From the Department of Medicine H7
Division of Gastroenterology and Hepatology

EXPERIMENTAL AND CLINICAL STUDIES ON LIVER REGENERATION AND HEPATOCELLULAR CARCINOMA

ROLES OF REDOX PROTEINS, IRON HOMEOSTASIS AND MULTIKINASE INHIBITION

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To my family

ABSTRACT

Compensatory liver regeneration is triggered by chronic liver injury or surgery and is crucial to maintain tissue homeostasis. The underlying mechanisms which include a whole battery of complex signaling events have been thoroughly studied for decades. The majority of hepatocellular carcinomas develop in a highly proliferative environment caused by underlying chronic liver disease in which lost liver tissue must be restored to meet the needs of the organism. The chronic inflammatory condition with chronic liver repair enhances the presence of free radicals leading to an increased risk of cell alterations.

This thesis includes four papers; the first three of which comprise studies regarding the importance of the regulation and endurance of cell proliferation, and also the sensitivity of the proliferating cells to compounds used in cancer prevention and treatment. In these studies we used a 2/3 partial hepatectomy (PH) rat model and also a chemically induced rat liver cancer model (The Solt and Farber Resistant hepatocyte model). The fourth paper is a human study in which we quantified immunohistochemical stainings for 6 different redox proteins in livers from patients resected for hepatocellular carcinoma (HCC) and colorectal carcinoma (CRC) liver metastases.

The specific aims were: (I) to characterize gene expression of the different pathways involved in hepcidin regulation after PH, until liver regeneration is complete; (II) to study the effects of sodium selenite on regenerative versus neoplastic liver cell proliferation in rat, and to investigate if TrxR1 is a constitutive tumour marker or an unspecific marker for cell growth in rat liver; (III) to study the effect of the anticancer agent sorafenib, a multikinase inhibitor, on normal liver regeneration after PH in rat; and (IV) to evaluate if redox protein (thioredoxins and glutaredoxins) expressions correlate to clinical features in human hepatocellular carcinoma and if they can be used as prognostic markers after liver surgery.

Our results showed that high serum levels of IL6 induced the levels of STAT3 and the expression of hepcidin mRNA during the acute phase after PH. The gene expressions of the iron sensing proteins HFE, hemojuvelin (HJV) and transferrin receptor 2 (TfR2) were decreased during the whole regeneration, gradually decreasing hepcidin gene expression and thereby mobilizing iron to the growing liver. The expression of genes involved in iron uptake; transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1) were increased thereby facilitating iron uptake (paper I).

After administration of sodium selenite in a tumour preventive, supranutritional dose followed by PH no effect on body weights or gain of liver mass was seen. In the hepatocarcinogenesis model the tumour volume was significantly decreased in animals supplied with selenium during the progression phase compared tumours in rats not treated with selenium. The expression of TrxR1 was exclusively seen in the neoplastic liver lesions but not in the remodelling preneoplastic lesions (paper II).

Treatment with sorafenib transiently suppressed liver regeneration and the gain of relative liver mass, but was followed by a delayed compensatory increase of liver cell proliferation one week after resection with the result that after 14 days the treated animals reached the same relative liver weights as the controls did in five days (paper III).

In the human study we saw an up-regulation of Trx1, Trx2 and Grx5 in HCC compared to its respective surrounding non-tumorous tissue. The same was observed in the CRC metastases where also the staining of Grx1 and Grx3 was significantly higher compared to non-tumorous

tissue. Trx1 expression correlated well to cell proliferation but not to tumour differentiation, micro-vascular invasion or tumour recurrence. A relative down regulation of Trx1 was seen in tumours compared to the surrounding liver in males, smokers and in patients with high alcohol consumption.

