liver transplantation (Brewer and Askari, 2005). In analogy to the treatment strategy in Wilson's disease, pharmacological intervention in hemochromatosis also aims at reducing the amount of metal ions in the body. Currently, medical phlebotomy is considered a safe and effective treatment for hemochromatosis (Pietrangelo, 2004).

2.2.5.4 Autoimmune-Mediated Liver Disease

Autoimmune-mediated liver disease can be divided into autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Whereas AIH and PBC mostly occur in women, 70% of the patients with PSC are men. Autoimmune diseases in general are the consequence of a breakdown of tolerance to self-antigens. For instance in AIH type II, high circulating levels of anti-CYP2D6 immunoglobulins can be detected, predominantly affecting hepatocytes. In type I and III, other cellular structures are targeted by antibodies, but it is not yet exactly clear which types of immunoglobulins are responsible. In PBC and PSC the small and medium-sized intrahepatic bile ducts are attacked, whereas in PSC especially the large extrahepatic ducts are affected. In all three forms of immune-mediated liver injury liver fibrosis develops, mainly of the periportal type (Bacon et al., 2003).

Although AIH, PSC and PBC are all autoimmune diseases, only AIH responds well to immunosuppressive therapy with corticosteroids and azathioprine. Conversely, for the treatment of PBC, corticosteroid therapy is explicitly not recommended because a worsening of osteoporosis can be expected, of which a higher incidence is associated with the disease already. Especially in PBC patients that are diagnosed in an early stage of hepatic injury, treatment with ursodeoxycholic acid (UDCA) inhibits histopathological progression significantly. However, when hepatic injury has already progressed further, most studies show no beneficial effect of treatment anymore. The mechanism of action of UDCA is probably related to the reduction of toxic effects of hydrophobic bile acids by a choleric effect and/or a direct inhibitory effect on bile acid-induced apoptosis of hepatocytes (Schoemaker et al., 2004).

Even less pharmacotherapeutical options exist for PSC. UDCA appears ineffective and to date no drugs have been identified that show significant therapeutic effects in PSC, except those drugs that provide symptomatic relief of the mainly cholestasis-associated symptoms (Czaja et al., 2005; Beuers, 2005; Kaplan and Gershwin, 2005; Cullen and Chapman, 2005).

2.2.5.5 Drug-Induced Liver Disease, Alcoholic and Non-Alcoholic Steatohepatitis

Although the use of certain drugs has been associated with the development of liver fibrosis, most hepatic drug reactions are followed by recovery upon withdrawal of the

injurious stimulus, without the occurrence of significant fibrosis. Examples of drugs which have been associated with drug-induced fibrosis are methotrexate, isoniazid, and valproic acid (Bacon et al., 2003; Kaplowitz, 2000; Larrey, 2000).

Clearly, best known are the detrimental effects of chronic alcohol abuse on the liver. In fact, this drug-induced form of liver fibrosis is the most common cause of cirrhosis in the western world. Chronic exposure to alcohol leads to benign macrovesicular steatosis of the liver in over 90% of alcohol abusers, and generally steatosis will spontaneously resolve upon alcohol abstinence. Nevertheless, in 20- 40% of the cases a more severe liver pathology develops as a result of chronic oxidative stress via alcohol metabolites (Sougioultzis et al., 2005). The resulting perivenous infiltration of neutrophils into the fatty liver, typically in combination with ballooning of hepatocytes and the formation of Mallory bodies, is termed alcoholic steatohepatitis (ASH). This is very often accompanied by pericellular fibrosis. Besides alcohol-induced oxidative stress, which results in a cytokine-mediated initiation and perpetuation of the inflammatory and fibrotic process, also antibodies against the ethanol metabolite acetaldehyde, as well as auto-antibodies against alcohol dehydrogenase and CYP2E1 have been implicated in the pathophysiology (Bacon et al., 2003; Tilg and Diehl, 2000). Patients with an acute exacerbation of ASH present with decompensated liver function, encephalopathy and gastrointestinal bleeding. The one-month survival rate of these patients is only 50% and the survivors of such an exacerbation will generally develop end stage liver cirrhosis within 5 years, despite withdrawal from alcohol use (Tilg and Diehl, 2000).

NASH is considered to be the hepatic manifestation of the “metabolic syndrome”. The metabolic syndrome is characterized by obesity, insulin resistance and hyperlipidemia and can be considered a typical welfare disease which is the consequence of an unhealthy diet and limited physical activity. The hepatic pathology of NASH is in many ways similar to that of ASH and typical features are ballooning of hepatocytes and lobular inflammation leading to pericellular fibrosis. It is estimated that 10-15% of the patients with NASH will progress to advanced fibrosis and cirrhosis. In the future, NASH may become a more important cause of liver fibrosis because its prevalence is expected to parallel the increase in patients suffering from the metabolic syndrome (Bacon et al., 2003; Wright and Rockey, 2004).

Currently, weight loss or withdrawals of alcohol or drugs are the main treatments available for patients suffering from NASH, ASH and drug-induced liver fibrosis. If this does not sort any effect and fibrosis still progresses, only symptomatic treatments can be initiated to ensure that the patient survives until a liver transplantation can be performed (Sougioultzis et al., 2005; Wright and Rockey, 2004).

2.2.6 Determinants of Fibrosis Progression

Fibrosis progression can vary tremendously between different liver diseases and even among patients with the same disease. Risk factors for disease progression can be acquired or genetic and are best characterized for hepatitis C, for which acquired factors include alcohol intake, adiposity, male gender, age at onset, duration of infection, concurrent liver diseases and immunosuppression. Animal studies (described above) have identified some genetic determinants of fibrosis progression, and these have now been complemented by large-scale studies in humans, which are intended to identify gene polymorphisms that predict progression rates in both hepatitis C and NASH (Bataller et al., 2003(a)). Accurate stratification of fibrosis progression risk will be vital for the identification of those patients meriting earlier or more aggressive therapies, and to optimize the design of clinical trials, enabling smaller enrollment and shorter study intervals.

The foremost challenge is to find the optimal method of diagnosing fibrosis. Although liver biopsy combined with connective tissue stains has been a mainstay of diagnosis, it is prone to sampling error and inter-observer variability. This may account for a one-stage difference in up to a third of patients when using conventional five- or six-point scoring scales (e.g. METAVIR, ISHAK). A potentially more quantitative and feasible application of liver biopsy might include the measurement of key fibrogenic mRNAs by Real-Time polymerase chain reaction (PCR). For example, changes in the expression of fibrogenic genes, including those correlating with stellate cell activation (e.g. the gene encoding PDGFB receptor) may reveal early evidence of regression even before the matrix content has changed. Efforts to make this application a reality are currently under development.

There has been significant progress in the development of noninvasive markers, which will be essential to obtain early biomarkers of efficacy for clinical trials and to guide clinical usage. Current clinical trials of antifibrotic drugs anticipate that at least

6-12 months will be required to demonstrate efficacy by biopsy, based on observed rates of regression in patients cured of their underlying liver disease (e.g. those infected with HBV or HCV); however, lengthy trials are costly and may discourage drug development.

The ideal noninvasive fibrosis marker must be reproducible and linear, reflecting fibrosis in all types of chronic liver disease, and correlating with matrix content and clinical outcome (Albanis and Friedman, 2002). It must also be sensitive enough in individual patients to discriminate between different stages of fibrosis, and reflect the response to antifibrotic therapy over time. There are several approaches currently being developed. First is the serum measurement of one or more circulating matrix proteins and/or serum biochemistries in combination. Second are serum proteomics or glycomics, which assess patterns of protein peaks or glycoprotein branching, respectively. A recent study combined glycomics with a serum marker panel (Callewaert et al., 2004). It achieved a high level of discrimination in diagnosing compensated and decompensated cirrhosis, however, these patients are also readily diagnosed by standard clinical means. Third are imaging methods [Computed tomography (CT), Magnetic resonance imaging (MRI), Positron emission tomography (PET), elastography, radionuclide receptor scanning], which can assess either intrahepatic blood flow patterns, organ texture, or the mass of activated stellate cells using cell-specific reagents to bind membrane receptors.

Currently, several of these approaches are already successful in distinguishing patients with little or no fibrosis, and those with advanced disease. However, they are less reliable at discriminating intermediate stages over time. Nonetheless, evidence of either minimal or advanced fibrosis can be extremely important in making treatment decisions, especially in patients infected with HCV, for which current antiviral therapies are often poorly tolerated or ineffective in a substantial fraction of patients.

Given the apparent importance of fibrosis in predicting prognosis and, moreover, data indicating that it is important to stage fibrosis prior to therapy (and to judge the effect of therapy), histologic assessment of the liver has taken on a major role in the management of patients with liver disease.

While liver histology remains an important part of the clinical assessment of hepatic injury and indeed is considered the current gold standard, a number of questions have

arisen as to the accuracy of liver biopsy in correctly staging disease. Thus, in recent years it has been recognized that noninvasive markers of hepatic fibrogenesis may be appropriate as alternatives to liver biopsy (NIH Consensus Statement on Management of Hepatitis C, 2002; ANAES, 2002). Although the concept of using radiological, clinical, and laboratory parameters of inflammation and fibrosis as alternatives to liver biopsy has been around for some time, the importance of accurate staging of fibrosis for guiding antiviral therapy and following disease progression in patients with chronic hepatitis C (and other types of chronic liver disease) has resulted in the development of newer algorithms employing noninvasive markers. A number of biochemical marker panels have been developed by cross-sectional study design and validated mostly in patients with chronic hepatitis C. Given the common downstream pathways of hepatic fibrosis, some of these panels also appear useful for disease staging in non-virally mediated hepatic injury. Promising newer approaches being developed for assessment of fibrosis in chronic hepatitis C patients include the use of transient elastography as well as emerging technologies such as proteomics and metabolomics.

2.2.6.1 Liver Biopsy

Liver biopsy provides useful information to the clinician for determining prognosis and the urgency of therapy, predicting response to treatment, and investigating the etiology of liver injury, as well as for providing a baseline to allow comparisons of future histologic outcomes (Table 2.1) (Bravo et al., 2001).

Table 2.1 Typical Advantages and Limitations of Liver Biopsy in Comparison to Noninvasive Biomarkers

	Liver Biopsy	Biomarkers
Diagnosis	Fibrosis stage, necro-inflammation and activity, steatosis, iron content, hepatotoxicity, and other chronic liver diseases	Fibrosis stage, activity
Prognosis and guide to therapy	Established role	Exclusion of significant disease in chronic viral hepatitis. To be determined in other liver disease
Posttransplant	Established role	None at this time

False results	Subcapsular biopsy, nonliver sampling	Common causes include acute hepatitis or other inflammation, hemolysis, cholestasis
Assessment	Semiquantitative and qualitative	Semiquantitative or quantitative
Adverse events	Mortality 3/10,000 Morbidity 3/1,000 Localized pain 3/10	None
Sampling error	33% for fibrosis stage	None known
Observer error	20% for fibrosis stage 40% for activity (METAVIR)	None
Sample requirements	Interpretation limited by quality of biopsy size and staining	Standardized assays and analyzers
Hospital stay	Usually 4–6 hours	None
Contraindications	Inability to cooperate Severe comorbid disease Coagulopathy Obesity	None
Cost	Very expensive (uncomplicated biopsy)	Inexpensive to moderately expensive

However, percutaneous liver biopsy is an invasive procedure and may be associated with significant complications in 3% of recipients, with a mortality rate of 0.03% (Perrault et al., 1978; Perrillo, 1997; Poynard et al., 2000). A study evaluating complication rates from 2,084 liver biopsies performed in France noted severe complications in 0.57% of patients and no deaths (Cadranel et al., 2000). Risk factors such as age and cirrhosis increase the likelihood of adverse events from liver biopsy. Furthermore, liver biopsy is costly: the direct costs of an uncomplicated liver biopsy in the United States are estimated at \$1,500–\$2,000, and do not account for lost productivity and time off work (Wong et al., 2000). For these reasons many patients are reluctant to undergo liver biopsies, further limiting the ability to assess and follow disease progression or determine efficacy of treatment.

An additional concern with liver biopsy is that it samples only 1/50,000 of the liver and thus is subject to sampling error, particularly in non-homogenously distributed chronic liver disease. Several studies have highlighted the inaccuracy of liver biopsy for staging of advanced liver disease. Single biopsies may misclassify cirrhosis in 10–30% of cases (Pagliaro et al., 1983; Poniachik et al., 1996). The number of biopsies performed also appears important. One study that obtained liver biopsies immediately

prior to autopsy demonstrated that the diagnostic accuracy of cirrhosis increased from 80% for a single biopsy to 100% with three samples (Abdi et al., 1979). Additionally, in patients with cirrhosis, even when three consecutive biopsies obtained through a single entry site were performed, the rate of concurrent findings about the histologic presence of cirrhosis was only 50% (Maharaj et al., 1986). Varying the site of biopsy also appears to result in significant discordance.

A recent study evaluated laparoscopic biopsies obtained from the right and left lobes in 124 patients with chronic hepatitis C. There was a discordance of at least one stage in one third of patients, and in 14% of cases cirrhosis was present in one lobe, and a diagnosis of bridging fibrosis only in the other lobe (Ragev et al., 2002). Most liver biopsies in clinical practice, however, are not obtained using laparoscopy. Further, the type and size of needle used may also be important. Compared to the Menghini needle, cutting-type instruments may increase the likelihood of making a correct diagnosis of cirrhosis (Vargas-Tank et al., 1985; Colombo et al., 1988).

Another important limitation of liver biopsy is inter- and intraobserver variation among pathologists (The French METAVIR Cooperative Study Group, 1994; Westin et al., 1999; Gronbaek et al., 2002). In chronic hepatitis C, the use of standardized grading systems such as Knodell, METAVIR, or Ishak (among others) results in good concordance rates between pathologists about fibrosis (there is concordance in 70–80% of samples), but there is generally less agreement about inflammation scores (Poynard et al., 2004(b)). Specimen size appears to be very important for the pathologist, with smaller samples leading to an underestimation of disease severity (Colloredo et al., 2003). A study from France that created digitized virtual image biopsy specimens of varying lengths from large picrosirius-stained liver tissue sections noted that 75% of 25-mm biopsy specimens were correctly classified using the METAVIR system, compared to only 65% of biopsies 15 mm in length (Bedossa et al., 2003). In clinical practice most needle biopsies are likely to be less than 25 mm; however, a recent study noted that the experience of the pathologist may have more influence than specimen length on interobserver agreement (Rousselet et al., 2005).

Given the limitations of liver biopsy, alternative methods for quantifying liver tissue in histologic specimens have been utilized with variable success. Computer-aided image analysis can provide an objective measurement of the proportion of liver with

fibrous tissue (O'Brien et al., 2000). However, the coefficient of variation for image analysis remains unacceptably high at approximately 45%, even for 25-mm biopsies, precluding its routine clinical use for assessing fibrosis (Bedossa et al., 2003). Nonetheless, there may be a potential role for image analysis in evaluating paired biopsies, or as a research tool in the development of noninvasive markers when quantitation of total matrix deposition is required. By comparison, histologic staging takes into account additional, subjective factors such as architectural distortion or nodule formation, although such morphologic assessment is clearly dependent upon the inherent bias of the individual pathologist.

Immunohistochemical analysis of ECM components on liver biopsy may provide useful information regarding disease progression (Afdhal and Nunes, 2004). For example, matrix glycoproteins such as tenascin are deposited early in the fibrogenesis cascade into relatively immature matrix tissue that has the potential for reversibility (Koukoulis et al., 1999). Conversely, vitronectin is a marker of mature fibrous tissue that is unlikely to have significant potential for reversal (Koukoulis et al., 2001). Alternatively, assessment of stellate cell activation may be an attractive approach to evaluating fibrogenesis. Both assessment of ECM and stellate cell activation could potentially be correlated with serologic markers, thus providing a reflection of the dynamic state of fibrogenesis, as opposed to the standard static histologic measurements of disease stage. ECM and stellate cell activation could be useful in assessing treatment response and monitoring the disease process.

Limitations in liver biopsy have important implications for the development and validation of newer, noninvasive measures of fibrosis. Both the quality of the biopsy and the skill of the pathologist have to be taken into account. Furthermore, the semiquantitative grading systems developed for histopathologic analysis do not reflect linearity of fibrosis deposition or actual matrix content. For example, the Ishak system stages fibrosis on an ordinal scale where scores of 1 and 2 indicate portal fibrosis, 3 and 4 bridging fibrosis, and 5 and 6 incomplete and established cirrhosis, respectively (Ishak et al., 1995). Likewise, the METAVIR classification scores fibrosis on a 5-point scale from F0 to F4, with F1 and F2 indicating portal fibrosis with and without portal septae, respectively, F3 bridging fibrosis, and F4 cirrhosis (Bedossa and Poynard, 1996). However, stage F2 does not imply twice as much fibrous tissue as F1.

These noncontinuous scales were developed to standardize and improve observer variability, and provide some assessment of the severity of chronic liver injury that could be used to determine thresholds for therapy in chronic hepatitis C in particular. Although certain ECM-specific serum markers are intuitively expected to reflect matrix deposition, their main limitation is that they are correlated with a semiquantitative morphologic assessment of fibrosis that is itself imperfect. Despite the obvious drawbacks of validating Noninvasive markers against a liver biopsy for staging of fibrosis, alternative measures of fibrosis that achieve predictive area under receiver operating characteristics (AUROC) curve values above 0.85 should be acceptable as being equivalent to a liver biopsy (Afdhal and Nunes, 2004).

2.2.6.1.1 Grading and Staging of Liver Fibrosis

There are a variety of ways to interpret a liver biopsy. The most common scoring methods include Metavir and histologic activity index (HAI) also called the Knodell. It is important to remember that the length of the liver specimen and the knowledge of the professional reading the biopsy can influence the interpretation of the report.

2.2.6.1.1.1 METAVIR

The Metavir scoring system was specially designed for patients with hepatitis C. The scoring consists of using a grading and a staging system. The grade gives an indication of the activity or amount of inflammation and the stage represents the amount of fibrosis or scarring (Brunt, 2000).

The grade is assigned a number based on the degree of inflammation, which is usually scored from 0-4 with 0 being no activity and 3 or 4 considered severe activity. The amount of inflammation is important because it is considered a precursor to fibrosis.

The fibrosis score is also assigned a number from 0-4:

- 0 = no scarring
- 1 = minimal scarring
- 2 = scarring has occurred and extends outside the areas in the liver that contains blood vessels
- 3=bridging fibrosis is spreading and connecting to other areas that contain fibrosis
- 4=cirrhosis or advanced scarring of the liver

2.2.6.1.1.1 KNODELL

The Knodell score or HAI is also commonly used to stage liver disease. It is a somewhat more complex process, but some experts believe that it is a better tool for defining the extent of liver inflammation and damage (Brunt, 2000). It is composed of four individually assigned numbers that make up a single score. The first component (periportal and/or bridging necrosis) is scored 0-10. The next two components (intralobular degeneration and portal inflammation) are scored 0-4. The combination of these three markers indicates the amount of inflammation in the liver:

- 0 = no inflammation
- 1-4 = minimal inflammation
- 5-8 = mild inflammation
- 9-12 = moderate inflammation
- 13-18 = marked inflammation

2.2.6.2 Non-Invasive Biochemical Markers of Liver Fibrosis

Laboratory markers of liver fibrosis should be the ideal diagnostic tool to assess the grade of fibrosis. They are supposed to provide accurate and reliable results in a simple, fast and cost-effective manner. Indeed, much effort has been dedicated in the past years to investigate non-invasive markers, which discriminate between low fibrotic stages such as Metavir <F2 and higher fibrotic stages F2–F4, in order to identify patients who are at risk of clinically relevant fibrosis progression. However a panel of routine laboratory markers, which are widely used in clinical practice and novel direct and indirect biochemical markers of hepatic fibrosis have been evaluated, but none of the tests met the expectations, and their superiority to standard clinical evaluation is still questionable.

2.2.6.2.1 Direct Serological Markers of Liver Fibrosis

These markers are supposed to be directly involved in the deposition and removal of ECM, i.e. in fibrogenesis and fibrolysis. They include markers of matrix metabolism as well as cytokines. Fibrosis markers can be classified according to their molecular structure (Table 2.2).

Table 2.2 Fibrosis markers

Type	Name
Collagens	Procollagen I C peptide (PICP)
	Procollagen III N peptide (PIIINP)
	Type IV collagen and its fragments (NC1 and PIVNP)
Glycoproteins and polysaccharides	Hyaluronic acid (HA)
	Laminin
	Tenascin
	YKL-40
Collagenases and their inhibitors	Metalloproteinases (MMPs)
	Tissue inhibitors of metalloproteinases (TIMPs)
Cytokines	TGF- β 1
	PDGF

It is very difficult to make a clear delimitation between markers of ECM deposition and degradation. Serum levels of direct markers reflect simultaneously both processes as well as the total mass of ECM undergoing remodeling (Afdhal and Nunes, 2004).

There are strong arguments for this supposition:

- the levels of direct markers are elevated in disease with rapidly progressing fibrosis severe alcoholic hepatitis or active hepatitis;
- the levels of these markers have a decreasing tendency in response to treatment of the disease, before reduction in the stage of fibrosis;
- there is an independent correlation between serum direct markers and the stage of fibrosis in chronic liver diseases (Ramadori et al., 1991; Pares et al., 1996; Camps et al., 1994; Guechot et al., 1995(a); McHutchison et al., 2000).