We concluded that the peak of hepcidin expression during the acute phase was eventually overruled by the downregulation of the iron sensing pathway in order to promote iron mobilization to the regenerating liver. We also concluded that selenium in a supranutritional dose impaired tumour growth without impairing the normal liver cell proliferation, and that the selenoprotein TrxR1 is a constituent of the neoplastic phenotype. Sorafenib prolonged liver regeneration in proportion to the length of treatment but the liver adapted to the early inhibitory effects of the drug. Thioredoxins and glutaredoxins were ubiquitously expressed in livers exposed to oxidative stress and various malignancies and can therefore not be used as diagnostic markers for HCC. Smoking and high alcohol consumption increased the Trx1 expression in tissue surrounding the HCCs, whereas expression of Trx1 in the HCCs correlated to cell proliferation. Redox protein expression in HCCs cannot be used as predictive markers for tumour recurrence after liver resection.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Levern är kroppens största körtel och kroppens näst största organ efter huden. Den är ett mångfacetterat organ med uppgifter som att producera essentiella proteiner, till exempel koagulationsfaktorer, lagra energi i form av glykogen, producera och utsöndra vissa hormoner, producera och transportera galla till tarmen för fettnedbrytning, reglera upptag av järn samt bryta ner toxiska kemiska föreningar. Utöver detta har levern också en viktig roll i immunförsvaret för att eliminera främmande partiklar såsom virus och bakterier.

Levern har en stor förmåga till anpassning efter behovet av funktionell levermassa men också en betydande reservkapacitet. Levern har förmågan att återuppbyggas efter skador, genom att levercellerna delar sig vilket kallas leverregeneration. Sådana skador kan uppstå på grund av en kronisk leverinflammation eller efter ett kirurgiskt ingrepp där en bit av levern opereras bort. Vid en kronisk leverskada orsakad exempelvis av hepatit B/C eller alkohol så ökar risken för ackumulerade mutationer, som på sikt kan leda till tumörer. Ett helt maskineri av komplexa signalvägar ligger bakom leverregeneration och om man i detalj kan kartlägga dessa skulle nya strategier för behandling av levercancer kunna utvecklas.
Vid primär levercancer kan symptomen vara vaga och uppstå i ett sent skede. Om cancern upptäcks i ett tidigt stadium kan patienten behandlas kirurgiskt om den upptäcks i ett intermediärt stadium kan cellgifter sprutas in i leverartären till tumören, men om den hittas i ett avancerat stadium kan man bara ge en ”bromsmedicin”, sorafenib, som hämmar tumörens tillväxt men inte ger bot.
I den här studien har vi använt en djurmodell på råtta för att kunna studera leverregeneration och hur den påverkas av olika behandlingar. Sjuttio procent av djurets lever tas bort i ett kirurgiskt ingrepp som kallas 2/3 partiell hepatektomi, (PH). Den återstående delen av levern kommer omedelbart att starta en kompensatorisk regenerationsprocess och på råtta ser man redan efter 5-7 dagar att vävnadsförlusten är ersatt och levern har nått sin ursprungliga funktionella massa för att möta organismens behov.

Det finns sjukdomar som leder till för högt järnupptag vilket på sikt är levertoxiskt och kan leda till levercancer. Vi har studerat hepcidin som är ett protein som styr järnupptaget i levern genom att blockera upptaget från tarmen. Hepcidinet självt produceras av levern och regleras av flera molekyler som känner av järnhalten i kroppen. Vid inflammation uppregleras hepcidin vilket ger en minskning av järnhalten i plasma. Detta ses som ett försvar mot mikroorganismer som har behov av järn för att kunna föröka sig. Efter partiell hepatektomi följer en akutfas-reaktion som bland annat leder till en ökad produktion av hepcidin. Den akuta fasen är över efter ett dygn och övergår i en regenerativ fas. Det var tidigare inte känt vad som sker med uttrycket av hepcidin och därmed regleringen av järnupptaget under själva regenerationsfasen av leverns återuppbyggnad, vilket föranledde oss att genomföra den här studien. Vi använde oss av PH-djurmodellen och analyserade uttrycket av hepcidinreglerande gener och proteiner och fann att hepcidin nedreglerades efter den akuta fasen för att underlätta upptaget av järn som är en viktig komponent under celldelning och celltillväxt.
Selen är ett annat grundämne som är ett viktigt spårämne i kroppen, och som är inblandat i många viktiga cellulära processer, bland annat celltillväxt och cellulär utmognad (differentiering). Det finns studier som har visat att selen har en hämmande effekt på olika

typer av tumörer. Vi studerade selenets inverkan på levercancer i råtta och fann att selen, i en dos som är högre än normalt dagssintag men som inte är toxisk, hämmade levertumörernas tillväxt utan att påverka den normala leverns tillväxt. Vi undersökte även förekomsten av ett selenoprotein, thioredoxin reduktas 1 (TrxR1) som är involverat i cellens försvar mot oxidativ stress, i tumörerna och fann att detta protein överuttrycktes i levertumörer men inte i de cellförändringar utgör ett förstadium till tumör och som var på väg att återbildas. Vi drog slutsatsen att TrxR1 är en del av tumörcellens karakteristika (vilket brukar kallas den ”neoplastiska fenotypen”) och skulle kunna användas som en vävnadsmarkör för cancer.

Vi studerade även sorafenib som är en relativt ny medicin som ges till patienter med avancerad inoperabel levercancer. Sorafenib verkar genom att hämma vissa cellulära signalvägar som är inblandade i celldelningen. Frågan har väckts om sorafenib skulle kunna användas i samband med kirurgi för att minska risken för återfall av sjukdomen. Ett problem är dock att sorafenib också kan hämma normala leverceller som ska dela sig för att återställa förlorad vävnad efter operation.
Av den anledningen valde vi att studera effekten av sorafenib på normala tillväxande leverceller hos råtta efter PH. Djuren fick sorafenib dagligen under en vecka innan PH och avlivades vid olika tidpunkter därefter. Vi studerade leverns tillväxt upp till 14 dagar efter operationen och fann att sorafenib hämmade tillväxten i proportion till längden på behandlingen efter PH samt att levern anpassade sig till drogen genom att öka sin celldelning i ett senare skede efter PH. Detta för att kompensera för den tidiga hämmande effekten. Efter 14 dagar var de sorafenib-behandlade djurens levrar lika stora som innan, medan detta tog 5 dagar hos de djur som inte fått sorafenib.

Uttrycket av vissa redoxproteiner har rapporterats vara ökat i bland annat tjocktarms- och gallblåsecancer, och dessutom korrelerats till en sämre prognos efter kirurgi. För att studera om detta även gäller levercancer så analyserade vi uttrycket av sex olika redoxproteiner i kirurgiskt avlägsnade levertumörer hos patienter med primär levercancer. Som jämförelsematerial använde vi dels omgivande levervävnad runt tumörerna och dels levermetastaser från tjocktarmscancer samt den tumörfria levern som omgav metastaserna. Vi fann att flera av dessa proteiner var signifikant uppreglerade i tumörerna jämfört med den omgivande vävnaden men att uppregleringen var svagare i primär levercancer jämfört med tjocktarmscancermetastaserna.
Vi fann även ett starkare uttryck av proteinerna i den omgivande vävnaden i levrarna med primär levercancer jämfört med uttrycket i den vävnad som omgav tjocktarmsmetastaserna. Detta tror vi speglar en högre grad av oxidativ stress (bildning av fria radikaler) hos levercancerpatienterna beroende på deras underliggande kroniska leversjukdom jämfört med levervävnaden hos patienterna med metastaser, där levervävnaden i grunden är frisk. Vi såg inget samband mellan uttrycket av redoxproteiner och risken att återfå sin levercancer efter operationen.