Also, there is a good correlation between different direct fibrosis markers, suggesting that they investigate a similar process.

The proposal to assess simultaneously markers of matrix deposition and degradation by using a different combination of these markers in an attempt to evaluate the whole process of matrix remodelling has added little diagnostic accuracy (Afdhal and Nunes, 2004).

The assessment of direct markers could be useful for:

- staging liver disease and for
- assessing the effect of treatment and predicting disease progression (Afdhal and Nunes, 2004).

2.2.6.2.1.1 Direct Individual Markers in Staging Liver Disease**2.2.6.2.1.1.1 Markers Associated With Matrix Deposition**

Several studies have investigated the value of procollagen peptides. During synthesis of collagen, procollagen suffers an enzymatic cleavage at both the carboxy - and aminoterminal ends by two different enzymes: procollagen-C-proteinase and procollagen-N-proteinase. The peptides released into the serum: procollagen type I carboxy - terminal peptide and procollagen type III amino-terminal peptide can be used as a measure of matrix deposition (Schuppan et al., 1991; Schuppan et al., 1995).

Procollagen type I carboxy terminal peptide (PICP)

PICP has little value in the diagnosis of chronic hepatitis and is elevated in cirrhosis, quantifying disease severity or indicating the alcoholic etiology (Fabris et al., 1997).

Procollagen type III amino-terminal peptide (PIIINP)

Serum levels of P III NP were extensively studied alone or in combination with different other markers and the results showed the correlation between their levels and histological stage of hepatic fibrosis in alcoholic liver disease, viral hepatitis and primary biliary cirrhosis (Schuppan et al., 1995; Fabris et al., 1997; Bentsen et al., 1987; Babbs et al., 1988).

When refining the methods of assessment by using two assays methods of PIIINP (for col 1-3 : collagen synthesis and for col 1: collagen degradation), some authors found a significant correlation between serum PIIINP (col 1-3 and col-1) and histological changes: fibrosis, periportal necrosis and histological activity index (Walsh et al., 1999(a)).

Serum type IV collagen

Type IV collagen is an important component of ECM. Unlike type I and III collagens, which are processed by proteolysis, type IV collagen is deposited intact in the matrix and the serum component of type IV collagen reflects matrixdegradation (Murawaki et al., 1995). The assay of fragments of type IV collagen in serum (carboxyterminal cross-linking domain - NC1 and aminoterminal 7S domain of procollagen type IV – PIVNP) are used most frequently in practice (Shahin et al., 1992; Hirayama et al., 1996; Hayasaka et al., 1990). Irrespective of the methods used for determination,

serum levels of type IV collagen had a positive correlation with the degree of hepatic fibrosis in patients with chronic viral hepatitis, alcoholic liver disease and were sensitive indicators of the presence of cirrhosis in haemochromatosis (Fontana and Lok, 2002; Afdhal and Nunes, 2004).

In hepatitis C, a cut-off value was established for diagnosing stages greater than F2 (110 ng/ml) and for predicting stage F3 (130 ng/ml) (Murawaki et al., 2001(b)).

Laminin

A major non-collagenous glycoprotein synthesized by HSC, laminin is deposited in the basement membrane of the liver and increases during fibrosis around the vessels, in the perisinusoidal spaces and the portal tract.

Serum laminin levels and pepsin-resistant fragment of laminin (laminin P1) are elevated in chronic liver diseases irrespective of etiology: viral or alcoholic and reflect an increase in perisinusoidal fibrosis (Kropf et al., 1988; Walsh et al., 2000).

Some studies suggest that the serum levels of laminin correlate with the severity of fibrosis and liver inflammation in chronic hepatitis C, and are superior to serum ALT in reflecting liver injury (Walsh et al., 2000), particularly in cirrhosis (Afdhal and Nunes, 2004). Also, these studies showed a good correlation of serum laminin with Child-Pugh's score, complications of liver cirrhosis, portal pressure and hepatic vein portal gradient (Korner et al., 1996).

Hyaluronic acid

Hyaluronic acid (HA) is a glycosaminoglycan, component of the ECM, synthesized by HSC. In normal circumstances the endothelial cells of the liver sinusoid are the site of HA uptake and degradation (Eriksson et al., 1983). Increased levels of HA are due to decreased hepatic removal, increased production or both.

High levels of serum HA have been detected in patients with liver diseases of different etiologies and particularly in those with cirrhosis (Engstrom-Laurent et al., 1985; Guechot et al., 1996(b)).

Serum levels of HA have been shown to be related not only to the stage of fibrosis (Guechot et al., 1996(b)) but also to the degree of necroinflammation (Murawaki et al., 1995).

The assessment of both laminin and HA concentration has a good prognostic value for complications of liver cirrhosis: hepatic encephalopathy stage III and IV, refractory ascites, portal vein thrombosis (Korner et al., 1996).

Serum HA at a level of < 60 mg/l excludes vein significant fibrosis or cirrhosis with a positive predictive value (PPV) of 93% and 99% respectively and has an important role in identification of early fibrosis, thus reducing the need for biopsy in this subgroup of patients (McHutchison et al., 2000).

At the cut-off value of 85 mg/l serum HA had a sensibility of 64.5% and specificity of 91.2% for discriminating patients with extensive liver fibrosis from those with no or mild fibrosis. At the cut-off value of 110mg/l the sensitivity was 79.2% and specificity 89.4% for discriminating patients with from those without liver cirrhosis (Guechot et al., 1996).

It appears that as an isolated marker, HA is the most useful diagnostic tool for both staging and grading in patients with chronic C virus infection (Murawaki et al., 1995).

YKL-40 (human cartilage glycoprotein)

YKL-40 is a mammalian member of a chitinase family (18- glycosylhydrolases). YKL-40 is produced in a wide variety of cell types and especially in cells located in tissues with increased remodelling/degradation or inflammation of the ECM. The cellular source in the liver is supposed to be activated HSC (Johansen et al., 2000). Its physiological function is unknown, but YKL-40 may contribute to tissue remodelling, acts as a growth factor for fibroblasts, acts synergistically with insulin-like growth factor, as a chemoattractant for endothelial cells and has a role in angiogenesis (Johansen et al., 2000; Malinda et al., 1999). In liver diseases, serum levels of YKL-40 were closely related to the degree of fibrosis histologically documented, the highest values being found in severe fibrosis (Johansen et al., 2000).

In chronic HCV infection, serum levels greater than 284.8 ng/ml predict cirrhosis with a sensitivity of 80% and specificity of 71% and have a negative predictive value (NPV) of 78% (Saitou et al., 2005). Unlike PIIINP and HA, serum YKL-40 is significantly elevated in the subset of alcoholic cirrhotic patients who have also alcoholic hepatitis and is the best of these serological markers in discriminating

between patients with mild fibrosis and those with no fibrosis (Johansen et al., 2000; Tran et al., 2000).

2.2.6.2.1.1.2 Markers Associated With Matrix Degradation

Products of matrix degradation result from the activity of a family of enzymes: matrix metalloproteinases (MMPs).

Synthesized intracellularly and secreted as pro-enzymes, MMPs are activated by a proteolytic cleavage by membranetype matrix metalloproteinase 1 (MT1-MMP) or plasmin and inhibited by binding to specific tissue inhibitors of metalloproteinase (TIMPs).

Considering their substrate specificity there are five categories of MMPs: interstitial collagenases (MMP-1, -8, -13), gellatinases (MMP-2, - 9 and fibroblast activation protein), stromelysins (MMP-3, -7, -10, -11), membrane type (MMP-14, - 15, - 16, - 17, -24, - 25) and metalloelastase (MMP-12) (Arthur, 1998; Friedman, 1999; Aparicio and Lehy, 1999). The MMPs and their inhibitors are involved in the control of matrix degradation (Friedman, 1999).

In chronic liver disease, the investigations have centered on MMP2 (gellatinase or 72 kDA type IV collagenase), membrane-type metalloproteinase-1 or - 2 which activate latent MMP2 and TIMP-1 and TIMP-2.

MMP-1 shows a substrate specificity for interstitial collagen type I and III, while MMP-2 has as substrate collagen type IV, V, VII, X elastin and fibronectin.

TIMPs can irreversibly bind the proenzyme or active forms of MMPs and inactivate them. Excess production of TIMPs relative to MMPs may be an important factor for progression of liver fibrosis (Iredale et al., 1992).

HSC are the principal source of MMP-2 in the human liver and activation of MMP-2 require interaction withhepatocytes. TIMP-1 is produced by HSC and hepatocytes (Arthur, 1998; Friedman, 1999; Iredale et al., 1992).

Regarding the diagnostic value of MMP-2 and TIMP-1, one study reported that MMP-2 levels were elevated only when cirrhosis had developed, while TIMP-1 had a diagnostic value in detecting earlier stage of fibrosis (Boeker et al., 2002). Also, this study revealed that TIMP-1 levels had a strong correlation with histological

inflammatory scores and that MMP-2 levels had no relationship to the stage of fibrosis in the noncirrhotic liver.

Other studies established that serum levels of MMP-1 had a declining tendency with the severity of liver fibrosis and inflammation and abnormal serum MMP-1 did not appear until the patients were in the advanced stages of fibrosis (Zhang et al., 2003(a)).

However, older studies which investigated the role of TIMP-1 in patients with various liver disease, comparing TIMP-1 with PIIINP, type IV collagen, laminin P1 and the histological aspect, suggested that the serum levels of TIMP1 may be useful to estimate hepatic fibrogenesis associated with active inflammatory activity (Ueno et al., 1996).

Also, the serum levels of TIMP-1 were shown to correlate positively with the degree of fibrosis and a striking increase in serum TIMP-1 levels was observed in the late stage of fibrosis, but not in the mild stage (Walsh et al., 1999(b)).

The ratio of TMP-1/MMP-1 could be useful in the diagnosis of hepatic fibrosis (Zhang et al., 2003(a)).

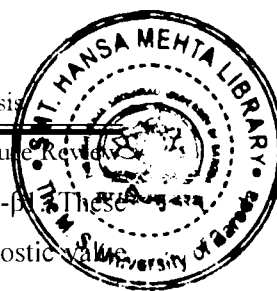
2.2.6.2.1.1.2 Cytokines and Chemokines Associated With Hepatic Fibrosis

TGF- β 1

Transforming growth factor- β 1 (TGF- β 1) is a homodimeric polypeptide that is secreted in an inactive form which requires activation. It has pleiotropic effects through membrane receptors on a wide variety of cells. In hepatic pathology, TGF- β 1 is the most important stimulus for the production of ECM by HSC (Sasaki et al., 1992) and it is also an inhibitor of hepatocyte growth and proliferation (Nakamura et al., 1985).

In the liver biopsy from patients with chronic liver disease, TGF- β 1, mRNA levels correlate with type I collagen mRNA (Breitkopf et al., 2001).

The value of serum TGF- β 1 levels has some limitations related to the contamination of the sample by TGF - β from platelets, the interference with plasmin activity in the plasma that increases the amount of TGF- β 1 through opening LAP-TGF- β complex, the binding of TGF- β at the sites of injury to ECM and to vascular endothelium, the



sequestration by soluble proteins and the complicated clearance of TGF- β . These factors explain why plasma levels of TGF β 1 are unlikely to be of diagnostic value (Zhang et al., 2003(a); Breitkopf et al., 2001).

However, some studies showed a good correlation between serum levels of total TGF - β , and Knodell scores (Nelson et al., 1997) and also a correlation with the rate of fibrosis progression (Kanzler et al., 2001).

Moreover, some authors established cut-off values with prognostic significance for patients with no progression of fibrosis and those with progressive disease. A TGF- β 1 level below 75 ng/ml was predictive for stable disease (Kanzler et al., 2001).

PDGF

Platelet derived growth factor (PDGF) is the main stimulus of HSC proliferation and migration and is upregulated following liver injury. PDGF-BB is the main subunit with the most important role for the signalling pathway in HSC (Gressner, 1998; Pinzani and Marra, 2001).

The serum level of PDGF-BB was found to have the highest value for assessment of hepatic fibrosis, when compared to other eight markers (Zhang et al., 2003(a)).

2.2.6.2.1.2 Combination of Direct Markers

The combination of direct markers to generate an algorithm capable of evaluating the existence of fibrosis and its stage is an alternative approach. There are several studies which made use of this approach.

Oberti et al. (1997) studied four specific markers of fibrosis: hyaluronic acid, PIII NP, laminin and TGF-b, together with other nonspecific markers: prothrombin index, GGT, apolipoprotein-A1 (PGA score) and α 2-macroglobulin in a prospective study. The best diagnostic accuracy was found for HA (86%), followed by laminin (81%), P III NP (74%) and TGF-b1 (67%).

Taken together, the results of the Oberti's study did not show any diagnostic advantage of the specific over nonspecific markers of fibrosis.

In another investigation, the serum markers of fibrosis: C-terminal peptide of procollagen I, PIIINP, collagen IV and serum prolylhydroxylase were studied in cirrhotic and noncirrhotic patients (Fabris et al., 1997). By stepwise logistic regression

analysis and ROC curves the authors established that collagen IV and PIIINP were independently associated with cirrhosis.

One study investigated the diagnostic value of PIIINP, PIVNP, HA, MMP-1, MMP-2 and TIMP-1 in order to assess by ROC the usefulness of serum direct markers for fibrosis staging and necroinflammatory grading in chronic hepatitis C (Murawaki et al., 2001(a)). The authors concluded that HA and MMP-2 were most useful in that order for diagnosing stages greater than F2, while serum HA and PIVNP for diagnosing moderate grade. Because of the great overlap among stages and grades they did not consider that the above mentioned investigated markers can replace liver biopsy for the assessment of liver histology, but have a value for the global clinical status judgement and for prognosis.

The European Liver Fibrosis Study, conceived as an international, multicenter, cross-sectional cohort study, compared the diagnostic performance of three direct serum markers: HA, PIIINP and TIMP-1 with liver biopsy to generate a diagnostic algorithm for estimating the severity of liver fibrosis. By adopting as thresholds sensitivity greater than 90% and specificity greater than 90%, this algorithm was found to exclude significant fibrosis. Also, this algorithm can detect cirrhosis with a sensitivity greater than 90% (Rosenberg et al., 2004).

Recently, another study investigated the diagnostic value of a combination of three markers: HA, TIMP-1 and α 2-macroglobulin, with the aim of generating an algorithm able to discriminate between significant and non-significant fibrosis. Establishing cut-off values for these markers, they may reliably differentiate chronic hepatitis C patients with moderate / severe fibrosis (F2 to F4 Metavir) from those with no or mild fibrosis (F0 to F1 Metavir) (Patel et al., 2004).

In an attempt to find a better combination of markers, serum levels of PDGF-BB, TGF- β 1, MMP-1, TIMP-1, HA, PC III, collagen IV, laminin and mRNA-TIMP-1 and mRNA-MMP-1 in peripheral blood mononuclear cells (PBMCs) were investigated in patients with chronic viral B infection. Serum levels of PDGF-BB, TIMP-1 mRNA, the ratio TIMP-1 mRNA/MMP-1 mRNA in PBMCs and serum levels of TIMP-1 and TIMP-1/MMP-1 ratio were valuable markers for fibrosis assessment. The combination of serum PDGF-BB, TIMP-1 mRNA and MMP-1 mRNA in PBMCs was the best test in screening of liver fibrosis (Zhang et al., 2003(a)).

2.2.6.2.1.3 The Role of Fibrosis Markers in Assessing Treatment Efficacy and Predicting Disease Progression

The dynamic assessment of direct markers of liver fibrosis: HA, PIIINP, YKL-60 and TIMP-1 showed decreased levels in patients who achieved a sustained biochemical or virological response and a good correlation with histological findings (Fabris et al., 1999; Yagura et al., 2000; Leroy et al., 2001; Nojgaard et al., 2003(b)).

The fall of the TGF- β levels after antiviral therapy suggests that interferon has also a direct antifibrotic effect through a direct inhibition of TGF- β expression (Castilla et al., 1991).

Noninvasive markers have also a prognostic value, by predicting clinical evolution and fibrosis progression. Serum HA levels have a great predictive value and correlate with Child –Pugh's score in patients with viral C cirrhosis.

HA and PIIINP were independently predictive of disease progression in primary biliary cirrhosis (Forns et al., 2002), serum laminin levels correlate with Child-Pugh's score of liver cirrhosis irrespective of etiology (Korner et al., 1996) and elevated levels of PIIINP and YKL-40 are predictive of shorter survival in alcoholic cirrhosis (Nojgaard et al., 2003(a)).

High basal levels of TGF β 1 allow for the identification of a subset of patients with chronic hepatitis C who will have progressive liver fibrosis (Kanzler et al., 2001), a statement that has been documented by serial evaluations of serum TGF- β 1. Patients with progressive hepatic fibrosis had a parallel increase in TGF- β 1 levels.

2.2.6.2.1.4 Limitations of the Serum Direct Markers of Liver Fibrosis

Using either a single marker or a combination of tests, direct markers have some limitations:

- they reflect the rate of matrix turnover, not only deposition, and have the tendency to be more elevated when there is an associated high inflammatory activity. As a consequence, extensive matrix deposition might not be detected in the presence of minimal inflammation;
- they are not liver-specific and their serum levels may be elevated in the presence of concomitant sites of inflammation;

- serum levels of markers depend on clearance rates, which are influenced by dysfunction of endothelial cells, impaired biliary excretion or renal function.

2.2.6.2.2 Indirect Markers of Liver Fibrosis

Liver fibrosis may be predicted by using a single routine laboratory test that reflects alteration in hepatic function, or a combination of such tests.

2.2.6.2.2.1 Individual Serum Indirect Markers of Fibrosis

Serum ALT levels

Although serum ALT levels generally reflect liver injury, the correlation between ALT levels and necroinflammatory and fibrosis score is poor, especially in chronic viral C infection. However, an extensive study established that ALT levels had a good sensitivity and specificity for the prediction of histologic substrate (Pradat et al., 2002).

ROC analysis showed that the best theoretical ALT threshold with the best histologic predictive value is 2.25 times the upper limit of normal, but it implies the overlooking of 28% of patients with a histologic score greater than A1F1 Metavir. At the same time, among patients with persistently normal ALT levels, about 26% have a histologic score greater than A1F1, and a liver biopsy must be taken into consideration (Pradat et al., 2002).

AST / ALT ratio

Assay of AST levels had a stronger correlation than ALT with hepatic fibrosis (Gordon et al., 2000). The increase in ALT levels is related to mitochondrial dysfunction and to reduced clearance of AST by hepatic sinusoidal cells. Reversal of the AST / ALT ratio was reported in patients who progress from chronic hepatitis to liver cirrhosis and the AST/ALT ratio of more than 1 had a good predictive value for advanced fibrosis or cirrhosis (Giannini et al., 2003). A good correlation with Child-Pugh's score, MELD score and monoethylglycinexylidide (MEGX) was found.

The AST/ALT ratio had also a predictive value. An AST/ALT ratio greater than 1.16 had 81.3% sensitivity and 55.3% specificity in identifying cirrhotic patients who died within 1 year of follow-up (Giannini et al., 2003).

Platelet count (PLT)

Trombocytopenia is a valuable marker of advanced liver disease, but it may be related to many mechanisms: hypersplenism, myelosuppression by HCV, decreased trombopoietin production, autoimmune process (Peck-Radosavljevic, 2001). Combined assessment of the AST/ALT ratio and PLT had a high diagnostic value for cirrhosis (Giannini et al., 2003).

Prothrombin index

Prothrombin time as an index that reflects the synthesis capacity of the liver is one of the earliest indicators of liver cirrhosis (Croquet et al., 2002). In the HALT-C study, a multivariate logistic regression model that comprised prothrombin time, PLT, AST/ALT ratio and alkaline phosphatase was predictive of cirrhosis. In another study, prolonged prothrombin time correlated with the presence and size of esophageal varices (Pilette et al., 1999). Prothrombin time is also a part of different composite indexes.

2.2.6.2.2.2 Multicomponent Indirect Fibrosis Tests

In order to improve the diagnostic value of different laboratory tests, several combinations of indirect tests have been developed (Table 2.3).