LIST OF PUBLICATIONS

I. **Annelie Mollbrink**, Petra Holmström, Mattias Sjöström, Rolf Hultcrantz, Lennart C. Eriksson and Per Stål.
Iron-regulatory gene expression during liver regeneration.
Scand J Gastroenterol, 2012; 47(5):591-600

II. Suvd Erkhembayar, **Annelie Mollbrink**, Lennart C. Eriksson.
The effect of sodium selenite on liver growth and thioredoxin reductase expression in regenerative and neoplastic liver cell proliferation.
Biochem Pharmacol. 2012 1;83(5):687-93

III. **Annelie Mollbrink**, Martin Augsten, Rolf Hultcrantz, Lennart C. Eriksson and Per Stål.
Sorafenib prolongs liver regeneration after hepatic resection in rats.
J Surg Res (under revision)

IV. **Annelie Mollbrink**, Rim Jawad, Alexios Vlamis, Pia Edenvik, Olof Danielsson, Per Stål and Aristi Fernandes. Characterization of redox proteins in human hepatocellular carcinoma.
Manuscript

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LIST OF ABBREVIATIONS

2-AAF	2-acetyl aminofluorene
AFP	Alpha-feto protein
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate aminotransferase
BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
CRC	Colorectal carcinoma
DEN	Diethylnitrosamine
DMT1	Divalent metal transporter 1
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FPN	Ferroportin
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HFE	Hereditary hemochromatosis gene
HGF	Hepatocyte growth factor
HJV	Hemojuvelin
IL6	Interleukin 6
IRE	Iron regulatory element
IRP	Iron regulatory protein
PDGF	Platelet derived growth factor
PH	Partial hepatectomy
RH-model	Resistant hepatocyte model
RNR	Ribonucleotide reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SeCys	Selenocysteine
SeMet	Selonomethionine
SeMSC	Selenomethylselenocysteine
SO	Sham operated
SMAD4	Mothers against DPP homologs
STAT3	Signal transducer and activator of transcription 3
TfR	Transferrin receptor
Trx	Thioredoxin
TrxR	Thioredoxin reductase
u-PA	Urokinase activating plasminogen factor
VGFR	Vascular endothelial growth factor

PREFACE

The liver is a fascinating and diverse organ with many challenging tasks. Positioned under the ribs on the right side of the abdomen, and with a direct contact with the intestine via the portal vein it is the first organ receiving and taking care of anything that is ingested. The liver is not only the biggest organ on the inside of our body with a weight of 1.2 -1.5 kilograms in adults, but also our largest gland.

It has a major role in the body's metabolism and produces important plasma proteins and lipids; it synthesizes and excretes bile, and functions moreover as a huge reservoir of several important biomolecules.

With its double blood supply the liver is fraught with venous and nutrient rich blood from the intestines and spleen brought via the portal vein whereas the hepatic artery supplies the liver with oxygenated blood. These vessels lie parallel and enter the liver through the *porta hepatis* where they divide into branches to the right and the left lobes. The left and the right hepatic bile ducts form the common hepatic duct which exits the liver where the vessels enter. The hepatic nerve plexus follows the hepatic artery and bile ducts into the hepatic parenchyma.

The gallbladder is a reservoir that collects the bile produced by the liver. The bile is emptied in the intestines to help breaking down lipids which are taken up by the epithelial cells that lines the intestinal mucosa for subsequently transportation via the portal vein to the liver for further processes.

The liver consists of two anatomical lobes; the right lobe is bigger and constitutes 60-65% of the whole liver. The lobes are separated by *ligamentum falciforme hepatis*. Each lobe is organized in functional segments where they have independent vascular and biliary stalks and venous drainage. In total the liver has 8 segments designated I-VIII.

One of the most unique features of this organ is its capability to regenerate; something which is a very important evolutionary adaptation for a life on our planet, with its variable environment that sometimes can be very hostile.

Already the ancient Greeks had some idea of liver regeneration, in Greek mythologies there is a story about Prometheus – an immortal titan- that was stealing the fire from the Gods in Olympia to give to the people. For this he was punished by Zeus for all eternity by being fettered to a rock and getting his liver eaten every day by an eagle. Each night a new liver grew back so that the eagle could feast on a fresh liver the next day and this endless torture went on until he was eventually released and the eagle was shot.

1 INTRODUCTION

The liver is a very plastic gland that in a changing environment is constantly adapting its functions to the needs of the body. It has an impressive capacity and flexibility that we hardly ever think of or has to consider in daily life. It is however also a target for environmental toxicity and disease. With efficient repair processes, including intracellular restoration of organelles as well as regeneration of new hepatocytes, the liver can compensate for a significant loss of liver cells and liver cell function. Acute toxicity is therefore usually not life threatening unless the entire liver is severely compromised. Chronic toxicity and chronic necroinflammatory diseases, involving cell degeneration and cell death induce chronic repair processes and a need for chronic regeneration. Increased compensatory cell proliferation in a toxic environment and in an environment with increased oxidative stress causes with time chronic liver damage, liver fibrosis and increases the risk of liver cancer.

In this thesis we have focused on the compensatory hepatocyte cell proliferation, involved in liver cancer development. We have investigated different aspects of importance for the regulation and endurance of cell proliferation and also the sensitivity of the proliferating cells to compounds used in cancer prevention and treatment. An important function of the liver cell is to be able to handle reactive compounds and oxidative stress. We have therefore also investigated the expression of redox proteins in liver cancer and in the surrounding non neoplastic liver in which the tumour has developed and that is of importance also for the growth of the cancer cells and cancer progression. The patient is dependent for its survival on the status of the surrounding liver tissue and a severely compromised surrounding liver tissue will also limit the options available for treatment.

The thesis covers the homeostasis of iron in a situation of sustainable restoration after removal of 2/3 of the liver tissue. We also investigate the effect on selenium on the growth of the normal hepatocyte and compare that with data on the selenium effect on liver cancer and liver cancer development. This is of interest in a situation where selenium is used to prevent liver cancer in a patient with chronic liver disease. Sorafenib is today the only compound used for treatment of advanced liver cancer with any effect on the survival. It has been suggested that this compound should be used in patients that has gone through a liver resection to remove the liver cancer. We have looked at the effect of sorafenib on the non-neoplastic growing hepatocyte. Finally we have investigated thioredoxins and glutaredoxins, proteins involved in cellular oxidative defense that could be involved in the resistant phenotype of liver cancer and have been suggested as potential markers for liver cancer.

1.1 THE CELLS OF THE LIVER

1.1.1 Parenchymal cells

The hepatocytes build up the liver parenchyma and constitute the functional units of the liver, and are therefore named “liver parenchymal cells”.
The hepatocytes are relatively large in size compared to other cells in the liver. About 80% of the liver volume is composed of hepatocytes, but in numbers, hepatocytes constitute only around 60% of the total number of cells. The shape of the nucleus of the hepatocyte is spherical and contains one or more nucleoli. About 25% of the hepatocytes are binuclear in an

adult healthy liver. The functions of the hepatocytes display a broad variety and they are one of the most complex and metabolically diverse cells in the body. They synthesize plasma proteins, glucose, lipoproteins, cholesterol, phospholipids and fatty acids. They take up, store and control the release of nutrients into the circulation. Through the bile secretion they help to digest and absorb dietary fats and furthermore they degrade, detoxify and excrete exogenous and endogenous compounds. The work load of the liver is under strict control and adapts to the need of the organism in a fine-tuned way. The mechanisms or adaptation includes hyperplasia and involution as well as hypertrophy and atrophy.
The hepatocytes are very rich in organelles such as endoplasmic reticulum (ER), mitochondria, lysosomes and peroxisomes. The ER is highly active and has many different functions in hepatocytes such as synthesis of structural membrane proteins, proteins and glycoproteins for secretion into the blood, metabolism of fatty acids, triglycerides and phospholipids, metabolism and production of cholesterol, metabolism of xenobiotics, ascorbic acid synthesis and degradation of heme. Heme is an important component of cytochromes and is synthesized by the granulated rough ER (RER) with ribosomes attached in polysome groups. The brown color of the liver is due to the high content of heme. Bilirubin will be conjugated by the hepatocytes to glucuronic acid and subsequently secreted into the bile.
There is more of rough ER (RER) in hepatocytes compared to smooth ER (SER) which are not coated with ribosomes. While RER is responsible for protein synthesis, endogenous as well as proteins for export, SER contain enzymes for drug metabolism, biosynthesis of cholesterol and conversion of cholesterol to bile acids. SER is also involved in posttranslational modifications of proteins, such as glycosylation and formation of lipoproteins.
The functional heterogeneity is facilitated by the morphology of the hepatocytes i.e. the exocrine function with secretion of bile to the canaliculus, as well as the endocrine function that involves the synthesis of proteins and other compounds that enter the sinusoids.