Table 2.3 Diagnostic accuracy of direct and indirect laboratory markers in distinguishing between low stages of ‘insignificant’ fibrosis (F0–F1 by METAVIR) and ‘significant’ fibrosis (F2–F4 by METAVIR)

Laboratory marker	Disease	Sensitivity	Specificity	AUC	References
Direct laboratory markers of liver fibrosis					
Hyaluronic acid	HCV	75–79	80–100	0.82–0.92	(McHutchison et al., 2000) (Murawaki et al., 2001(a)) (Guechot et al., 1996) (Walsh et al., 2000)
	AFLD	87	93	0.79–0.91	(Pares et al., 1996) (Naveau et al., 2005)
	NAFLD	66–85	68–91	0.78–0.87	(Suzuki et al., 2005) (Santos et al., 2005)
YKL-40	AFLD	88.5	50.8	m.d.	(Tran et al., 2000)
	HCV	78	81	0.81	(Saitou et al., 2005)
Laminin	HCV	80	83	0.82	(Walsh et al., 2000)
	NAFLD	82	89	m.d.	(Santos et al., 2005)
Type IV collagen	HCV	73–80	81–85	0.83	(Walsh et al., 2000) (Murawaki et al., 2001(b))
Type IV collagen-7S	HCV	74–83	75–88	m.d.	(Murawaki et al., 2001(b))
	NAFLD	70	81	0.83	(Sakugawa et al., 2005)

Procollagen III	HCV	60-78	75	0.69	(Guechot et al., 1996) (Saitou et al., 2005)
MMP-2	HCV	7-75	70-100	0.59	(Murawaki et al., 2001(b)) (Boeker et al., 2002)
TIMP-1	HCV	67	68	0.71	(Boeker et al., 2002)
Indirect laboratory markers of liver fibrosis					
PGA	HCV	91	81	m.d.	(Teare et al., 1993)
Fibrotest	HCV	65-87	59-81	0.74-0.87	(Imbert-Bismut et al., 2001) (Sebastiani et al., 2006) (Rossi et al., 2003) (Poynard et al., 2004(a))
	HCV/HIV	90	60	0.85	(Myers et al., 2003(a))
	AFLD	88	60	0.84	(Naveau et al., 1994)
APRI	HCV	41-91	47-95	0.69-0.88	(Sebastiani et al., 2006) (Wai et al., 2003)
Forns' Index	HCV	80-94	95-98	0.78-0.86	(Sebastiani et al., 2006) (Forns et al., 2002) (Patel et al., 2003) (Thabut et al., 2003)
	HCV/HIV	43	96	0.77	(Macias et al., 2006)

Two other studies are reported. Fortunato et al (2001) combined the determination of pseudocholinesterase, fibronectin, prothrombin, ALT, N-acetyl-b-glucosaminidase, manganese superoxide dismutase and obtained a correct classification of cirrhosis in 81% of cases.

With the aim of limiting the need for liver biopsy in patients with chronic hepatitis C, the MULTIVIRC group developed a panel of biochemical markers that combines six markers: α 2-macroglobulin, haptoglobin, GGT, total bilirubin, apolipoprotein A1 and ALT with the patient's age and gender to generate a measure of fibrosis stage (FibroTest) and of necroinflammatory grade (ActiTest) of the liver (Imbert-Bismut et al., 2001).

The choice of these markers was justified by their significance in liver disease. α 2-macroglobulin is an acute phase protein, that is a feature of HSC activation and as a consequence is related to hepatic fibrosis (Tiggelman et al., 1997). It is also a proteinase inhibitor that can inhibit catabolism of matrix proteins, enhancing fibrotic process. Haptoglobin is negatively associated with fibrosis (Bacq et al., 1993). The complex role of hepatocyte growth factor and TGF- β 1 on the synthesis of these two markers explains the different behaviour of these proteins (101). GGT is associated with fibrosis and early cholestasis and an increase of epidermal growth factor may be the cause of increased GGT levels, parallel with the stage of fibrosis (Edwards, 1987). Apolipoprotein A1 is trapped in ECM and decreases in liver fibrosis (Paradis et al., 1996).

Activated HSCs express α -smooth muscle actin (α -SMA) and produce an excess of collagen and other ECM components. As a result, liver fibrosis is developed.

In conclusion, a number of laboratory tests have been developed, but their utility in clinical practice is still a matter of debate. While the current body of literature allows the use of laboratory parameters and test panels in advanced fibrosis/cirrhosis, these tests are adopted slowly in clinical routine. The following reasons may be responsible for the restricted use of laboratory tests in assessing fibrosis.

Mild and moderate stages of fibrosis cannot be detected by any of the test systems. However, the early detection of patients at risk of fibrosis progression, e.g. in viral hepatitis or autoimmune liver disease, has an impact on the necessity and intensity of therapy.

The precision of tracking intra-individual changes, e.g. in therapy, has not been sufficiently investigated.

The superiority of any direct laboratory marker to routine laboratory parameters has still to be demonstrated. This is also with respect to the cost of medical diagnostics an important factor.

2.2.6.2.1 Ideal Features for a Marker of Liver Fibrosis

- Liver-specific
- Inexpensive and easy to perform
- Measures:
 - Stage of fibrosis (or mass of extracellular matrix)
 - Activity of matrix deposition
 - Activity of matrix removal
- Levels not altered by changes in liver, renal, or reticuloendothelial function
- Reproducible performance characteristics
- Follows dynamic changes in fibrogenesis

2.2.6.3 Imaging Methods

2.2.6.3.1 Ultrasound, CT and MRI

Ultrasound, CT and MRI are inadequate to diagnose and differentiate early stages of fibrosis. Even diagnosis of cirrhosis is often based only on signs of advanced liver

cirrhosis e.g. signs of portal hypertension, reduction of the right liver lobe with enlargement of the left liver lobe or caudate lobe resulting in a high specificity but lower sensitivity of the methods (Honda et al., 1990). Ultrasound studies (Aube et al., 1999) combining up to 11 ultrasound parameters and Doppler measurements achieved accuracies for the diagnosis of cirrhosis up to a maximum of 88%.

In recent years special MRI and ultrasound techniques have been developed for non invasive detection and quantification of liver fibrosis.

2.2.6.3.1 Magnetic Resonance Elastography

Diffusion-weighted MR imaging technique is based on the measurement of the apparent diffusion coefficient (ADC), which is influenced by the hydration and metabolic status of the liver. Studies showed a reduction of the ADC values in fibrotic liver tissue, but the differences were relatively small (Aube et al., 2004; Boulanger et al., 2003).

Another approach evaluating liver elasticity uses an external probe at the back of the patient sending low frequency vibrations (60 Hz) through the liver and measuring the MRI spin-echo sequence. With this technique shear elasticity and viscosity maps are obtained. A study comparing MR elastography of 30 healthy volunteers and 50 patients with chronic liver disease with liver histology showed a sensitivity of 86% and a specificity of 85% for discrimination between patients with moderate and severe fibrosis (Metavir F2–F4) and those with mild fibrosis (Yin et al., 2007). Up to now there is only a small number of studies dealing with MRI detection and quantification of liver fibrosis, and one has to wait for further studies for assessing the diagnostic potential of the method more precisely.

2.2.6.3.1 Transient Elastography (Fibroscan)

Transient elastography (Fibroscan, EchoSens) evaluates liver fibrosis based on measuring the elasticity of the liver using ultrasound (Sandrin et al., 2002). The system consists of an ultrasound transducer combined with a vibration probe, which is positioned intercostally and transmits a low frequency vibration wave with mild amplitude (50 Hz) into the right liver lobe. The vibration induces an elastic shear wave that propagates through the liver tissue. Pulse-echo ultrasound waves then measure the velocity of the shear wave in the liver tissue at a distance of 2.5–6.5 cm

under the skin level. This corresponds to a measured distance of 4 cm in the liver tissue. The velocity correlates with liver tissue stiffness and therefore the degree of fibrosis. The stiffer the tissue, the faster the shear wave propagates. The values are recorded in kilopascal (kPa). The mean value is established from ten valid measurements. The learning curve is steep with a low inter- and intra-observer variability after at least 50 prior fibroscan examinations (Kettaneh et al., 2007). Technical limitations of the method are the presence of ascites and obesity. Currently a new elastography probe, which can penetrate chest wall fat, is being evaluated in obese patients to overcome this.

The method has been widely evaluated in studies on patients with chronic liver disease in comparison to histological fibrosis staging. In most studies the liver tissue samples were obtained by percutaneous liver biopsy.

The main focus of the initial tests was the evaluation of liver fibrosis in chronic hepatitis C. A prospective study of 327 patients with chronic hepatitis C (Ziol et al., 2005) compared transient elastography with liver histology for detection of relevant fibrosis or cirrhosis. The AUCs were 0.79 for $>F2$; 0.91 for $<F3$ and 0.97 for histological Metavir score F4. The optimum cut-off value as the highest product of sums for sensitivity and specificity for F4 was 14.6 kPa. For this value a sensitivity of 86%, specificity of 96%, a positive predictive value of 0.78 and a negative predictive value of 0.97 were achieved. After showing high diagnostic accuracy in chronic hepatitis C the method has also been used for determination of graft fibrosis after liver transplantation due to recurrent hepatitis C, with comparable high diagnostic accuracy for the diagnosis of severe fibrosis or cirrhosis (AUC 0.90 for Metavir score ≥ 4) (Harada et al., 2008). First results of transient elastography in post-liver transplant follow up in a small number of patients (Rigamonti et al., 2008) showed promising concordance of stiffness changes with histological staging and grading over a time interval of 6 months. This indicates a possible role of the method in the follow-up of liver fibrosis in chronic liver diseases.

Recently a meta-analysis (Friedrich-Rust et al., 2008) screened and weighted published studies from 2002 to 2007 comparing transient elastography with liver histology in chronic liver diseases. Fifty studies fulfilled quality requirements and were included in the analysis. The mean AUC for the diagnosis of significant fibrosis

($F \leq 1$ vs. $F \geq 2$), severe fibrosis ($F \leq 2$ vs. $F \geq 3$) and cirrhosis ($F \leq 3$ vs. $F = 4$) were 0.84 (CI 0.82–0.86); 0.89 (CI 0.88–0.91) and 0.94 (CI 0.93–0.95) in a recent meta-analysis (Friedrich-Rust et al., 2008). Transient elastography performed excellently at the differentiation of cirrhosis vs. no cirrhosis with an optimal cut-off value of 13.01 kPa. For the diagnosis of cirrhosis no significant difference in AUC was found between the different underlying liver diseases. These results support transient elastography to be able to diagnose cirrhosis with a high diagnostic accuracy independent from the underlying liver disease eventually replacing liver biopsy.

The diagnostic accuracy of the method decreases in the presence of low degrees of fibrosis (Metavir score F1 and F2), as the ascertained values often overlap. In the meta-analysis (Friedrich-Rust et al., 2008) the AUC for $F \geq 2$ (diagnosis of significant fibrosis) varied between the different studies with a range of 68–100%, a mean AUC of 0.84 and an adjusted AUC of 0.91. Here a significant reduction of heterogeneity was achieved by differentiating between the underlying liver diseases. The optimal cut-off value suggested from the SROC was 7.65 kPa. Probably, focusing to the underlying liver disease with certain AUC and cut-off values may reduce this heterogeneity but one has to interpret the results in this range with caution.

Further studies evaluated the correlation of liver stiffness with complications of liver cirrhosis in patients with HCV. The reported AUC for the prediction of portal hypertension (Vizzutti et al., 2007) correlated with a HVPG ≥ 10 mmHg and ≥ 12 mmHg were 0.99 and 0.92, respectively. However prediction of oesophageal varices regardless of grading them resulted in a rather low sensitivity (90%) and specificity (43%) at the chosen cut-off value of 17.6 kPa. Other data (Foucher et al., 2006) ascertained the occurrence of complications in liver cirrhosis at a 90% negative predictive value with high cut-off values of 27.5 kPa, indicating already an advanced stage of cirrhosis for the presence of oesophageal varices and 53.7 kPa for the presence of hepatocellular carcinoma. Assuming that liver stiffness reflects an increase in portal pressure, it does not record all the complex hemodynamic changes occurring in the development of portal hypertension. Liver stiffness measurement may therefore be able to indicate a portal hypertension, e.g. with correlation to a HVPG of 10 mmHg, but is limited in gradation between further complications of portal hypertension.

Acute inflammation (Arena et al., 2008) (Sagir et al., 2008) and extrahepatic cholestasis (Millonig et al., 2008) have been shown to increase liver stiffness values. During the acute phase of liver damage in toxic hepatitis (Arena et al., 2008) and acute viral hepatitis (Sagir et al., 2008), liver stiffness values exceeded the cut-off values for prediction of significant fibrosis or cirrhosis. In the follow-up examinations liver stiffness values returned to baseline with the decline of aminotransferases and bilirubin. For reliable estimation of transient elastography biochemical activity of chronic liver disease has to be taken in account; liver stiffness values in acute hepatitis with acute liver damage lead to an overestimation of liver fibrosis.

Summarising the imaging data, transient elastography allows diagnosis of liver cirrhosis and significant fibrosis with a high degree of diagnostic accuracy, while it has to be remarked that percutaneous liver biopsy as the reference method, used as gold standard, can also coincide with an underestimation of the degree of fibrosis (Denzer et al., 2007).

The diagnostic accuracy of transient elastography decreases in the presence of acute liver inflammation or extrahepatic cholestasis and in the presence of low degrees of fibrosis (Metavir score F1 and F2), as the ascertained values often overlap. Probably, the combination of transient elastography and serum markers of fibrosis may differentiate milder degrees of fibrosis more accurately in the future.

Nevertheless, due to the uncomplicated and rapid procedure and the non-invasive character, transient elastography appears to be an attractive alternative for liver biopsy, if the aetiology of liver disease is clear, e.g. in chronic hepatitis C. The method seems also suitable for monitoring the course of chronic liver disease, as it is widely accepted by patients.

- Ultrasound, CT and MRI are inadequate to diagnose and differentiate early stages of fibrosis. Even diagnosis of cirrhosis is often based on signs of advanced liver cirrhosis, e.g. signs of portal hypertension resulting in a high specificity but lower sensitivity of the methods.
- Diffusion-weighted MR imaging technique and MR elastography need to be evaluated by further studies for assessment of the diagnostic potential of the methods more precisely.

- Transient elastography allows the diagnosis of liver cirrhosis and significant fibrosis. Diagnostic accuracy decreases in the presence of low degrees of fibrosis (Metavir score F1 and F2). Referring to the reported data, the technique may be of practical relevance in the diagnosis of significant fibrosis and in the follow-up of chronic liver diseases.

2.2.7 Reversibility of Fibrosis and Cirrhosis

The demonstration that hepatic fibrosis and even cirrhosis may regress (Bonis et al., 2001; Desmet and Roskams, 2004) has overturned a longstanding dogma, and has accelerated enthusiasm for developing antifibrotic therapies. Regression has been documented in patients with hepatitis B, hepatitis C (Poynard et al., 2002), delta hepatitis, metabolic diseases and cholestasis, among others, although so far improvement has only been seen when the underlying disease has been eradicated. A key determinant of fibrosis reversion is clearance of activated HSCs through apoptosis, which requires downregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) (Murphy et al., 2002).

Although the point at which human cirrhosis becomes truly irreversible is unknown, animal and human studies suggest that more prolonged injury leads to increasingly thickened fibrotic septae with enhanced crosslinking that is mediated by tissue transglutaminase (Wanless et al., 2000; Issa et al., 2004). These septae become insoluble and may be more resistant to proteolysis by metalloproteinases, which limits complete regression. Interstitial collagen within these septae might provide an important survival signal to activated stellate cells, preventing their clearance through apoptosis. Moreover, it is uncertain whether complete or partial matrix regression will restore normal portal blood flow, because the distorted vascularity and shunting characteristic of advanced liver disease may not be reversible.

2.2.8 Clinical Trials

The choice of patients and endpoints for clinical trials of antifibrotic drugs presents a conundrum. Current efforts focus mainly on patients with hepatitis C and advanced fibrosis or cirrhosis who have failed antiviral therapies, in part because of the large number of such patients and their well characterized natural history. On the one hand, antifibrotics are more urgently needed in patients with HCV cirrhosis and are more likely, if successful, to yield improved survival and reduced morbidity. On the other

hand, cirrhosis is probably less reversible than earlier fibrosis stages and will require longer treatment, and these patients will be less able to tolerate any idiosyncratic hepatotoxicity from an investigational agent. They are also at higher risk for hepatocellular cancer. Thus, initial trials should be aimed at demonstrating the capacity for matrix resorption without insisting that complete reversal of fibrosis or improved morbidity and mortality be documented; findings from such studies will support the rationale for longer, larger trials with 'hard' clinical endpoints including survival and decreased complications. Other potential target populations include either children with neonatal fibrosis, or patients with hepatitis C post-liver transplantation, because fibrosis is accelerated in these groups, or those with cholestatic liver disease or NASH.

2.2.9 Emerging Treatments for Hepatic Fibrosis

The clarification of the mechanisms of fibrosis has led to a surge in enthusiasm for treating hepatic fibrosis, and animal models strongly support the rationale of this approach. In fact, much like the paradigm of cancer treatment, success in animal models has been far easier to achieve than in human trials, possibly because the duration of injury and the extent of matrix deposition and crosslinking are greater in humans. Key obstacles remain to be overcome before antifibrotic therapies can reach widespread clinical use.

2.2.9.1 Reducing Injury and Inflammation

1.1 Antiviral therapy for viral hepatitis

1.2 Anthelmintic therapy for schistosomiasis

1.3 Chelation/venesection, treatment of copper/iron overload disease

1.4 AT1 receptor antagonists, ACE inhibitors

1.5 Hepatoprotectants:

1.5.1 Caspase inhibitors

1.5.2 HGF/HGF mimetics

2.2.9.2 Attenuating Stellate-Cell Activation

2.1 Alpha interferon

2.2 Antioxidants:

2.2.1 Vitamin E, PDTC

2.2.2 AT1 receptor antagonists

2.3 Cytokine-directed therapy:

2.3.1 TGF- β antagonists

2.3.2 Endothelin receptor antagonists

2.3.3 HGF/HGF mimetics

2.4 PPAR agonists

2.5 FXR agonists

2.6 Aldosterone antagonists

2.7 Pentoxifylline

2.2.9.3 Inhibiting Properties of Activated Stellate Cells

3.1 Antiproliferative:

3.1.1 PDGF receptor antagonists

3.1.2 Sodium exchange inhibitors

3.1.3 HMG CoA reductase inhibitors

3.1.4 Plasmin/thrombin receptor antagonists

3.2 Anticontractile:

3.2.1 Endothelin/endothelin receptor antagonists

3.2.2 Nitric oxide donors

3.3 Antifibrogenic:

3.3.1 Collagen synthesis inhibitors

3.3.2 TGF- β inhibitors (including soluble receptors and neutralizing antibodies)

3.3.3 HGF/HGF mimetics

3.3.4 AT1 receptor antagonists

3.3.5 ACE inhibitorsIntegrin antagonists

3.3.6 CTGF/CCN antagonists

3.3.7 SMAD7 agonists

2.2.9.4 Promoting Specific Apoptosis of Hepatic Stellate Cells

4.1 Gliotoxin

4.2 NGF agonists

4.3 TIMP antagonists

2.2.9.5 Degrading Scar Matrix

5.1 Direct collagenase administration

5.2 Inhibitors of transglutaminase or collagen crosslinking

5.3 TIMP antagonists

5.4 TGF- β inhibitors

Table 2.4 describes the classification of a number of pharmacological agents or strategies whose therapeutic potential has been demonstrated in carefully and/or originally conducted studies (Pinzani et al., 2005).

Table 2.4 Anti-fibrogenic agents and strategies

Agent or strategy	Mode of action	Ref.
Direct anti-fibrogenic effect		
Colchicine	Inhibition of pro-collagen secretion and conversion to collagen synthesis. Suppression α -SMA and TGF β_1 expression in activated HSC	(Lee et al., 2004; Poupon et al., 1996)
Prostaglandins	Inhibition of both basal and TGF β_1 -mediated induction of collagen synthesis by HSC	(Hui et al., 2004)
OPC-13013: a cyclic nucleotide phosphodiesterase type III, inhibitor	Augmentation of forskolin-induced increase in intracellular cyclic AMP level (inhibitory effect on HSC activation)	(Shimizu et al., 1999)
6-Ethyl chenodeoxycholic acid (6- ECDCA): a FXR ligand	Induction of SHP expression, down-regulation of $\alpha 1(I)$ collagen and TGF β_1 mRNA steady state level in HSC	(Fiorucci et al., 2004)
Pentoxifylline	Down-regulation of pro-collagen type I, TGF β_1 and CTGF expression in HSC	(Romanelli et al., 1997; Raetsch et al., 2002)
HDAC inhibitors: trichostatin A and sodium butyrate	Inhibition of mRNA and de novo protein synthesis of pro-collagens type I, III and α -SMA	(Niki et al., 1999)

All trans retinoic acid	Inhibition of steady state mRNA and de novo protein synthesis of pro-collagen type I, III, IV and fibronectin and laminin	(Hellemans et al., 1999)
Lovastatin, simvastatin: HMG-Co reductase inhibitors	Inhibition of protein synthesis of pro-collagens type I, III and IV	(Rombouts et al., 2003)
Canrenone	Inhibition of TGF β_1 -induced de novo protein synthesis of pro-collagens type I and IV and fibronectin. Indirectly: inhibition of PDGF-induced proliferation and migration of HSC	(Caligiuri et al., 2003)
Tetrandrine	Reduction of HSC activation and collagen accumulation in liver fibrosis induced by biliary obstruction. Indirectly: promotion of HSC apoptosis	(Zhao et al., 2004)
Butein	Down-regulation of α -SMA and collagen type-I protein expression. Reduction of $\alpha 1(I)$ collagen and TIMP-1 mRNA expression and induction of MMP-13 mRNA expression	(Woo et al., 2003)
Hepatocyte growth factor (HGF)	Reduction of TGF β_1 levels in the liver	(Ikeda et al., 1998; Ueki et al., 1999)
Halofuginone	Reduction of total collagen content, $\alpha 1(I)$ collagen gene expression, TIMP-2 content, α -SMA expression and inhibition of the proliferation of other cell types of the fibrotic liver in vivo	(Bruck et al., 2001)
IL-10	Upregulation of mRNA steady state expression of interstitial collagenase and inversely down-regulation of $\alpha 1(I)$ collagen	(Zhang et al., 2004; Wang et al., 1998(b))
Dilinoleoylphosphatidylcholine (DLPC)	Down-regulation of collagen accumulation induced by TGF β_1 in cultured HSC	(Cao et al., 2002)
Relaxin	Reduction of the levels of total collagen by inhibition of new collagen synthesis. Downregulation of α -SMA expression and up-regulation of expression and secretion of MMP-13, TIMP-1 and TIMP-2	(Bennett et al., 2003)
Camostat mesilate, a serine protease inhibitor	Down-regulation of the activity of TGF β , which blocked in vitro activation of HSC. Markedly attenuation of plasmin and TGF β levels, HSC activation and hepatic fibrosis in an in vivo model	(Okuno et al., 2001)
HOE 077 and S4682: prolyl 4-hydroxylase inhibitors	Reduction of the stability of the collagen triple helix by inhibiting prolyl-4 hydroxylase. Inhibition of collagen synthesis in HSC	(Aoyagi et al., 2002; Bickel et al., 1998)
Malotilate	Down-regulation of collagen synthesis in CCl $_4$ -induced hepatic fibrogenesis	(Nojgaard et al., 2003(a))
LU 135252: a non-peptide ET-A receptor antagonist	Reduction of $\alpha 1(I)$ collagen and TIMP-1 mRNA steady state levels	(Cho et al., 2000)
S-Farnesylthiosalicylic acid (FTS)	Augmentation in MMP-2 and MMP-9-induced collagenolytic activity and up-regulation of TIMP-2 expression	(Reif et al., 2004)
PPAR γ ligands: 15dPGJ(2) and troglitazone	Reduction in $\alpha 1(I)$ pro-collagen, α -SMA and MCP-1 mRNA levels and up-regulation of MMP-3 and CD36. Inhibition of $\alpha 1(I)$ pro-collagen promoter activity. Modulation of pro-fibrogenic and proinflammatory actions in	(Miyahara et al., 2000; Marra et al., 2000)

	HSC	
TTNPB: an RAR agonist, AGN194204: an RXR agonist	Inhibition of collagen and fibronectin synthesis	(Hellemans et al., 2004)
Indirect anti-fibrogenic effect		
NS-398: a COX-2 inhibitor	Inhibition of α -SMA protein and PCNA expression. Reduction in cell growth: Downregulation of the generation of PGE2, IL-8, IL-6 and hyaluronan in HSC	(Cheng et al., 2002)
Non-specific COX inhibitors: indomethacin and ibuprofen	Inhibition MCP-1 gene and protein expression in HSC	(Efsen et al., 2001)
Gliotoxin	Induction of apoptosis in HSC through a thiol redox-dependent interaction with adenine nucleotide transporter (ANT)	(Orr et al., 2004)
Ethylisopropylamiloride	Inhibition of the activity of Na ⁽⁺⁾ H ⁽⁺⁾ exchanger 1 and reduction in PDGF-BB induced proliferation of HSC	(Yang et al., 2003(a); Benedetti et al., 2001) (Di Sario et al., 2003)
Cariporide	Inhibition of the activity of Na ⁽⁺⁾ H ⁽⁺⁾ exchanger 1 and reduction in PDGF-BB induced proliferation of HSC	(Baik et al., 2003)
Losartan: an angiotensin II receptor inhibitor	Reduction in the contraction and growth of HSC	(Yoshiji et al., 2002)
Perindopril, an ACE inhibitor	Suppression of activated HSC proliferation and TIMP-1 expression	(Okuyama et al., 2001)
N-acetyl-L-cysteine (NAC)	Induction of extracellular proteolysis of PDGF receptor- β and down regulation of type II TGF- β receptor	(Iwamoto et al., 1999(b))
Arg-Gly-As peptides	Induction of apoptosis in HSC through integrin antagonism and stimulation of collagenase expression by HSC	(Iwamoto et al., 1999(a); Gutierrez-Ruiz et al., 2001)
Anti-oxidants		
Metadoxine	Inhibition of acetaldehyde-induced increase in collagen and attenuation of TNF- α secretion	(Lee et al., 2001)
α -Tocopherol	Down-regulation of collagen type I, α -SMA and PCNA expression. Protection against CCl ₄ -induced chronic liver damage and cirrhosis	(Parola et al., 1992)
Quercetin: a flavonoid	Reduction of collagen content, iNOS expression and lipid peroxidation in total liver. Augmentation of total peroxyl radical- trapping anti-oxidant capacity of liver. Antioxidant and free radical-scavenging activities	(Pavanato et al., 2003)
Epigallocatechin-3-gallate	Inhibition of the TGF β signal transduction pathway and inhibition of the expression of α 1(I) collagen, fibronectin and α -SMA genes	(Chen et al., 2002)
Trans-resveratrol	Inhibition of type I collagen mRNA. Inhibition of proliferation and down- regulation of α -SMA expression. Reduction of the secretion of MMP-2	(Godichaud et al., 2000)
Herbes		
Da Ding Feng Zhu	Down-regulation of serum indexes of liver fibrosis	(Li et al., 2003(c))
Trichilia roka root	Hepatoprotective agent preventing a preferential deposition of collagen around the sinusoidal cell layer	(Germano et al., 2001)

Sylimarin	Down-regulation of pro-collagen $\alpha 1(I)$, TIMP-1 and TGF β_1 mRNA levels	(Jia et al., 2001)
Sho-saiko-to	Suppression of pro-collagen type I and III mRNA expression and III and cell cycle	(Kayano et al., 1998)
Herbal Compound 861	Inhibition of the cell cycle and activation of HSC by reduction of the expression levels of α -SMA mRNA in HSC	(Wang et al., 2004)
Salvionolic acid-A (SA-A): active compound of <i>Salvia miltiorrhiza</i>	Inhibition of cell proliferation and collagen production in HSC	(Liu et al., 2000(a))
Monomer IH764-3: active compound of <i>Salvia miltiorrhiza</i>	Inhibition of HSC proliferation and induction of HSC apoptosis	(Zhang et al., 2002)
Qinggan Huoxue recipe (QGHR)	Reduction of the levels of liver fibrosis markers and cytokines, alleviation of the antilipid superoxidation damage in liver, and markedly improvement in the degree of fatty liver	(Ji et al., 2004)
Hujin pill	Down-regulation of the retardation of liver fibrosis	(Huang et al., 2000)
Dahuang Zhechong pill	Inhibition of HSC proliferation and secretion of TGF β_1 - Reduction in collagen synthesis	(Li et al., 2003(b))
HanDanGanLe	Up-regulation of collagenase activity in immune hepatic fibrosis	(Lu et al., 2000; Zhang et al., 2003(b))
Inchin-ko-to (TJ-135)	Regulation of PDGF-BB-dependent signaling pathways of HSC. Down-regulation of collagen and fibronectin synthesis	(Imanishi et al., 2004)
Maotai liquor	Inhibition of proliferation of HSC and reduction in collagen gene and protein expression	(Cheng et al., 2004)
Aloe emodin	Inhibition of type I collagen production and α -SMA expression	(Woo et al., 2002)
Curcumin	Reduction in cell proliferation, induction of apoptosis and suppression of ECM gene expression through PPAR γ activity in activated HSC	(Zheng and Chen, 2004)
Glycyrrhizin	Reduction of serum ALT and AST values, inhibition of the NF-kappaB binding activity in CCl $_4$ and ethanol-induced chronic liver injury	(Park et al., 1997; Wang et al., 1998(a))
Biotechnologies		
Soluble decoy receptors for TGF β_1	Reduction of collagen type I expression during hepatic fibrosis	(Muriel and Castro, 1998)
Anti-sense oligonucleotides phosphorthioate (AdCATb-TR),	Inhibition of TIMP-1 gene expression	(Muriel et al., 1994)
adenovirus-mediated local expression of a dominant-negative type II TGF- β receptor	Reduction of liver fibrosis and hydroxyproline content, serum levels of HA and transaminases	(George et al., 1999)
(Ad5-CMV-asPDGF), an adenoviral vector serotype 5 (Ad5) expressing an anti-sense mRNA of the PDGF B-chain	Regulation of endogenous PDGF B-chain and PDGFR β mRNA in culture-activated HSC and rat livers. Attenuation of experimental liver fibrogenesis by reduction of α -SMA and collagen type I expression	(Nie et al., 2001)

Anti-sense oligonucleotides against TGF- β 1	Inhibition of HSC activation, secretion of TGF β 1, and downregulation of collagen synthesis	(Qi et al., 1999)
Anti-sense TIMP-1 recombinant plasmid	Inhibition of collagens type I and III synthesis	(Borkham-Kamphorst E et al., 2004)
Peptide-modified albumin (pPB-HSA)	Reduction of PDGF-BB-induced fibroblast proliferation	(Liu et al., 2000(b))
Anti-sense T beta R I and T beta R II recombinant plasmids	Blocks mRNA and protein expression of T beta R I and T beta R II and decreases collagen types I and III	(Liu et al., 2003)
Miscellaneous		
NCX-1000	Inhibition of HSC contraction. Metabolization of NCX-1000 and secretion of and release nitrite/nitrate in cell supernatants	(Beljaars et al., 2003)
Nitroglycerin (NTG): an NO-donor	Inhibition of proliferation, motility, and contractility of HSC and reduction of fibrillar ECM accumulation	(Jiang et al., 2004)
S-Adenosyl-L-methionine (SAME)	Hepatoprotective agent by restoring transmethylation loss in liver fibrosis	(Fiorucci et al., 2001; Failli et al., 2000)

2.3 PPAR γ LIGANDS AND LIVER FIBROSIS

The PPARs belong to the superfamily of ligand-dependent transcription factors that is predominantly expressed in adipose tissue, where it has been shown to have a key role in adipogenesis (Kliwer et al., 1994; Tontonoz et al., 1995; Green and Wahli, 1994). PPAR forms heterodimers with the retinoid X receptor and binds to specific response elements to induce transcription in response to a variety of endogenous and exogenous ligands, including fatty acids, arachidonic acid metabolites, and synthetic drugs (Forman et al., 1996). Of the PPAR isoforms, PPAR- γ is the most widely studied (Auwerx, 1999). Previous studies indicated that expression of PPAR- γ inhibited PDGF-induced proliferation and migration of vascular smooth muscle cells (Fu et al., 2001). Recently, additional functions such as regulation of inflammation; control of cell cycle and apoptosis were attributed to PPAR γ , suggesting a more pleiotropic role in multiple fundamental pathways with wide-ranging biomedical implications (Rocchi and Auwerx, 1999; Everett, 2000). Three recent studies independently demonstrated that the level of PPAR- γ and its trans-activating activity were diminished during HSC activation in vitro, whereas NF- κ B and activator protein-1 (AP-1) activities were increased (Galli et al., 2000; Marra et al., 2000; Miyahara et al., 2000). PPAR- γ ligands inhibited cell proliferation and collagen- α_1 (I) expression in primary HSC (3–4 days) (Miyahara et al., 2000). The dramatic reduction in the abundance of PPAR- γ results in a significant decline in response to exogenous PPAR- γ ligands in activated HSC (Galli et al., 2000; Marra et al., 2000;

Miyahara et al., 2000). These findings implied a potential therapeutic value of PPAR- γ ligands in treatment of liver fibrosis if the expression of PPAR- γ can be induced in activated HSC.

2.4 ANGIOTENSIN II RECEPTOR ANTAGONIST AND LIVER FIBROSIS

Drugs modulating the action of vasoactive substances are currently used in the treatment of different types of human fibrosis. These vasoactive substances include vasoconstrictors (angiotensin-II, aldosterone, and ET-1) and vasodilators (prostaglandins and nitric oxide). Among these factors, angiotensin-II appears to play an important role. Drugs interfering with angiotensin-II synthesis (i.e., angiotensin-converting enzyme inhibitors or Angiotensin-I antagonists) reduce the progression of cardiac and renal fibrosis in patients with severe arterial hypertension. Angiotensin-II induces contraction and is a mild mitogen toward HSCs. Moreover, in a preliminary study, an Angiotensin-I antagonist inhibited liver fibrosis in bile duct-ligated rats (Figure 2.4) (Bataller et al., 2000; Jonsson et al., 2001).

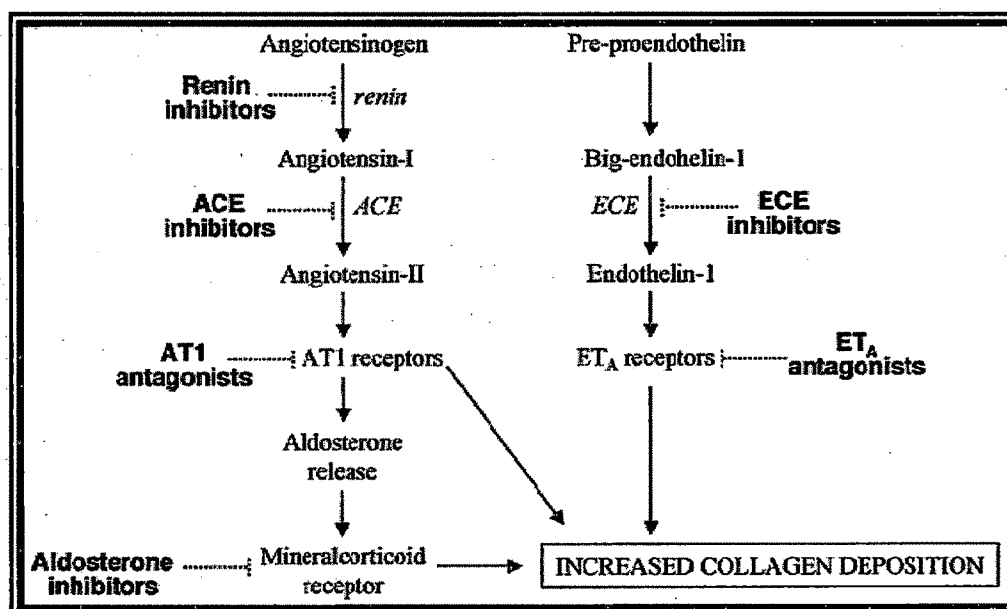


Figure 2.4 The renin-angiotensin-aldosterone and endothelin pathways and potential sites for therapy of liver fibrosis (ACE = angiotensin-converting enzyme; ECE = endothelin-converting enzyme)

2.5 DRUG TARGETING TO HSC

A large number of currently tested antifibrotic drugs aim, either direct or indirect, at activated HSC. In vivo, however, effectiveness of these compounds is often limited due to the lack of the specificity for HSC. Selective delivery of compounds to the HSC in the fibrotic liver by means of specific drug carriers is an alternative for traditional treatments.

Due to selective accumulation in the HSC, high drug concentrations can be achieved, while at the same time adverse effects will be avoided in other tissues and cells. In order to target HSC, protein based carriers were developed using chemically modified human serum albumin (HSA) with specific groups that are recognised by receptors expressed on the cell membrane of activated HSC. The introduction of mannose 6-phosphate (M6P-HSA) groups (Beljaars et al., 1999; Beljaars et al., 2001) into the albumin molecule yielded a carrier which was recognized by the mannose 6-phosphate/insulin like growth factor II receptor that is abundantly expressed on activated HSC. The best homing properties of this carrier were achieved when around 30 M6P groups were attached per albumin molecule. In two other carriers, specific sequences of cyclic peptides, that mimic the binding-sites of natural ligands such as PDGF (Beljaars et al., 2003) and collagen type VI (Beljaars et al., 2000) to their receptors, were attached to HSA. Both PDGF receptor and collagen type VI receptor are up-regulated on activated HSA and the association of these carriers with activated HSC was demonstrated. In vivo, it was shown that all these carriers accumulate in HSC in the fibrotic livers of bile duct ligated rats.

Modified HSA can function as a drug carrier, as was recently proved by coupling several drugs, including pentoxifyline (Gonzalo et al., 2006), mycophenolic acid (Greupink et al., 2005), doxorubicine (Greupink et al., 2006) and gliotoxin (Hagens et al., 2006) to M6P-HSA. These drug-carrier constructs retained their antifibrotic properties, as was demonstrated in cultured HSC, and were also delivered to HSC in the fibrotic liver. Another application of modified HSA would be to serve as a homing ligand for other of drug carrier systems such as liposomes, cationic lipoplexes or polymers (Figure 2.5).

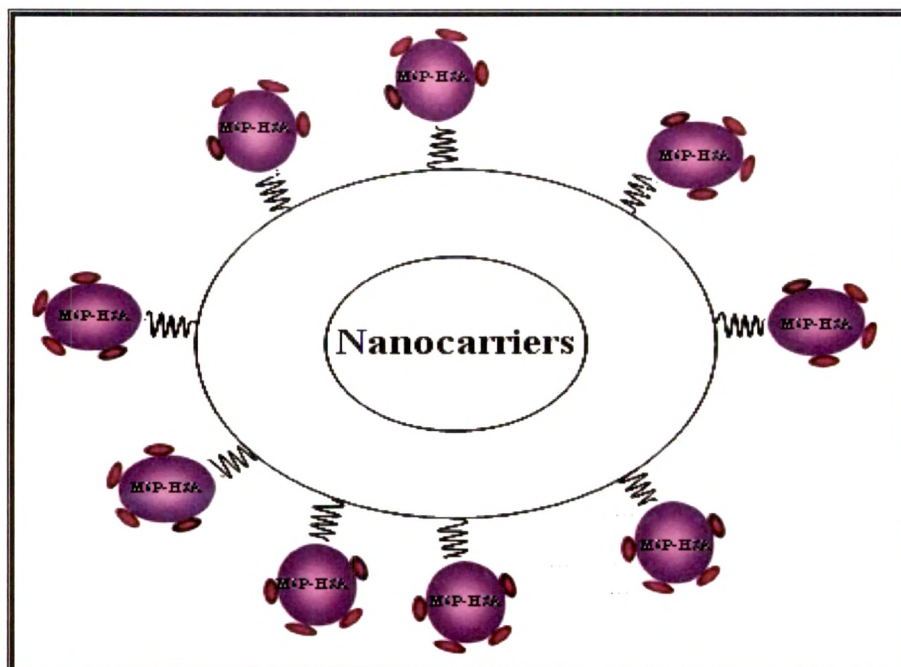


Figure 2.5 Schematic representation of M6P-HSA conjugated carrier

2.6 LIPOSOMES

Liposomes are synthetic, single or multi-compartmental vesicles having lipid membranes enclosing aqueous chambers. Liposomes are vesicles composed of phospholipids bilayers surrounding aqueous compartments as described by Bangham et al (1965) (Bangham et al., 1965). They consist of one or more bilayers. The driving force for bilayer assembly is the amphiphilic nature of phospholipid molecules. Liposomes are composed of phospholipid/s or lipids or and glycerides with or without sterols. Phospholipid typically consists of a hydrophilic head group attached to two hydrophobic fatty acid chains. When suspended in an excess of aqueous solution, phospholipid molecules orient themselves in ordered bilayers so that the polar heads are hydrated and hydrophobic tails are excluded from the aqueous environment (Figure 2.6). Although suspended phospholipids may also assume other geometric(s) such as micelles and tubular aggregates in hexagonal phases, this can be controlled by several factors including lipid composition and method of preparation. Entrapment of compounds is highly influenced by their physiochemical properties. Generally hydrophobic molecules are incorporated into the lipid bilayers whereas hydrophilic compounds are entrapped in the internal aqueous volume (Stamp and Juliano, 1979).

2.6.1 Composition of Liposomes

2.6.1.1 Phospholipids

Glycerol containing phospholipids are by far, the most commonly used component of liposome formulations and represent more than 50% of the weight of lipid present in biological membranes (Riaz et al., 1988). As examples of potentially useful lipids can be mentioned natural lipids such as egg lecithin, soya lecithin, and synthetic lipids such as phosphoglycerolipids, sphingolipids, and digalactosylglycerolipids. Amongst natural lipids may be mentioned sphingolipids such as sphingomyelin, ceramide and cerebroside; galactosylglycerolipids such as digalactosyldiacylglycerol; phosphoglycerolipids such as egg-yolk phosphatidylcholine and soya-bean phosphatidylcholine; and lecithins such as egg-yolk lecithin and soya-bean lecithin. Amongst synthetic lipids may be mentioned dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine, dilauryl phosphatidylcholine, 1-myristoyl-2-palmitoyl phosphatidylcholine, 1-palmitoyl-2-myristoyl phosphatidylcholine, dioleoyl phosphatidylcholine, hydrogenated soyaphosphatidylcholines (HSPC), and the like. Some naturally occurring phospholipids include phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylglycerol (PG) while dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylserine (DPPS), dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidic acid (DPPA), dipalmitoyl phosphatidylglycerol (DPPG), dioleoyl phosphatidylcholine (DOPC) and dioleoyl phosphatidylglycerol (DOPG) are some synthetic phospholipids.

2.6.1.2 Sterols

Sterols such as cholesterol, ergosterol, nanosterol, or its derivatives are often included as components of liposomal membrane. Cholesterol has been called the “mortar” of bilayer because by virtue of its molecular shape and solubility properties, it fills in empty spaces among the phospholipid molecules, anchoring them more strongly into the structure. Its inclusion in liposomal membranes has 3 effects (i) increasing the fluidity or microviscosity of the bilayer (ii) reducing the permeability of the membrane to water-soluble molecules and (iii) solubilizing the membrane in the presence of biological fluids such as plasma.