1.1.2 Non-parenchymal cells

The non-parenchymal cells (Kupffer cells, stellate cells, endothelial cells, bile duct cells, fibroblasts and lymphocytes) constitute approximately 40% of cells in the liver. Kupffer cells are specialized macrophage-like cells that are important in the phagocytosis and break-down of cell debris. Like other reticuloendothelial macrophages they lyse red blood cells and heme and the globin chains are catabolized into polypeptides for re-use and heme is broken down into biliverdin and iron. Biliverdin will be further converted to bilirubin and iron will be bound to transferrin for transport or to ferritin for storage (see the iron section on page 17). Like other immune cells Kupffer cells play a role in the defense against infections and they respond highly to acute-phase proteins. They also play important roles in signaling during liver regeneration and inflammation and release a variety of immunoregulatory compounds like IL-1, IL6, TGFβ and TNFα. They also express MHC class II molecules and they process and present antigens to lymphocytes.
The stellate cells are pericytes which also are called Ito cells or fat-storing cells. These cells are found in the space of Disse; they store vitamin A under normal conditions and form extracellular matrix like laminin, collagen type IV and fibronectin. They also regulate the tonus in the sinusoids and thereby regulate the blood flow. In response to liver damage they are activated, transforms into myofibroblasts and form scar tissue by changing their components of secreted matrix proteins to collagen type I and III and simultaneously break down the normal matrix proteins. By these mechanisms stellate cells are involved in the formation of

fibrosis and eventually cirrhosis in chronic liver disease. The sinusoidal endothelial cells are the cells lining the sinusoids in the liver, and in contrast to endothelial cells in capillaries in other tissues they lack a basement membrane and are fenestrated to facilitate passage of molecules [1]. In cirrhosis, the fenestrations are lost, and fibrotic tissue in the space of Disse reduces the exchange of metabolites between the blood and hepatocytes *(Figure 1*).