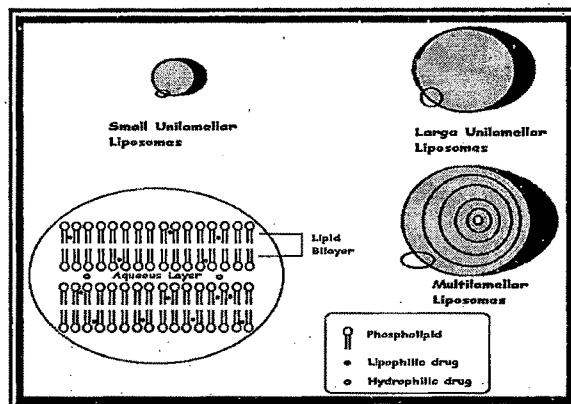


Figure 2.6 The structure of multilamellar vesicles showing the organization of phospholipid bilayers and the encapsulation of lipophilic and hydrophilic compounds

2.6.1.3 Other Non-Structural Components

Charge inducer materials which provides a negative charge, for example phosphatidic acid, dicetyl phosphate or beef brain ganglioside etc, or one which provides a positive charge for example stearylamine acetate or cetylpyridinium chloride etc. have been incorporated into liposomes so as to impart either a negative or a positive surface charge to these structures. Many single chain surfactants of number of single and double chain lipids having fluorocarbon chains and also compounds like quaternary ammonium salts and dialkyl phosphates (Ringdorf et al., 1988) can also be used to form liposomes.

2.6.2 Types of Liposomes

Different types of liposomes can be prepared and are classified by the size and structure. Different types of liposomes are small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), oligolamellar vesicles (OLV), and multi-lamellar vesicles (MLVs). MLVs consist of numerous concentric bilayers separated by aqueous spaces and range up to 15 μm in diameter. Vesicles consisting of a single bilayer encompassing a central aqueous compartment are referred to as small unilamellar vesicles (SUVs), which range upto 100 nm in diameter and large unilamellar vesicles (LUVs) ranging from 100 to 500 nm in diameter (Figure 2.6).

2.6.3 Methods of Preparation of Liposomes

Numerous procedures have been developed to prepare liposomes. There are at least fourteen Major published methods for making liposomes (Ostro, 1988; Martin, 1990). The seven, most commonly employed methods are, Lipid film hydration method (Bangham et al., 1965), Ethanol injection method (Batzri and Korn, 1973), Ether infusion method (Deamer and Bangham, 1976), Detergent dialysis method (Kagawa and Racker, 1971), French press method (Barenholz et al., 1979), Rehydration-dehydration techniques (Shew and Deamer, 1985) and Reverse phase evaporation method (Cortesi et al., 1999).

2.6.4 Characterization of Liposomes (New, 1990)

The behavior of liposomes in both physical and biological systems is determined to a large extent by factors such as physical size, chemical composition, quantity of entrapped solutes etc. Hence, liposomes are characterized with respect to the following parameters:

2.6.4.1 Size and Size Distribution

There are number of methods reported in the literature to determine size and its distribution of the vesicles (Bangham et al., 1974; Meeren et al., 1992). The most commonly used ones are light microscopy preferably using electron microscope, laser light scattering or cryoelectron microscopy.

2.6.4.2 Lamellarity

The lamellarity, the average number of bilayers present in liposomes, can be determined either by ^{31}P -NMR spectroscopy or freeze fracture electron microscopy.

2.6.4.3 Determination of Percentage Capture

The quantity of material entrapped inside liposomes can be determined more commonly by mini-column centrifugation method, protamine aggregation method, dialysis technique or by gel chromatography.

2.6.5 Stability of Liposomes

A prerequisite for the successful introduction of liposomes in therapy is the long-term stability of the formulation. The stability of drug-laden liposome dispersions preferably should meet the standards of conventional pharmaceutical product. A 1-

year shelf life is considered to be an absolute minimum. Both chemical and physical determines the shelf life of a product.

In the literature, on the physical stability of liposomes, attention has been focused on two processes affecting the quality and therefore acceptability of liposomes (Talsma and Cormmelin, 1993). First, the encapsulated drug can leak from the vesicles into the extra-liposomal compartment (reduced retention). Second, liposomes can aggregate and/or fuse, forming larger particles. Both these processes change the disposition of the drug in vivo and thereby presumably affect the therapeutic index of the drug involved. Besides, other physical parameters may also change during storage. For instance, hydrolysis of phospholipids causes the formation of fatty acids and lysophospholipids. These compounds considerably affect the physical properties of the bilayer (Talsma and Cormmelin, 1993). Apart from this, chemical degradation process may influence the safety of liposomes. Solid experimental data on the safety of partially hydrolyzed liposomes are not yet available; lysophospholipids alone have been reported to be toxic.

Several approaches have been developed to ensure the physical stability of liposomes on storage.

9. 1. For storage of aqueous dispersions, the lipid composition of the bilayer and the aqueous solvent can be adjusted to induce optimum stability by reducing permeability/leakage. Phospholipids with long and saturated alkyl chains (distearoyl phosphatidyl choline and dipalmitoyl phosphotidyl choline or saturated hydrogenated soyabeen or egg phosphotidyl choline) provide rigid bilayers with low permeabilities for small, non-bilayer-interacting compounds (Talsma and Cormmelin, 1993). The incorporation of the bovine serum albumin in the liposomal membrane and treatment with glutaraldehyde has been reported to prevent leakage of the entrapped contents (Law et al., 1994). Crommelin has reported the effect of bilayer composition on permeability of carboxyfluorescein in (Crommelin and Van Bommel, 1984).

To formulate drugs in liposomes it is necessary to reduce the leakage of an entrapped drug. The rate of leakage of a molecule from liposomes is governed by the physio-chemical properties of a molecule. Liposomes are freely permeable to water, but cations are released at a slower rate than anions (Bangham et al., 1965), whereas

aqueous hydrogen bonding may determine the leakage rate of non-electrolytes (Cohen, 1975).

Phospholipids in the liquid-crystalline state are more permeable to entrapped material than when they are in the gel state. Thus, loss of entrapped material is temperature dependent, generally being greatest around the phospholipid phase transition temperature (T_c) (Papahadjopoulos et al., 1973). The stability of liposomes in terms of retention of dideoxyinosine triphosphate (ddITP) was measured by Betageri (Betageri, 1993) at 4°, 25°, and 37°C. He observed that retention of ddITP in liposomes was maximum when stored at 4°C followed by 25°C and 37°C.

Another way to control stability is to incorporate cholesterol into the lipid structure, since it is known to reduce leakage of various solutes through the lipid bilayer when the membrane is in a fluid-like state (Gregoriadis and Davis, 1979; Scherphof et al., 1984), or by polymerization of phospholipid molecules (Johnston and Chapman, 1984; Scherphof et al., 1981). The introduction of cholesterol in liposomes of 5,6-carboxyfluorescein (CF) has been reported to reduce the rate of leakage during storage (Hernandez-Caselles et al., 1990). He also observed that CF retention was greater in liposomes stored at 4°C in the presence of O_2 than those of room temperature, although liposomes stored at room temperature but in O_2 -free atmosphere were more stable than those stored at room temperature in the presence of O_2 . Key

2. Freezing the liposome dispersion is also an approach to achieve prolonged liposome shelf-life (Talsma et al, 1992(b)). Lyophilization and rehydration, which include a freezing and thawing cycle, represent another method, used by many laboratories for better stability of liposomal formulations (Venkataram et al., 1990). Several groups have published reports on freezing, drying (Hauser and Strauss, 1987) or freeze-drying of liposomes. Cryoprotectants play an important role in the physical stabilization of liposomes during freezing, drying or freeze-drying. The 100% CF retention could be found (Talsma and Cormmelin, 1993) using cryoprotectant after a full freezing-thawing cycle. Studies made on the stability of liposomes with time, when they were either freeze-dried or in solution have been reported (Crommelin and Van Bommel, 1984).

3. In addition, two other techniques can solve the problem of drug leakage during storage, proliposomes and remote loading (Talsma and Cormmelin, 1993) that permit liposome dispersion preparation in situ. Several reports have been published in this context. Chemical analysis mainly concerns hydrolysis of the ester bonds in phospholipids and oxidation of their unsaturated acyl chains if present. Hydrolysis of phospholipid to free fatty acid and lysophospholipids can disturb the phospholipid bilayer structure and may disrupt it, leading to leakage of encapsulated products. Oxidation of unsaturated phospholipids and cholesterol may be initiated by the action of light and heavy metals (New, 1990). According to Hernandez-caselles (Hernandez-Caselles et al., 1990), the presence of A-tocopherol decreased the breakdown of phosphatidyl choline to lysophosphatidyl choline and also reduced the level of peroxidation. Although the mechanism of the action of α -tocopherol is not clear, it is suggested that this may happen through specific binding to the phospholipid molecule (Villalain et al., 1986). α -tocopherol acetate was found to be much less effective than α -tocopherol in preventing lipid peroxidation (Fukuzawa et al., 1981). Further information about chemical stability can be found in reviews of hydrolytic and oxidation reactions in phospholipids (Talsma and Cormmelin, 1993).

2.6.6 Liposomes as Drug Delivery Systems

In the recent past, controlled release concept and technology have received increasing attention in the face of growing awareness to toxicity and ineffectiveness of drugs when administered or applied by conventional methods. Liposomes as drug delivery systems are among research topics that are being vigorously investigated in both academic and industrial laboratories, with different outlooks and common goals and end products. The scientific literature is rich with comprehensive review of liposomes as drug delivery systems (Gulati et al., 1998; Perugini and Pavanetto, 1998).

Over the last twenty years, the liposome has changed its status from being a novel plaything for the laboratory worker to a powerful tool for an industrialist with the gap between the ideal desired characteristics of liposomes and what is technically feasible becoming narrower all the time. Vastly improved technology in terms of drug capture, vesicle stability on storage, scale-up production and the design of formulations for special tasks has facilitated the application of a wide range of drugs in the treatment and prevention of diseases in experimental animals and clinically.

Liposomes may prove to be efficient carrier for targeting the drug to the site of action because of the following properties: Amphiphilic nature, flexibility in structural characteristics, localized drug effect, controllability of drug release rate, stability in vivo, direct cell liposome interaction, sterilizability, ability to protect drug and body from each other, non-toxicity, non immunogenicity, biocompatibility and biodegradability and accommodation of molecules with wide range of solubility and molecular weight. At the same time, there are certain problems associated with liposome as drug delivery systems (Deasy, 1984) such as difficulty in procuring pure phospholipids, difficulty in scale-up, poor stability over a long shelf-life, expensive, batch to batch variation in performance, low drug loading, difficulty in avoiding the reticulo-endothelial system and possibility of unwanted vascular obstruction caused by large liposomes. However, research into the use of liposomes in drug delivery has led to vastly improved technology in terms of drug capture, vesicle stability, storage, scaled up production and the design of formulations for specialized tasks. Table 2.5 shows the liposome application according to their mode of action.

Due to their high degree of biocompatibility, liposomes were initially considered as delivery systems for intravenous administration. The first parenterally applied formulation Ambisome (Vestar Inc., San Dimas, CA), a liposomal amphotericin formulation for the treatment of disseminated fungal infections that frequently occur in immunosuppressed patients, was launched in Ireland in 1990 that showed both high therapeutic activity and reduced toxicity (Talsma and Cormmelin, 1992(a)) as compared to the original product. More recently in 1995, a sterically stabilized liposomal formulation containing the anticancer drug, doxorubicin (Lasic, 1993) has been launched in United States.

It has since become apparent that liposomes can also serve as an effective tool for other delivery systems that include oral (Sveinsson and Holbrook, 1993), ophthalmic (Velpandian et al., 1999), aerosol (Conley et al., 1997), dermal/transdermal (Fresta and Puglisi, 1997; Trafny et al., 1999) applications, as immunological adjuvants, as carriers of antigens, leishmaniasis, lysosomal storage diseases, cell biological application etc. The recent research is concentrated on the use of liposomes to deliver hemoglobin and act as red blood cell substitutes. The scientists are also engaged in designing of liposomal prodrug using principle of specific enzyme cleavage and

facilitated spontaneous hydrolysis. Another field of liposomal research in producing sterically stabilized liposomes for prolonged circulation in blood stream. Liposomes are currently being studied as drug carriers for a variety of drugs that include recombinant proteins (Sugarman and Perez-Soler, 1992), gene transfer and immuno diagnostic applications (Tolstoshev, 1993). Of these, non-invasive route of administration continuously demands significant efforts in designing the liposomes that will no doubt continue to contribute significantly to more efficient use of "old drugs" with better and established therapeutic index vis-a-vis minimum side effects.

Table 2.5 Major modes of liposomal action and related applications

Mode of action	Application
Intracellular uptake (lysosomes, endosomes/cytoplasm)	Microbial disease, Metal storage-disease, Gene manipulation, uptake by some tumour, cells, macrophage activation to a tumoricidal/microcidal state, efficient antigen presentation by antigen presenting cells (vaccines).
Slow release of drugs near the target area	Tumors near fixed macrophages.
Avoidance of tissue, sensitive to drugs	Cardio toxicity of doxorubicin
Circulating reservoirs	Blood surrogates
Facilitation of drug uptake by certain routes	Drug delivery to skin, lungs, eyes, mucosal tissues.

2.6.7 Liposomes in Drug Delivery to Liver Cells

Liposomes are formed through hydration of amphiphilic lipids. In these microscopic vesicles, an aqueous lumen is surrounded by one or more lipid bilayers. Most preparations of liposomes for drug delivery purposes are made from neutral or anionic phospholipids with addition of cholesterol to stabilise the liposomal membrane. This liposomal composition resembles the naturally occurring cell membrane, and thus liposomes are considered to be biocompatible, i.e. they are biodegradable, non-immunogenic and nontoxic. Because the liposomal structure comprises an aqueous phase and a lipid phase, it can accommodate both water- and lipid-soluble substances. Water-soluble compounds can be encapsulated in the aqueous inner part of the vesicle while lipophilic drugs can be accommodated in the lipid bilayer. Additionally, molecules such as antibodies, proteins and sugar groups can be coupled relatively easily to the surface of the liposomes to target them to specific tissues and cells. Polymers such as polyethyleneglycol (PEG) attached to the liposomal membrane prolong the circulation time of these particles in the blood. In principle, liposomes

change the pharmacokinetic properties and the biodistribution of the encapsulated drugs; they often prolong the circulation time in the blood and may enhance the deposition and internalisation at the target site. In addition, liposomes protect the carried drug from degradation in the blood stream while they safeguard the rest of the body from the encapsulated potentially toxic drugs.

Intravenously injected liposomes smaller than 1 μm readily accumulate in the liver, where they end up predominantly in KCs. However, depending on their size and lipid composition as well as on surface modification, they can be re-targeted to other types of liver cells, including hepatocytes and LEC. Therefore, in liver diseases such as fibrosis, showing a pathology that involves all major population of liver cells, delivery of drugs to particular types of the cells, using specifically targeted liposomes, provide the possibility to interfere simultaneously with different processes that occur during disease development.

2.6.7.1 Liposomes Targeted to Hepatocytes

To reach hepatocytes from the blood stream, liposomes have to pass the fenestrations in the endothelial cells. This limits the size of the liposomes that can be taken up by hepatocytes to about 150 nm. Indeed, small unilamellar vesicles with diameters around 50 nm and composed of neutral lipids, accumulate predominantly in hepatocytes (Spanjer et al., 1986). The interaction of liposomes with hepatocytes might be mediated by the apolipoprotein E (ApoE) remnant receptor, leading to the endocytosis of liposomes, and the scavenger receptor B-1 (also known as the HDL receptor) resulting in selective transfer of lipids (Scherphof et al., 2001; Yan et al., 2004; Yan et al., 2005(a)).

Interestingly, liposomes with a relatively large size of 200 – 400 nm, containing the negative lipid phosphatidylserine (PS), accumulated in large amounts in hepatocytes (Daemen et al., 1997). This phenomenon was not observed when PS was replaced by another negatively charged lipid, phosphatidylglycerol (PG). The uptake mechanism of large PS-containing liposomes by hepatocytes is not fully understood, but it is possible that either PS exerts a pharmacological effect on the dimensions of the endothelial fenestrations or that PS-containing liposomes, due to their weak interaction with endothelial cells are squeezed through the fenestrations by the blood cells (Romero et al., 1999).

The active targeting of liposomes to hepatocytes can be achieved by modifying the liposomal surface with ligands of the asialoglycoprotein receptor (ASGPr), such as galactose and N-acetylgalactosamine (Hattori et al., 2000; Rensen et al., 2001; Sliedregt et al., 1999). ASGPr on the surface of hepatocytes mediates the clearance process of desialylated proteins from the blood. The efficiency of the uptake of galactosylated liposomes by hepatocytes depends on the density of galactose groups attached to liposomes. In addition, the size of liposomes modified with galactose moieties seems to be critical for the interaction with hepatocytes through ASGPr. Galactosylated liposomes smaller than 70 nm are taken up by hepatocytes, larger ones do accumulate in KCs (Rensen et al., 2001). In the optimal interaction with ASGPr, parameters like the clustering of galactose moieties (tetraantennary or monoantennary) and an appropriate spacing of the sugar residues play a role as well (Sliedregt et al., 1999).

Recently, targeting of liposomes to hepatocytes was demonstrated using a peptide sequence originating from a surface protein of *Plasmodium*, a protozoan causing infections in humans (Longmuir et al., 2006). The 19-amino acid peptide from the circumsporozoite protein contained a heparan sulphate proteoglycan binding sequence which is recognised by the highly sulphated heparan sulphate proteoglycans located on the basolateral side of hepatocytes. Systemically injected liposomes modified with this peptide mainly accumulated in hepatocytes.

2.6.7.2 Liposomes and Kupffer Cells

KCs are specialised cells that clear the blood from foreign particles, microorganisms, and senescent blood cells. Also, most of the liposomal preparations injected intravenously are readily taken up by these cells. As a result, researchers tend to make an effort to develop strategies which allow liposomes to escape from Kupffer cell accumulation, rather than actively target liposomes to these cells. Reduction of liposome sizes from 800 nm to around 100 nm decreases Kupffer cell uptake but it does not eliminate accumulation in these cells. The mechanism underlying the fast blood elimination of liposomes by KCs is called opsonization. Once liposomes enter the blood circulation, they absorb a broad spectrum of plasma proteins. The amount and type of adhered proteins is determined by the physicochemical properties of the liposomes, including the size, lipid composition and surface charge. The adsorbed

plasma proteins that mediate the specific interaction of liposomes with receptors on the macrophage are called opsonins. In principle, opsonins adsorbed on the surface of liposomes mark them for fast recognition and enhanced uptake by KCs. Liposomal opsonins are classified in two groups: immune opsonins and non-immune opsonins (Yan et al., 2005(b)). The immune opsonins mainly include complement proteins and immunoglobulins (antibodies), which identify liposomes as foreign particles and mark them for uptake by the MPS. The second class of opsonins are proteins such as fibronectin, α 2-macroglobulin and apolipoproteins which are ligands that direct liposomes to specific receptors on the macrophage cell membrane.

In order to prevent rapid blood elimination and accumulation in KCs, liposomes can be surface-grafted with a hydrophilic polymer, polyethylene glycol (PEG). The flexibility of PEG allows a relatively small number of surface-grafted polymer molecules to create a protective layer. Thus PEGylated liposomes are characterised by a significantly extended circulation time. This property is attributed to the decreased adsorption of opsonins from the blood on the liposomal surface, due to the highly flexible hydrophilic steric barrier provided by the polymer brushes. The development of long-circulating liposomes in the early 1990's was considered a breakthrough in the liposomal field, and resulted, among others, in a liposomal formulation of doxorubicin approved for regular clinical use (Doxil/Cealyx) (Allen and Cullis, 2004).

2.6.7.3 Targeting Liposomes to Liver Endothelial Cells

Although LEC have direct contact with blood, significant accumulation of conventional liposomes in these cells does not occur (Spanjer et al., 1986). Interesting results were observed when the uptake of negatively charged liposomes containing PS in vivo was compared with that in primary cultures of LEC. The contribution of LEC in the uptake of PS-containing liposomes after intravenous injection was minimal, while accumulation of these liposomes by cultured LEC was almost equal to that of KCs (Kamps et al., 1999). These in vitro observations were confirmed when uptake of PS-containing liposomes was studied in the serum-free perfused rat liver (Rothkopf et al., 2005). Polyinosinic acid, a competitive inhibitor of ScR reduced the association of PS containing liposomes with cultured LEC, as well as the uptake in the serum-free perfused livers, suggesting involvement of ScR in this process. These findings demonstrated that LEC have a high capacity to take up liposomes. However,

accumulation of PS-containing liposomes by LEC in vivo might be inhibited by “dysopsonins”, that mask the PS for receptor recognition.

LEC are known to abundantly express different classes of scavenger receptors (ScR) including class A I and II, class B ScR-B I and CD 36, all known to recognise anionic domains. As a matter of fact, massive targeting of liposomes to LEC in vivo was achieved by coupling a poly-anionic molecule, aconitylated human serum albumin (AcoHSA), to the liposomal surface (Kamps et al., 1997). Inhibition of the in vivo uptake of AcoHSA by polyinosinic acid also indicated that this association is specifically mediated by ScR. Application of AcoHSA as a targeting ligand to LEC was successfully applied in the preparation of stabilised lipid coated lipoplexes, that were shown to efficiently deliver functionally active antisense oligonucleotides to LEC in vivo (Bartsch et al., 2004).

Injured hepatocytes, activated KCs and endothelial cells release broad spectrum of cytokines and other substances such as ROS which induce inflammation and oxidative stress as well as activate HSC in the fibrotic liver. These processes perpetuate development of fibrosis and contribute to the liver failure. Liposomal drugs selectively targeted to hepatocytes, KCs and endothelial cells may be used for specific inhibition of proinflammatory actions in these cells simultaneously with antifibrotic compounds directed to HSC.

2.7 FORMULATION OPTIMIZATION

An experimental approach to Design of Experiment (DoE) optimization of drug delivery systems (DDS) comprises several phases. (Kannan et al., 2003(a); Singh et al., 2004(b); Doornbos and de Haan, 1995; Myers et al., 2003(b)). Broadly, these phases can be sequentially summed up in seven salient steps. Figure 2.7 delineates these steps pictographically.

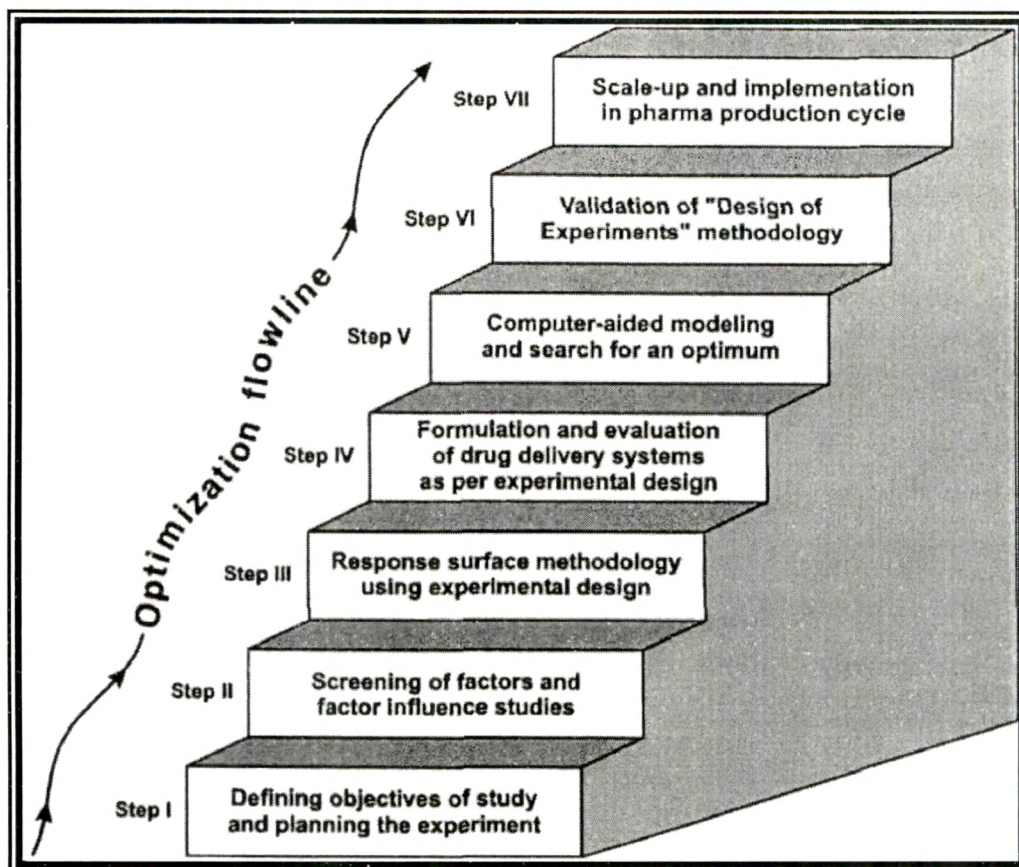


Figure 2.7 Seven-step ladder for optimizing drug delivery systems

The optimization study begins with:

- **Step I**, where an endeavor is made to ascertain the initial drug delivery objective(s) in an explicit manner. Various main response parameters, which closely and pragmatically epitomize the objective(s), are chosen for the purpose.
- In **Step II**, the experimenter has several potential independent product and/or process variables to choose from. By executing a set of suitable screening techniques and designs, the formulator selects the "vital few" influential factors among the possible "so many" input variables. Following selection of these factors, a factor influence study is carried out to quantitatively estimate the main effects and interactions. Before going to the more detailed study, experimental studies are undertaken to define the broad range of factor levels as well.

- During **Step III**, an opposite experimental design is worked out on the basis of the study objective(s), and the number and the type of factors, factor levels, and responses being explored. Working details on variegated vistas of the experimental designs, customarily required to implement DoE optimization of drug delivery, have been elucidated in the subsequent section. Afterwards, response surface modeling (RSM) is characteristically employed to relate a response variable to the levels of input variables, and a design matrix is generated to guide the drug delivery scientist to choose optimal formulations.
- In **Step IV**, the drug delivery formulations are experimentally prepared according to the approved experimental design, and the chosen responses are evaluated.
- Later in **Step V**, a suitable mathematical model for the objective(s) under exploration is proposed, the experimental data thus obtained are analyzed accordingly, and the statistical significance of the proposed model discerned. Optimal formulation compositions are searched within the experimental domain, employing graphical or numerical techniques. This entire exercise is invariably executed with the help of pertinent computer software.
- **Step VI** is the penultimate phase of the optimization exercise, involving validation of response prognostic ability of the model put forward. Drug delivery performance of some studies, taken as the checkpoints, is assessed vis-a-vis that predicted using RSM, and the results are critically compared.
- Finally, during **Step VII**, which is carried out in the industrial milieu, the process is scaled up and set forth ultimately for the production cycle.

The niceties of the significance and execution of each of these seven steps is discussed in greater detail below.

The foremost step while executing systematic DoE methodology is to understand the deliverables of the finished product. This step is not merely confined to understanding the process performance and the product composition, but it usually goes beyond to enfold the concepts of economics, quality control, packaging, market research, etc.

The term objective (also called criterion) has been used to indicate either the goal of an optimization experiment or the property of interest (Schwartz and Connor, 1996; Doornbos and de Haan, 1995). The objectives for an experiment should be clearly

determined after discussion among the project team members having sound expertise and empiricism on product development, optimization, production, and/or quality control. The group of scientists contemplates the key objectives and identifies the trivial ones. Prioritizing the objectives helps in determining the direction to proceed with regard to the selection of the factors, the responses, and the particular design (Kannan et al., 2003(a); Myers et al., 2003(b); Kannan et al., 2003(b)). This step can be very time consuming and may not furnish rapid results. However, unless the objectives are accurately defined, it may be necessary to repeat the entire work that is to follow. The response variables, selected with dexterity, should be such that they provide maximal information with the minimal experimental effort and time. Such response variables are usually the performance objectives, such as the extent and rate of drug release, or are occasionally related to the visual aesthetics, such as chipping, grittiness, or mottling (Singh et al., 2004(b)).

The word 'optimize' simply means to make as perfect, effective, or functional as possible (Lewis et al., 2002; Schwartz and Connor, 1996). The term optimized has been used in the past to suggest that a product has been improved to accomplish the objectives of a development scientist. However, today the term implies that DoE and computers have been used to achieve the objective(s). With respect to drug formulations or pharmaceutical processes, optimization is a phenomenon of finding the best possible composition or operating conditions (Lewis et al., 2002; Lewis et al., 1999). Accordingly, optimization has been defined as the implementation of systematic approaches to achieve the best combination of product and/or process characteristics under a given set of conditions (Singh and Ahuja, 2004(a)).

2.7.1 Variables

Design and development of any drug formulation or pharmaceutical process invariably involves several variables (Lewis et al., 2002; Schwartz et al., 1973; Stetsko, 1986). The input variables, which are directly under the control of the product development scientist, are known as independent variables - e.g., drug content, polymer composition, compression force, percentage of penetration enhancer, hydration volume, agitation speed. Such variables can either be quantitative or qualitative (Doornbos and de Haan, 1995; Bolton, 1997(a)). Quantitative variables are those that can take numeric values (e.g., time, temperature, amount of polymer,

osmogen, plasticizer, super disintegrants) and are continuous. Instances of qualitative variables, on the other hand, include the type of polymer, lipid, excipient, or tableting machine. These are also known as categorical variables (Lewis et al., 1999; Anderson et al., 2002). Their influence can be evaluated by assigning discrete dummy values to them. The independent variables, which influence the formulation characteristics or output of the process, are labeled factors (Lewis et al., 1999; Cochran and Cox, 1992; Bolton, 1997(a)). The values assigned to the factors are termed levels - e.g., 100 mg and 200 mg are the levels for the factor, release rate controlling polymer in the compressed matrices. Restrictions imposed on the factor levels are known as constraints (Schwartz and Connor, 1996; Bolton, 1997(a)).

The characteristics of the finished drug product or the in-process material are known as dependent variables - e.g., drug release profile, percent drug entrapment, pellet size distribution, moisture uptake (Lewis et al., 1999; Doornbos and de Haan, 1995; Box et al., 1960). Popularly termed response variables, these are the measured properties of the system to estimate the outcome of the experiment. Usually, these are direct function(s) of any change(s) in the independent variables.

Accordingly, a drug formulation (product), with respect to optimization techniques, can be considered as a system whose output (Y) is influenced by a set of input variables via a transfer function (T) (Montgomery, 2001). These input variables may either be controllable (X; signal factors) or uncontrollable (U; noise factors) (Doornbos and de Haan, 1995; Taguchi, 1987). Figure 2.8 depicts the same graphically.

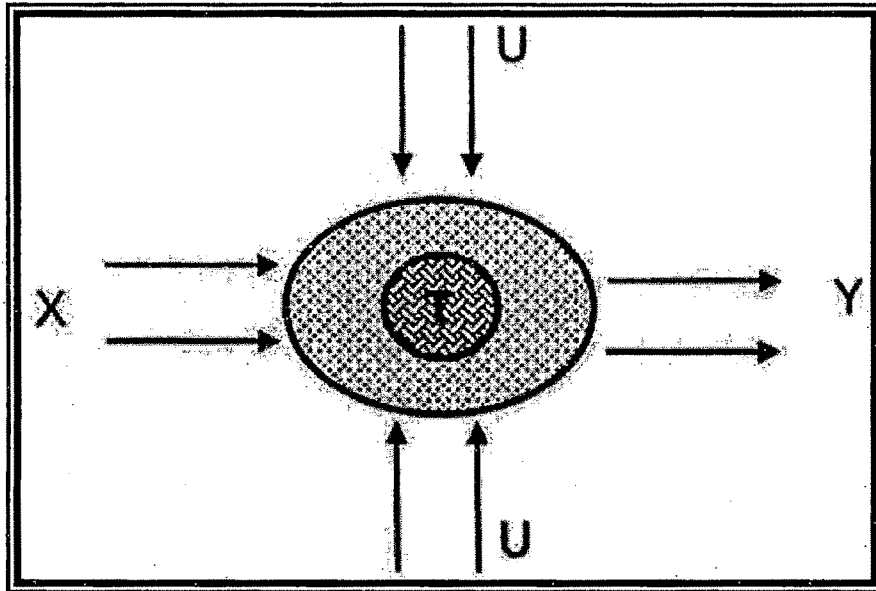


Figure 2.8 System with controllable input variables (X), uncontrollable input variables (U), transfer function (T), and output variables (Y)

The nomenclature of T depends upon the predictability of the output as an effect of the change of input variables. If the output is totally unpredictable from the previous studies, T is termed the black box. The term white box is used for a system with absolutely true predictability, while the term gray box is used for moderate predictability. Using optimization methods, the attempt of the formulator is to attain a white box or nearly white box status from the erstwhile black or gray box status observed in the traditional studies (Singh and Ahuja, 2004(a)). The greater the number of variables in a given system, the more complicated becomes the job of DoE optimization. Nevertheless, regardless of the number of variables, a distinct relationship exists between a given response and the factors studied (Lewis et al., 1999).

2.7.2 Effect, Interaction, and Confounding

The magnitude of the change in response caused by varying the factor level(s) is termed as an effect (Cochran and Cox, 1992; Bolton, 1997(a)). The main effect is the effect of a factor averaged over all the levels of other factors.

However, an interaction is said to occur when there is "lack of additivity of factor effects." This implies that the effect is not directly proportional to the change in the

factor levels (Bolton, 1997(a)). In other words, the influence of a factor on the response is nonlinear (Lewis et al., 2002; Lewis et al., 1999; Montgomery, 2001; Stack, 2003). In addition, an interaction may said to take place when the effect of two or more factors are dependent on each other - e.g., the effect of factor A changes on changing factor B by one unit. The measured property' of the interacting variables depends not only on their fundamental levels, but also on the degree of interaction between them. Depending upon whether the change in the response is desired (positive) or undesired (negative), the phenomenon of interaction may be described as synergism or antagonism, respectively (Lewis et al., 1999; Bolton, 1997(a)). Figure 2.9 illustrates the concept of interaction graphically.

Effects plot is plotted between the magnitude of various coefficients for the effects and/or interactions against the response variable (Lewis et al., 1999). The plot is drawn during the initial stages of DoE to determine the influence of each term.

The term orthogonality is used if the estimated effects are due to the main factor of interest and are independent of interactions (Kettaneh-Wold, 1991; Bolton, 1997(a); Myers and Montgomery, 1995; Box and Draper, 1987). Conversely, lack of orthogonality (or independence) is termed confounding or aliasing (Bolton, 1997(a); Stack, 2003). When an effect is confounded (or aliased, or mixed up, or equalled), one cannot assess how much of the observed effect is due to the factor under consideration. The effect is influenced by other factors in a manner that cannot easily be explored. The measure of the degree of confounding is known as resolution (Montgomery, 2001; Myers and Montgomery, 1995). At times, there is confusion between confounding and interaction. Confounding, in fact, is a bias that must be controlled by suitable selection of the design and data analysis. Interaction, on the other hand, is an inherent quality of the data, which must be explored. Confounding must be assessed qualitatively, while interaction may be tested more quantitatively (Stack, 2003).

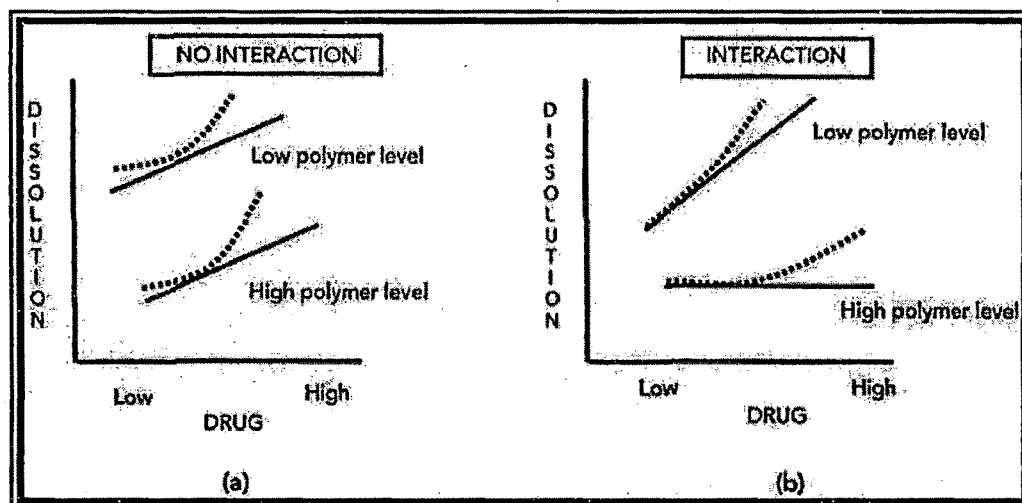


Figure 2.9 Diagrammatic depiction of interaction. Unparallel lines in (b) describe the phenomenon of interaction between the levels of drug and polymer amount affecting drug dissolution [(—); Linear response-factor relationship; (....): nonlinear response-factor relationship]

Subsequent to ascertaining the study objectives and responses, "several possible" factors are envisioned and screening of a "few important" ones is done. The influence of the important factors - i.e., the main effects and the possible interactions are also studied. Collectively, screening and factor influence studies are also known as factor studies (Lewis et al., 2002). Often carried out as a prelude to finding the optimum, these are sequential stages in the development process. Screening methods are used to identify important and critical effects (Lewis et al., 1999; Myers et al., 2003(b)). Factor studies aim at quantitative determination of the effects as a result of a change in the potentially critical formulation or process parameter(s). Such factor studies usually involve statistical experimental designs, and the results so obtained provide useful leads for further response optimization studies. 1. Screening of Influential Factors

As the term suggests, screening is analogous to separating "rice" from "rice husk," where rice is a group of factors with significant influence on response, and husk is a group of the rest of the noninfluential factors (Singh et al., 2004(b)). A product development scientist normally has numerous possible input variables to be investigated for their impact on the response variables. During the initial stages of optimization, such input variables are explored for their influence on the outcome of

the finished product to see if they are factors (Lewis et al., 2002; Lewis et al., 1999; Murphy, 2003). The process, called screening of influential variables, is a paramount step. An input variable, identified as a factor, increases the chance of success, while an input variable that is not a factor has no consequence (Doornbos and de Haan, 1995). Furthermore, an input variable falsely identified as a factor unduly increases the effort and cost, while an unrecognized factor leads to an erroneous picture, and a true optimum may be missed.

Principally, screening embarks upon the phenomenon of sparsity effect - i.e., only a few of the factors among the numerous envisioned ones truly explain a larger proportion of the experimental variation (Montgomery, 2001; Anonymous, 2002). The factors responsible for the variability are the active or influential variables, while others are termed inactive or less influential variables. The entire exercise aims solely at selecting the active factors and excluding the redundant variables, but not at obtaining complete and exact numerical data on the system properties. Such a reduction in the number of factors becomes necessary before the pharmaceutical scientist invests the human, financial, and industrial resources in more elaborate studies (Lewis et al., 2002; Myers et al., 2003(b)). This phase may be omitted if the process is known well enough from the analogous studies. Even after elimination of the non influential variables, the number of factors may, at times, still be too large to optimize in terms of available resources of time, money, manpower, equipment, etc (Lewis et al., 2002). In such cases, more influential variables are optimized, keeping less influential ones as constant at their best levels. The number of experiments is kept as small as possible to limit the volume of work carried out during the initial stages.

2.7.2.1 Screening Designs

The experimental designs employed for this purpose are commonly termed screening designs (Myers et al., 2003(b); Murphy, 2003). Screening presumes considerable approximation of the additivity of the different factors and the absence of interaction. Therefore, the primary purpose of the screening design is to identify significant main effects, rather than interaction effects. Thus, these are usually first-order designs with low resolution (Armstrong and James, 1990). These designs are also sometimes termed main effects designs or orthogonal main effect plans or simply orthogonal arrays (Lewis et al., 1999). The number of experiments in the screening process is

kept small, but it must at least be equal to the number of independent coefficients (P) required to be calculated, as in following equation:

$$P = 1 + \sum_{i=1}^k (S_i - 1)$$

where S_i is the number of levels of the i^{th} factor, when there are k factors in all (Lewis et al., 1999). The estimators of the coefficients should be orthogonal and be estimated with minimum possible error. In general, in order to determine main effects independently, the number of runs should be four times the number of factors to be estimated. The experimental designs are said to be saturated if the number of runs equals the number of model terms to be estimated (Anonymous, 2002). In cases where a larger number of factors need to be screened, the number of runs becomes exorbitantly high. In such circumstances, supersaturated designs, which possess fewer runs than factors, are used. Supersaturated designs can be attractive for factor screening, especially when there are many factors and/or the experimental runs are expensive. A supersaturated design can examine dozens of factors using fewer than half the number of runs. This is usually at the expense of the precision and accuracy of the information. The mathematical models normally considered for screening include the linear and interaction models already described by Eqs. (1) and (2). (Lewis et al., 2002; Myers et al., 2003(b); Murphy, 2003). A two-level screening design can be augmented to a high-level design by adding axial points along with center points.

2.7.2.2 Factor Influence Study

Having screened the influential variables, a more comprehensive study is subsequently undertaken, with the main aim to quantify the effect of factors and to determine the interactions, if any (Lewis et al., 2002; Lewis et al., 1999; Myers and Montgomery, 1995). Herein, the studied experimental domain is less extensive, as many fewer active factors are studied. The models used for this study are neither predictive nor capable of generating a response surface. The number of levels is usually limited to two (i.e, the factors are investigated at the extreme values). However, sufficient experimentation is carried out to allow for the detection of interactions among factors (Lewis et al., 1999; Box et al., 1978). The experimental designs used are generally of the same kind as used for screening. The experiments

conducted at this step may often be "reused" during the optimization or response modeling phase by augmenting the experimental designs with additional design points at the center or the axes. Central points (i.e., at the intermediate level), if added at this stage, are not included in the calculation of model equations (Lewis et al., 2002). Nevertheless, they may prove to be useful in identifying the curvature in the response, in allowing the reuse of the experiments at various stages, and if replicated, in validating the reproducibility of the experimental study.

2.7.3 Coding

The process of transforming a natural variable into a nondimensional coded variable, X_i , so that the central value of experimental domain is zero is known as coding (or normalization) (Cochran and Cox, 1992; Bolton, 1997(a); Das and Giri, 1994).

Generally, the various levels of a factor are designated as -1, 0, and +1, representing the lowest, intermediate (central), and highest factor levels investigated, respectively (Lewis et al., 1999; Bolton, 1997(a)). For instance, if sodium carboxymethyl cellulose, a hydrophilic polymer, is studied as a factor in the range of 120-240 mg, then codes -1 and +1 signify 120 mg and 240 mg amounts, respectively. The code 0 would represent the central point at the arithmetic mean of the two extremes - i.e., 180 mg. Alternatively, for convenience, the factors and their levels have been denoted by alphabetic notation (symbol) to express various combinations investigated in the study. For example, a factor is denoted by a capital alphabet letter (say factor A), the high level by a, and low level as (-1). Table 2.6 illustrates the alphabetic denotations used in pharmaceutical literature for coding factors and their factor combinations at the respective levels.

Although the terminology for factors as A and B and their levels as (1), a, b, etc. is comprehensive in the text format, their translation into mathematical equation(s) is neither practical nor easy to comprehend (Singh and Ahuja, 2004(a)). Therefore, the symbol X_k is normally used for representing the factor X, where the subscript k depicts the number of factors (Doornbos and de Haan, 1995). Analogously, the subscripted p values are employed to denote the coefficient values in the mathematical equations.

Coding involves the orthogonality of effects and depicts effects and interaction(s) using (+) or (-) signs (Schwartz and Connor, 1996; Bolton, 1997(a)). It assigns equal

significance to each axis and allows not only easier calculation of coefficients and coefficient variances, but easier depiction of response surfaces as well.

Table 2.6 Denotation of various levels of two factors

Factor	Level notation	
	Low level	High level
A	-1	a
B	-1	b
AB	-1	ab

To circumvent any anomaly in factor sensitivity with change in levels, it is recommended that the factor coding must be carried out judiciously (Doornbos and de Haan, 1995; Bolton, 1997(a)). For instance, in the case of microsphere production, if one factor is stirring speed (say, within the range of 1500-3000 rpm) and the other is pH (say within the range of 1-5), a change of 1 pH unit is far more significant than a change of 1 rpm.

2.7.4 Experimental Domain

The dimensional space defined by the coded variables is known as factor space (Lewis et al., 1999; Armstrong and James, 1990). Figure 2.10 illustrates the factor space for two factors on a bi-dimensional (2-D) plane during the formulation of controlled release microspheres. The part of the factor space, investigated experimentally for optimization, is the experimental domain (Lewis et al., 1999; Das and Giri, 1994). Also known as the region of interest, it is enclosed by the upper and lower levels of the variables, the factor space covers the entire figure area and extends even beyond it, whereas the design space of the experimental domain is the square enclosed by $X_1 = \pm 1$, $X_2 = \pm 1$.

2.7.5 Experimental Design

The conduct of an experiment and the subsequent interpretation of its experimental outcome are the twin essential features of the general scientific methodology (Lewis et al., 2002; Armstrong and James, 1990). This can be accomplished only if the experiments are carried out in a systematic way and the inferences are drawn accordingly.

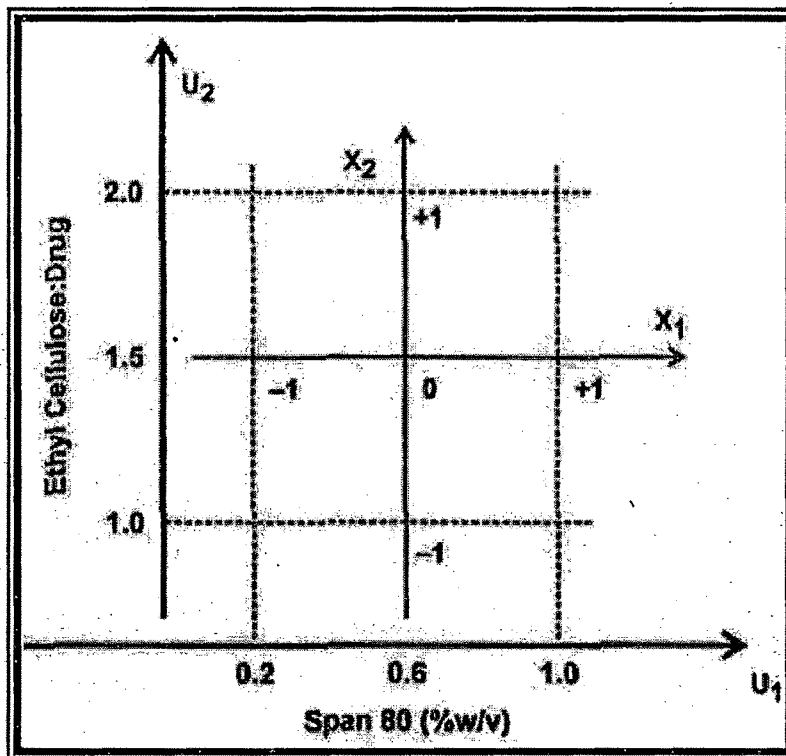


Figure 2.10 Quantitative factors and factor space (The axes for the natural variables, ethyl cellulose:drug ratio and Span 80 are labeled U_1 and U_2 and those of the corresponding coded variables X_1 and X_2)

An experimental design is the statistical strategy for organizing the experiments in such a manner that the required information is obtained as efficiently and precisely as possible (Haaland, 1989; Kettaneh-Wold, 1991; Cochran and Cox, 1992; Wehrle and Stamm, 1994). Runs or trials are the experiments conducted according to the selected experimental design (Lewis et al., 1999; Doornbos and de Haan, 1995). Such DoE trials are arranged in the design space so that the reliable and consistent information is attainable with minimum experimentation. The layout of the experimental runs in a matrix form, according to the experimental design, is known as the design matrix (Lewis et al., 1999). The choice of design depends upon the proposed model, the shape of the domain, and the objective of the study. Primarily, the experimental (or statistical) designs are based on the principles of randomization (i.e., the manner of allocations of treatments to the experimental units), replication (i.e., the number of units employed for each treatment), and error control or local control (i.e., the

grouping of specific types of experiments to increase the precision) (Montgomery, 2001; Cochran and Cox, 1992; Das and Giri, 1994).

DoE is an efficient procedure for planning experiments in such a way that the data obtained can be analyzed to yield valid and unbiased conclusions (Tye, 2004; Porter et al., 1997). An experimental design is a strategy for laying out a detailed experimental plan in advance to the conduct of the experimental studies (Araujo and Brereton, 1996; Armstrong and James, 1990; Haaland, 1989). Before the selection of experimental design, it is essential to demarcate the experimental domain within the factor space - i.e., the broad range of factor studies. To accomplish this task, first a pragmatic range of experimental domain is embarked upon and the levels and their number are selected so that the optimum lies within its realm (Singh and Ahuja, 2004(a)). While selecting the levels, one must see that the increments between them should be realistic. Too wide increments may miss finding the useful information between the levels, while a too narrow range may not yield accurate results (Singh et al., 2004(b)).

There are numerous types of experimental designs. Various commonly employed experimental designs for RSM, screening, and factor-influence studies in pharmaceutical product development are

- a) Factorial designs
- b) Fractional factorial designs
- c) Plackett-Burman designs
- d) Star designs
- e) Central composite designs
- f) Box-Behnken designs
- g) Center of gravity designs
- h) Equiradial designs
- i) Mixture designs
- j) Taguchi designs
- k) Optimal designs
- l) Rechtschaffner designs
- m) Cotten designs

For a three-factor study, an experimental design can invariably be envisaged as a "cube," with the possible combinations of the factor levels (low or high) represented at its respective corners (Tye, 2004). The cube thus can be the most appropriate representation of the experimental region being explored. Most design types discussed in the current article are, therefore, being depicted pictorially using this cubic model, with experimental points at the corners, centers of faces, centers of edges, and so forth. Such depiction facilitates easier comprehension of various designs and comparisons among them. For designs in which more than three factors are adjusted, the same concept is applicable except that a hypercube represents the experimental region. Such cubic designs are popular because they are symmetrical and straightforward for conceptualizing and envisioning the model.

2.7.5.1 Factorial Designs

Factorial designs (FDs) are very frequently used response surface designs (Araujo and Brereton, 1996; Bolton, 1997(a); Li et al., 2003(a)). A factorial experiment is one in which all levels of a given factor are combined with all levels of every other factor in the experiment (Lewis et al., 1999; Bolton, 1997(a); Acikgoz et al., 1996). These are generally based upon first-degree mathematical models. Full FDs involve studying the effect of all the factors (k) at various levels (x), including the interactions among them, with the total number of experiments being x^k . FDs can be investigated at either two levels (2^k FD) or more than two levels. If the number of levels is the same for each factor in the optimization study, the FDs are said to be symmetric, whereas in cases of a different number of levels for different factors, FDs are termed asymmetric (Lewis et al., 1999).

2.7.5.2 2^k Factorial Designs

The two-level FDs are the simplest form of orthogonal design, commonly employed for screening and factor influence studies (Bolton, 1997(a); Das and Giri, 1994). They involve the study of k factors at two levels only - i.e., at high (+) and low (-) levels. The simplest FD involves investigation of two factors at two levels only. Characteristically, these represent first-order models with linear response, as demonstrated in Figure 11 portrays a 2^2 and 2^3 FD, in which each point represents an individual experiment.

The design matrix for a two-level full factorial with k factors in the standard order can be generated in the following manner. The first column (X_1) starts with -1 followed by alternate sign for all 2^k runs. The second column (X_2) starts with -1 repeated twice, then alternates with 2 in a row of the opposite sign until all 2^k places are filled. The third column (X_3) starts with -1 repeated four times, then four repeats of +1, and so on. In general, the i^{th} column (X_i) starts with 2^{i-1} repeats of -1 followed by 2^{i-1} repeats of +1.

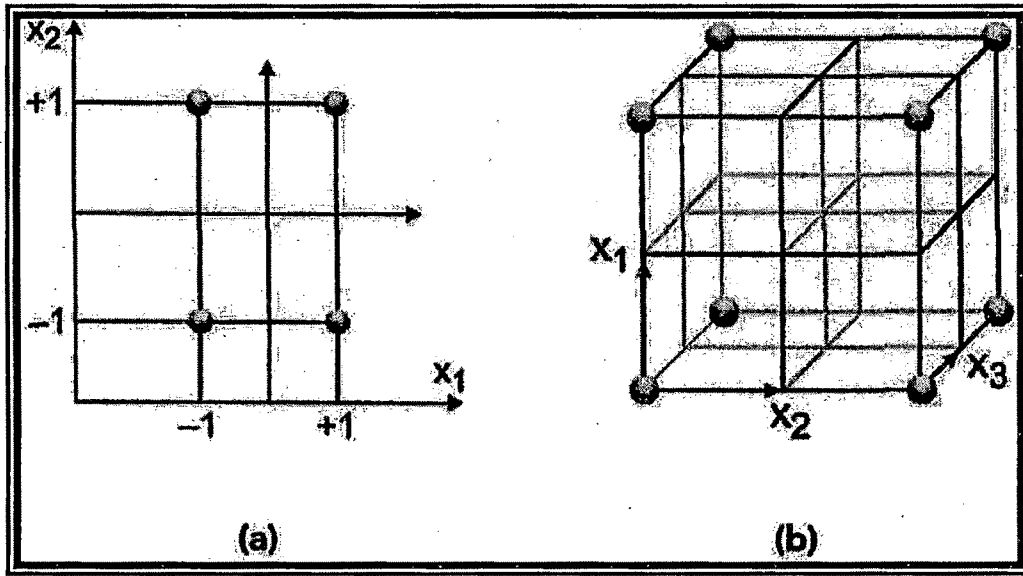


Figure 2.11 Diagrammatic representation of (a) 2^2 factorial design; (b) 2^3 factorial design

The mathematical model associated with the design consists of the main effects of each variable plus all the possible interaction effects - i.e., interactions between the two variables, and in fact, between as many factors as there are in the model (Lewis et al., 2002; Lewis et al., 1999; Box and Draper, 1987). Below equation is the general mathematical relationship for the FDs involving main effect and interaction terms.

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \sum_{j=i+1}^n \beta_{ij} X_i X_j + \sum_{i=1}^n \sum_{j=i+1}^n \sum_{k=i+2}^n \beta_{ijk} X_i X_j X_k$$

where n is the number of factors (3 in the above equation), X is +1 or -1 as per coding, Y is the measured response, and β_i , β_{ij} , β_{ijk} represent the coefficients

computed from the responses of the formulation in the design. For a 2^3 FD, the above equation can be written as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3$$

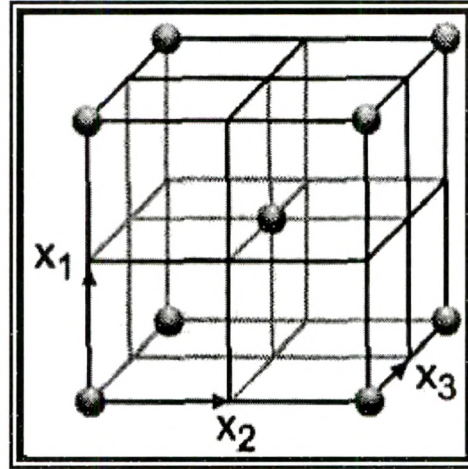
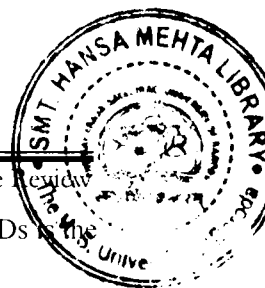


Figure 2.12 Diagrammatic representation of 2^3 factorial design with added center point

Center points can be added to 2^k FDs to allow identification of the curvature in the response and, upon replication, validate the reproducibility of the experimental study (Li et al., 2003(a)). Figure 2.12 shows the cubic model for 2^3 FD with an added center point.

2.7.5.3 Higher Level Factorial Designs

FDs at three or more number of levels are employed mainly for response surface optimization (Doornbos and de Haan, 1995; Myers and Montgomery, 1995; Li et al., 2003(a)). Simple to generate, these designs can detect and estimate nonlinear or quadratic effects. The main strength of the design is orthogonality, because it allows independent estimation of the main effects and interactions (Bolton, 1997(a); Li et al., 2003(a)). On the other hand, the major limitation associated with high-level FDs is the increased number of experiments required with the increase in the number of factors (k). Even at a modest number of factors, the number of runs is quite large. For instance, the absolute minimum number of runs required to estimate all the terms present in a four-factor, three-level quadratic model is 15, involving the intercept term, four main effects, six two-factor interactions, and four quadratic terms. The



corresponding 3^k FD for $k = 4$ requires 81 runs. Another disadvantage of x^k FDs is the lack of rotatability (Montgomery, 2001).

For deriving maximal benefits from DoE, an experimenter has invariably to know, comprehend and apply some or all of the following aspects.

2.7.5.4 Blocking in Experimental Designs

Often the estimation of "effects" and "interaction" becomes complicated as a result of variability in the results caused by some uncontrollable factors, commonly termed nuisance factors or extraneous factors (Montgomery, 2001). Although these nuisance factors are the factors that may affect the measured result, they are not of primary interest. In such situations, blocks are generated in the experimental domain. Each block is a set of relatively homogenous experimental conditions, wherein every level of the primary factor occurs the same number of times with each level of nuisance factor (Montgomery, 2001; Box and Draper, 1987). These uncontrollable factors, therefore, are usually taken as the blocking factors. This technique of blocking is used to reduce or eliminate the variability transmitted by the nuisance factors. Accordingly, the analysis of the experiment focuses on the effect of varying levels of the primary factor "within each block" of the experiment. Runs are distributed over blocks in such a way that any difference between the blocks does not bias the results for the factors of interest. This is accomplished by treating the blocking factor as another factor in the design. The inclusion of blocking factors as additional factors in the design results in loss of estimation of some interaction terms, eventually lowering the resolution of the design. Nonetheless, the technique of blocking makes the design statistically more powerful. It allows simultaneous estimation and control of variability stemming from the difference(s) between the blocks during optimization of a process or formulation. Blocking considerably improves the precision with which comparisons are made among the factors of interest.

2.7.5.5 Resolution of Experimental Designs

One of the important features of the experimental designs is their resolution - i.e., the ability to describe the degree to which the estimated main effects are aliased (or confounded) with the estimated two-, three-, or higher level interactions (Lewis et al., 1999; Montgomery, 2001; Singh et al., 2004(b); Myers and Montgomery, 1995). In general, the resolution of a design is one more than the smallest order interaction that

some main effect is confounded with (Anderson et al., 2002). For instance, if some main effects are confounded with some two-level interactions, then the resolution is III. The most prevalent design resolutions in the pharmaceutical arena are III, IV, and V (Lewis et al., 1999). These designs imply that

- a) **Resolution III Designs:** In such designs, the main effects are confounded (aliased) with two-factor interactions.
- b) **Resolution IV Designs:** No main effects are aliased with two-factor interactions, but two-factor interactions are aliased with each other.
- c) **Resolution V Designs:** No main effect or two-factor interaction is aliased with any other main effect or two-factor interaction, but two-factor interactions are aliased with three-factor interactions.

The orthogonal designs, where the estimation of main effects and interactions are independent of each other, are said to possess “infinite resolution.” For most practical purposes, when the number of factors is quite large in pharmaceutical product development, a resolution IV design may be adequate, while a resolution V design is an excellent choice. Resolution III designs, on the other hand, are also useful in conditions where the number of factors is large and interactions among them are assumed to be negligible.

The resolution of experimental designs can be improved upon by the fold over technique (Montgomery, 2001; Box and Draper, 1987; Loukas, 1997). The procedure involves the generation or addition of a second block of experiments, in which the levels of each factor are reversed from the original block. For a resolution III design, this will improve the alias structure for all the factors. Fold over designs can either be mirror-image fold over designs (resulting in complete dealiasing of main effects and all interactions) or alternative fold over designs (involving break-up of specific alias patterns).

2.7.5.6 Design Augmentation

In the whole DoE endeavor, a situation sometimes arrives in which a study, conducted at some stage, is found to be inadequate and needs to be investigated further, or when the study carried out during the initial stages needs to be “reused” (Singh et al., 2004(b)). In either situation, more design points can be added systematically to the erstwhile design. Thus, the erstwhile primitive design can be enhanced to a more

advanced design furnishing more information, better reliability', and higher resolution. This process of extension of a statistical design, by adding some more rational design points, is known as design augmentation (Anderson et al., 2002). For instance, a design involving study at two levels can be augmented to a three-level design by adding some more design points. A design can be augmented in a number of ways, such as by replicating, adding center points to two-level designs, adding axial points (i.e., design points at various axes of the experimental domain), or by folding over.

2.7.6 Response Surfaces

During this crucial stage in DoE, one or more selected experimental responses are recorded for a set of experiments carried out in a systematic way to develop a mathematical model (Araujo and Brereton, 1996; Podczek, 1996; Haaland, 1989; Bolton, 1997(b); Das and Giri, 1994; Abu-Izza et al., 1996; Wehrle et al., 1993). These approaches comprise the postulation of an empirical mathematical model for each response, which adequately represents change in the response within the zone of interest. Rather than estimating the effects of each variable directly, response surface modeling (RSM) involves fitting the coefficients into the model equation of a particular response variable and mapping the response over the whole of the experimental domain in the form of a surface (Lewis et al., 1999; Singh and Ahuja, 2004(a); Doornbos, 1981; Box and Draper, 1987; Myers et al., 2003(b)).

Principally, RSM is a group of statistical techniques for empirical model building and model exploitation (Box and Draper, 1987; Myers et al., 2003(b)). By careful design and analysis of experiments, it seeks to relate a response to a number of predictors affecting it by generating a response surface, which is an area of space defined within upper and lower limits of the independent variables depicting the relationship of these variables to the measured response.

Experimental designs, which allow the estimation of main effects, interaction effects, and even quadratic effects, and, hence, provide an idea of the (local) shape of the response surface being investigated, are termed response surface designs (Lewis et al., 1999; Doornbos and de Haan, 1995; Myers and Montgomery, 1995; Wehrle et al., 1993). Under some circumstances, a model involving only main effects and interactions may be appropriate to describe a response surface. Such circumstances arise when analysis of the results reveals no evidence of "pure quadratic" curvature in

the response of interest - i.e., the response at the center approximately equals the average of the responses at the two extreme levels, +1 and -1.

In each part of Figure 2.13 (a, b, and c), the value of the response increases from the bottom of the figure to the top and those of the factor settings increase from left to right. If a response behaves as in Figure 2.13a, the design matrix to quantify that behavior needs only to contain factors with two levels - low and high. This model is a basic assumption of simple two-level screening or factor-influence designs. If a response behaves as in Figure 2.13b, the minimum number of levels required for a factor to quantify that behavior is three.

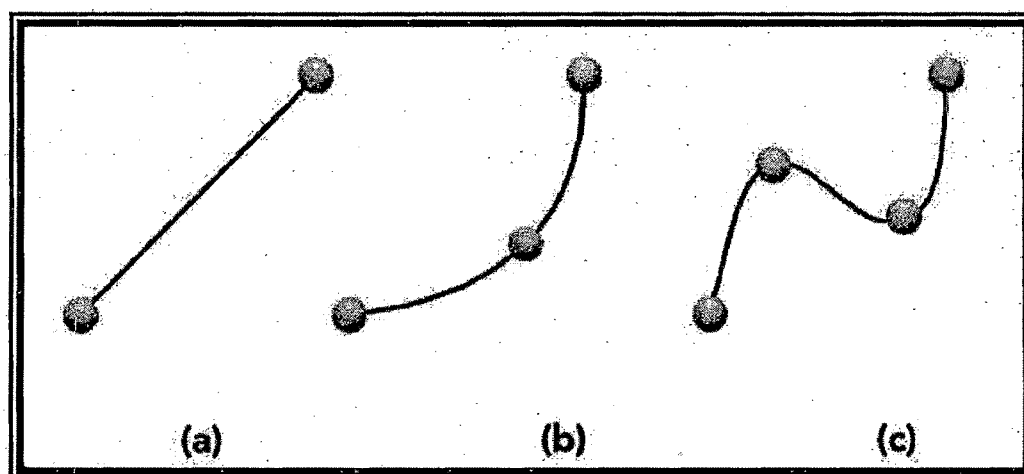


Figure 2.13 Different types of responses as functions of factor settings; (a) linear; (b) quadratic; (c) cubic

Addition of center points to a two-level design appears to be a logical step at this point, but the arrangement of the treatments in such a matrix may confound all the quadratic effects with each other (Myers and Montgomery, 1995; Box and Draper, 1987). A two-level design with center points can only detect the quadratic nature of the response, but not estimate the individual pure quadratic effects. Generally, the quadratic models are proposed for optimization of drug delivery devices (Lewis et al., 2002; Lewis et al., 1999; Armstrong and James, 1990). Therefore, response surface designs involving studies at three or more than three levels are employed for DoE optimization purposes. These response surface designs are used to find improved or optimal process settings, troubleshoot the process problems and weak points, and make a formulation or process more robust (i.e., less variable) against external and

non-controllable influences (Myers and Montgomery, 1995). Relatively more complicated cubic responses (Figure 2.13c) are quite infrequent in pharmaceutical practice (Lewis et al., 1999; Armstrong and James, 1990).

The prediction ability of response surface designs can be determined by prediction variance, which is a function of experimental variance (σ^2) and variance function (d) as described by equation (Lewis et al., 1999; Montgomery, 2001; Myers and Montgomery, 1995):

$$\text{var}(\hat{y}) = d \cdot \sigma^2$$

where $\text{var}(\hat{y})$ is the prediction variance. The variance function (d) further depends upon the levels of a factor and the experimental design. When the prediction variance of a response is constant in all the directions at a given distance from the center point of the domain, the design is termed rotatable (Montgomery, 2001; Araujo and Brereton, 1996). Ideally, all response surface designs should possess the characteristic of rotatability - i.e., the ability of a design to be run in any direction without any change in response prediction variance.

Conduct of DoE trials, according to the chosen statistical design, yields a series of data on the response variables explored. Such data can be suitably modeled to generate mathematical relationships between the independent variables and the dependent variables. Graphical depiction of the mathematical relationship is known as a response surface (Singh and Ahuja, 2004(a); Myers and Montgomery, 1995; Wehrle and Stamm, 1994). A response surface plot is a 3-D graphical representation of a response plotted between two independent variables and one response variable. The use of 3-D response surface plots allows us to understand the behavior of the system by demonstrating the contribution of the independent variables.

The geometric illustration of a response obtained by plotting one independent variable against another, while holding the magnitude of response and other variables as constant, is known as a contour plot (Doornbos and de Haan, 1995). Such contour plots represent the 2-D slices of the corresponding 3-D response surfaces. The resulting curves are called contour lines. Figure 2.14 depicts a typical response surface and contour plot for a diffusional release exponent (Korsemeyer et al., 1983) as the response variable, reported with mucoadhesive compressed matrices of atenolol (Singh et al, 2003). For complete response depiction among k independent variables,

a total of kC_2 number of response surfaces and contour plots may be required. In other words, 1, 3, 6, or 10 number of 3-D and 2-D plots are needed to provide depiction of each response for 2, 3, 4, or 5 number of variables, respectively (Singh et al., 2004(b)).

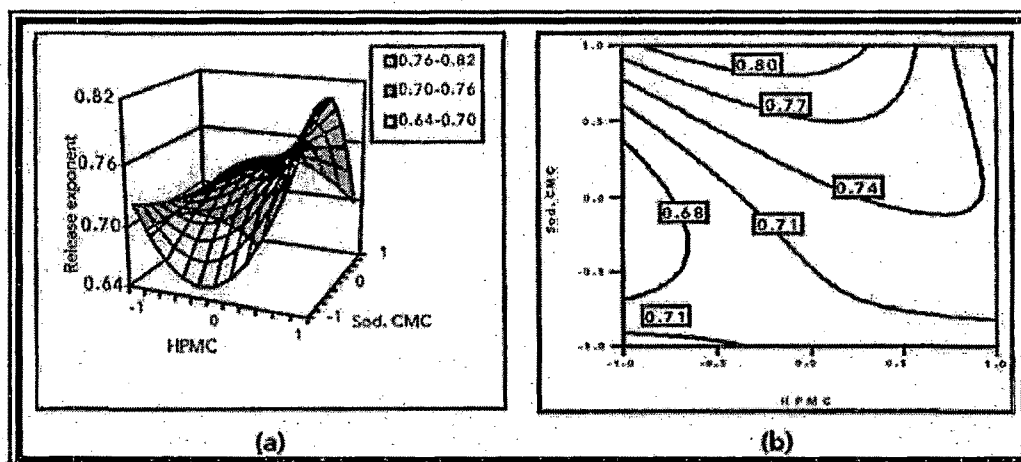


Figure 2.14 (a) A typical response surface plotted between a response variable, release exponent, and two factors, HPMC and sodium CMC, in case of mucoadhesive compressed matrices; (b) the corresponding contour plot

2.7.7 Mathematical Models

The mathematical model, simply referred to as the model, is an algebraic expression defining the dependence of a response variable on the independent variable(s) (Box and Draper, 1987; Box et al., 1978). Mathematical models can either be empirical or theoretical (Doornbos and de Haan, 1995). An empirical model provides a way to describe the factor/response relationship. It is most frequently, but not invariably, a set of polynomial equations of a given order (Montgomery, 2001; Box and Draper, 1987). Most commonly used linear models are shown in following equations:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \epsilon$$

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \dots + \epsilon$$

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \dots + \epsilon$$

where y represents the estimated response, sometimes also denoted as $E(y)$. The symbols X , represent the value of the factors, and β_0 , β_i , β_{ii} and β_{ij} are the constants representing the intercept, coefficients of first-order (first-degree) terms, coefficients

of second-order quadratic terms, and coefficients of second-order interaction terms, respectively. The symbol implies pure error. First two equations are linear in variables, representing a flat surface and a twisted plane in 3-D space, respectively. Third equation represents a linear second-order model that describes a twisted plane with curvature, arising from the quadratic terms.

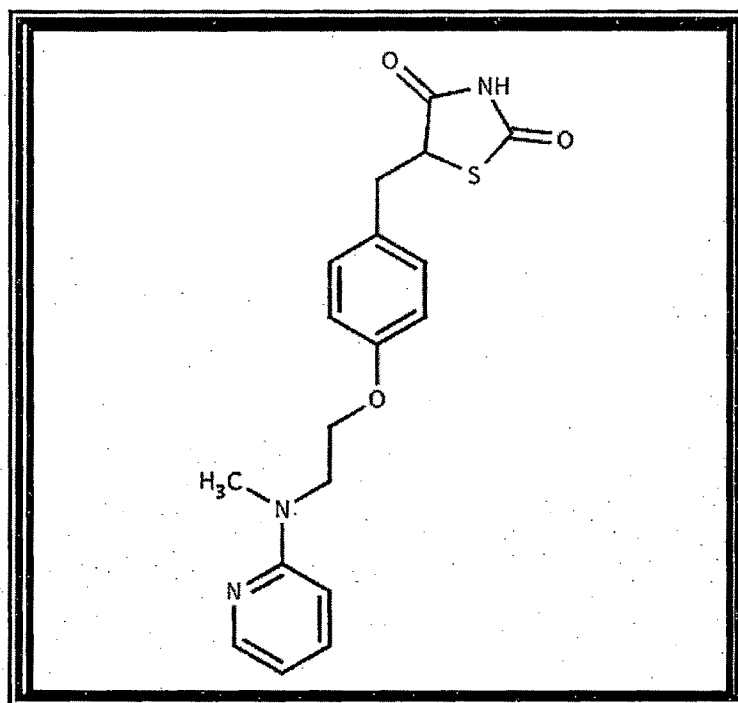
A theoretical model or mechanistic model may also exist or be proposed. It is most often a nonlinear model, where transformation to a linear function is not usually possible (Doornbos and de Haan, 1995). Such theoretical relationships are, however, rarely employed in pharmaceutical product development.

2.8 DRUG PROFILE

2.8.1 Rosiglitazone (RGZ)

Rosiglitazone is an oral diabetes medicine that helps control blood sugar levels. Rosiglitazone is for people with type-2 (non insulin dependent) diabetes. Rosiglitazone is sometimes used in combination with insulin or other medications, but it is not for treating type-1 diabetes. Taking rosiglitazone may increase your risk of serious heart problems, such as heart attack or stroke. Therefore, rosiglitazone is available only to certain people with type 2 diabetes that cannot be controlled with other diabetes medications. Rosiglitazone is not recommended for use with insulin. Apart from its effect on insulin resistance, it appears to have an anti-inflammatory effect: nuclear factor kappa-B (NFκB) levels fall and inhibitor (IκB) levels increase in patients on rosiglitazone. Recent research has suggested that rosiglitazone may also be of benefit to a subset of patients with Alzheimer's disease not expressing the ApoE4 allele. Thiazolidinediones class drugs also modulate the biology of fibrogenic cells and hence, studied to treat liverfibrosis. This is the subject of a clinical trial currently underway. The chemical name is 5-[(4-{2-[methyl(pyridin-2-yl)amino]ethoxy}phenyl)methyl]-1,3-thiazolidine-2,4-dione with the following structural formula:

2.8.1.1 Chemical Structure



Empirical Formula: C₁₈H₁₉N₃O₃S

CAS Number: 122320-73-4

Mol. Wt.: 357.43

2.8.1.2 Physical Properties

It is a white or almost white, crystalline, odorless, tasteless powder, practically insoluble in water (30µg/ml), freely soluble in organic solvents such as ethanol, DMSO, dimethyl formamide, methanol and chloroform. It has Log P value of 2.95 and melting point 153-155^o C.

2.8.1.3 Mechanism of Action

Rosiglitazone acts as an agonist at PPAR in target tissues such as adipose tissue, skeletal muscle, and liver. PPAR_γ activation reduces expression of interstitial collagens and other matrix proteins, downregulates the ability to proliferate and migrate in response to PDGF, blocks the secretion of proinflammatory chemokines such as monocyte chemoattractant protein 1, and induces apoptosis. Moreover, exposure TNF-α or PDGF reduces PPAR_γ expression or transcriptional activity while

PPAR γ ligands counteract these effects. In some systems the actions of TZDs have been shown to be independent of PPAR γ . However, ectopic expression of PPAR γ in activated HSCs recapitulates the effects of TZD treatment, indicating that these drugs modulate the biology of fibrogenic cells due to their ability to ligate PPAR γ .

2.8.1.4 Pharmacokinetics

The absolute bioavailability of rosiglitazone is 99%. Peak plasma concentrations are observed about 1 hour after dosing. Administration of rosiglitazone with food resulted in no change in overall exposure (AUC), but there was an approximately 28% decrease in C_{max} and a delay in T_{max} (1.75 hours). These changes are not likely to be clinically significant; therefore, rosiglitazone may be administered with or without food. About 99.8% of rosiglitazone is bound to plasma proteins, primarily to albumin and hence, volume of distribution is 6L. Rosiglitazone is extensively metabolized in the liver to inactive metabolites via N-demethylation, hydroxylation, and conjugation with sulfate and glucuronic acid. In vitro data have shown that Cytochrome (CYP) P450 isoenzyme 2C8 (CYP2C8) and to a minor extent CYP2C9 are involved in the hepatic metabolism of rosiglitazone. Following oral or intravenous administration of [¹⁴C] rosiglitazone, approximately 64% and 23% of the dose was eliminated in the urine and in the feces, respectively with elimination half life of 3-4 hours.

2.8.1.5 Side Effects

serious side effect includes: feeling short of breath, even with mild exertion; swelling or rapid weight gain; chest pain or heavy feeling, pain spreading to the arm or shoulder, sweating, general ill feeling; nausea, stomach pain, low fever, loss of appetite, dark urine, clay-colored stools, jaundice (yellowing of the skin or eyes); blurred vision; increased thirst or hunger, urinating more than usual; or pale skin, easy bruising or bleeding, weakness.

Less serious side effects may include: sneezing, runny nose, cough or other signs of a cold; headache; gradual weight gain; mild diarrhea; or back pain.

2.8.1.6 Interaction

Rosiglitazone has following drug interactions:

Drug	Interaction
Gemfibrozil	Increases the effect and toxicity of rosiglitazone/pioglitazone
Ketoconazole	Ketoconazole increases the effect of rosiglitazone
Pregabalin	Increased risk of edema
Rifampin	Rifampin reduces levels and efficacy of rosiglitazone
Somatropin recombinant	Somatropin may antagonize the hypoglycemic effect of rosiglitazone. Monitor for changes in fasting and postprandial blood sugars.
Tretinoin	The moderate CYP2C8 inhibitor, Rosiglitazone, may decrease the metabolism and clearance of oral Tretinoin. Monitor for changes in Tretinoin effectiveness and adverse/toxic effects if Rosiglitazone is initiated, discontinued to dose changed.

2.8.1.7 Contraindication

RGZ is contraindicated in patients with known hypersensitivity to RGZ and in conditions like Diabetic ketoacidosis.

2.8.1.8 Methods for Estimation

2.8.1.8.1 Ultraviolet Spectroscopy

UV spectra of RGZ gave absorption maxima at 311.8 nm in methanol and at 313.8 nm in diffusion medium (50 mM HPBCD, 20 mM HEPES, pH 7.4).

2.8.1.8.2 Chromatographic Analysis:

HPLC

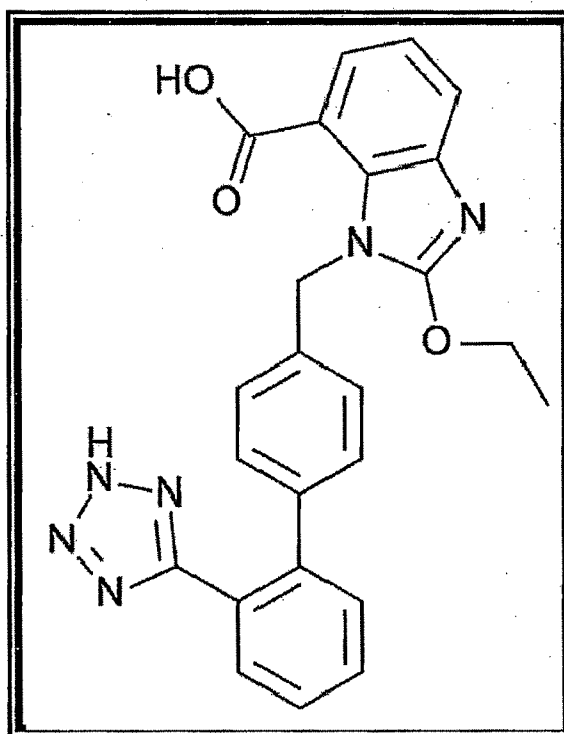
The chromatographic analysis was carried out on a GRACE Brava™ BDS C18 (5 μ m, 25 cm \times 4.6 mm) column maintained at $30 \pm 0.5^\circ\text{C}$. RGZ was eluted using a mobile phase composition of 10mM sodium acetate (pH 5): acetonitrile: methanol (40:40:20, v/v/v) at a flow rate $1.0 \text{ mL} \cdot \text{min}^{-1}$. The mobile phase was premixed, filtered through a 0.45 mm Nylon 66 membrane filter and degassed before use. RGZ was detected at 245.0 nm and was eluted in 6.7 min after injection.

2.8.2 Candesartan (CDS)

Candesartan, a angiotensin-receptor blocker (ARB), is used alone or with other antihypertensive agents to treat hypertension. Candesartan competes with angiotensin II for binding at the AT1 receptor subtype. Recent research has suggested that HSCs with contractile and fibrogenic properties are the principal site of ECM production.

These cells are stimulated by fibrogenic cytokines, one of which is angiotensin II (ATII). The role of the renin-angiotensin system is supported by the observation that angiotensin type 1 (AT1) receptor antagonists attenuate the progression of liver fibrosis and reduce portal pressure. The chemical name is 2-ethoxy-1-({4-[2-(2H-1,2,3,4-tetrazol-5-yl)phenyl]phenyl}methyl)-1H-1,3-benzodiazole-7-carboxylic acid with the following structural formula:

2.8.2.1 Structure



Empirical Formula: $C_{24}H_{20}N_6O_3$

CAS Number: 139481-59-7

Mol. Wt.: 440.45

2.8.2.2 Physical Properties

It is a white or almost white crystalline powder; odorless; tasteless; practically insoluble in water ($7.7\mu\text{g/ml}$), freely soluble in chloroform; soluble in acetone, sparingly soluble in methanol, slightly soluble in ethanol. It has Log P value of 4.02 and melting point $183\text{--}185^\circ\text{C}$.

2.8.2.3 Mechanism of Action

Angiotensin II, the principal pressor agent of the renin-angiotensin system, is responsible for effects such as vasoconstriction, stimulation of synthesis and release of aldosterone, cardiac stimulation, and renal reabsorption of sodium. Candesartan selectively blocks the binding of angiotensin II to the AT1 receptor, which in turn leads to multiple effects including vasodilation, a reduction in the secretion of vasopressin, and reduction in the production and secretion of aldosterone. The resulting effect is a decrease in blood pressure. Candesartan's action is independent of the pathways for angiotensin II synthesis. The drug blocks the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor in many tissues. This action is different from ACE inhibitors, which block the conversion of angiotensin I to angiotensin II, meaning that the production of angiotensin II is not completely inhibited, as the hormone can be formed via other enzymes. AT-II has also been shown to induce contraction and proliferation of HSCs, which play a pivotal role in liver fibrosis development. Accordingly, it has been suggested that AT-II plays a role in liver fibrosis development. The inhibition of angiotensin II synthesis and/or blockade of AT1 receptors attenuate experimental liver fibrosis.

2.8.2.4 Pharmacokinetics

Oral absorption of Candesartan is found to be $45\% \pm 11$. Volume of distribution is found to be 0.131/kg and plasma protein binding is 99.8%. Renal Excretion accounts for 52% and plasma half life is 9.7hr.

2.8.2.5 Side Effects

serious side effect includes: rash; hives; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue; hoarseness; change in the amount of urine produced; chest pain; dark urine; difficulty swallowing; fast, slow, or irregular heartbeat; fever, chills, or persistent sore throat; muscle pain or cramps; severe or persistent stomach pain (with or without nausea or vomiting); symptoms of low blood pressure (eg, fainting, lightheadedness, severe dizziness); unusual bruising or bleeding; yellowing of the eyes or skin.

Less serious side effects may include: Back pain; dizziness; upper respiratory tract infection.

2.8.2.6 Interaction

Candesartan has following drug interactions:

Drug	Interaction
Amiloride	Increased risk of hyperkalemia
Drospirenone	Increased risk of hyperkalemia
Lithium	The ARB increases serum levels of lithium
Potassium	Increased risk of hyperkalemia
Spironolactone	Increased risk of hyperkalemia
Tobramycin	Increased risk of nephrotoxicity
Trandolapril	The angiotensin II receptor blocker, Candesartan, may increase the adverse effects of Trandolapril.
Treprostinil	Additive hypotensive effect. Monitor antihypertensive therapy during concomitant use.
Triamterene	Increased risk of hyperkalemia

2.8.2.7 Contraindication

CDS is contraindicated in patients with hypersensitivity and in pregnancy (2nd and 3rd trimester) and lactation.

2.8.2.8 Methods for Estimation

2.8.2.8.1 Ultraviolet Spectroscopy

UV spectra of FRL gave absorption maxima at 304.8 nm in methanol and at 306.2 nm in diffusion medium (100 mM HPBCD, 20 mM HEPES, pH 7.4).

2.8.2.8.2 Chromatographic Analysis:

HPLC

The chromatographic analysis was carried out on a GRACE Brava™ BDS C18 (5 μ m, 25 cm \times 4.6 mm) column maintained at $30 \pm 0.5^\circ\text{C}$. CDS was eluted using a mobile phase composition of methanol: acetonitrile: 10mM sodium acetate pH 5 (74 : 16 : 10, v/v/v) (pH 2.5) at a flow rate $1.0 \text{ mL}\cdot\text{min}^{-1}$. The mobile phase was premixed, filtered through a 0.45 μ m Nylon 66 membrane filter and degassed before use. CDS was detected at 260.0 nm and was eluted in 4.5 min after injection.

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