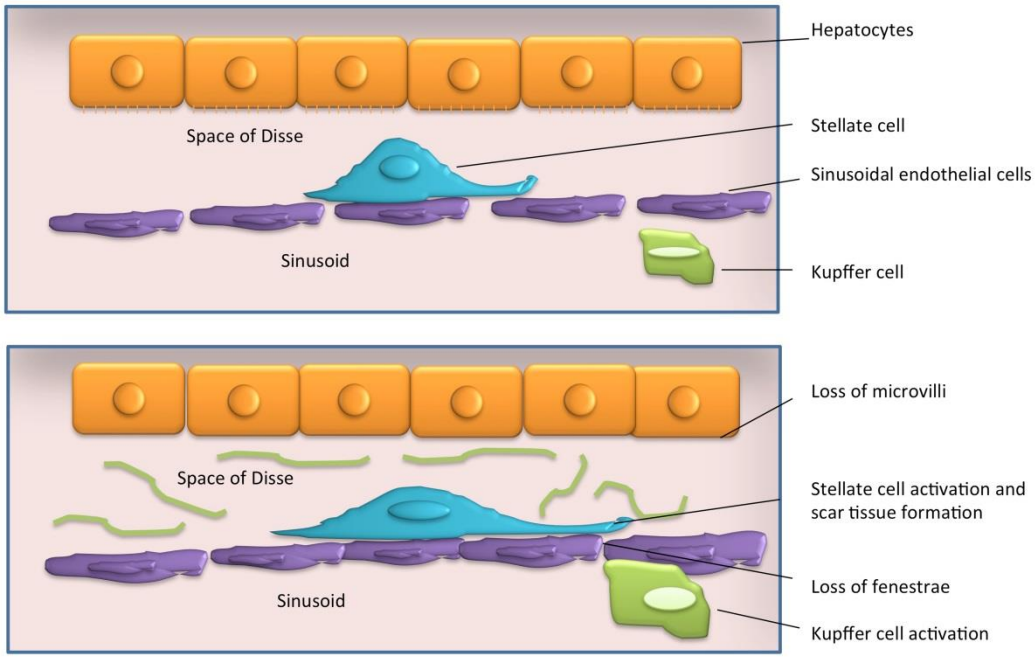


Figure 1. A simplified picture of the microscopic architecture of the liver sinusoid in a normal condition and in fibrosis. The upper panel demonstrates normal hepatocytes with microvilli, gap junctions and tight junctions. The stellate cells and the Kupffer cells are inactive, the extracellular matrix is normal, and there are fenestrations between and through the endothelial cells. The lower panel demonstrates a fibrotic liver; the microvilli of the hepatocytes are lost, the activated stellate cells have been transformed to myofibroblasts and produces collagen. The Kupffer cells will also migrate to the damaged area and release cytokines that will further enhance the inflammation.

1.1.3 Hepatocellular metabolism

The liver is the major site for protein and amino acid metabolism in the body and the uptake of ingested proteins starts by degradation in the gastro-intestinal tract. Proteins are cleaved to shorter chains by proteolytic enzymes and will by efficient hydrolysis yield free amino acids. The amino acids are absorbed by the gut mucosa and transported to the liver via the portal vein. Humans have the capacity to synthesize 10 of the 20 amino acids needed to build up proteins. Those 10 amino acids that we cannot make ourselves are thus essential and have to

be ingested via the food. About 400 g of protein is turned over daily in a normal-sized man where 75% is degraded and recycled through constituent amino acids [2]. The remaining 25% is degraded via oxidation or converted into glucose.

For the uptake of amino acids into the hepatocyte there are several sodium-dependent membrane transport systems on the sinusoidal side of the cell [3, 4]. The liver can also take up large proteins from plasma with a very efficient receptor-mediated endocytosis; for example it is estimated that one single hepatocyte can engulf 5 million molecules of asialoglycoprotein per hour [5, 6]. The endocytosed proteins are directed to the lysosomal compartment for further degradation. Examples of proteins that are taken up either via specific receptors or by fluid-phase endocytosis are: IgA, EGF, TGFβ, low-density lipoproteins (LDL) asialoglycoproteins, hemopexin, albumin and immunoglobulins.

The liver synthesizes the essential amino acids needed and it produces and secretes more than 90% of circulating plasma proteins, the most prominent of which is albumin. The hepatocytes also produce enzymes, receptors, membrane channels and transport proteins to be used within the cell. Other plasma proteins that are secreted by hepatocytes are blood clotting factors, ceruloplasmin and α-1-antitrypsin to mention some.

Ingested fatty acids, mostly triglycerides, are broken down to free fatty acids and monoglycerides by pancreatic lipase to allow absorption by the intestine. Once inside the body they will be reformed into triglycerides and package with apoproteins and cholesterol into blood-soluble complexes called chylomicrons. The chylomicrons move across the blood vessel membrane and into the bloodstream. These complexes will bind to the membranes of hepatocytes, adipocytes and muscle fibers and will within the cells be either oxidized forming CO_2 and ATP or stored as fat droplets in adipose tissue. Free fatty acids (FFAs) are transported to the muscles via serum albumin as an energy source. Also, free fatty acids are transported from splanchnic adipose tissue via the portal blood into the liver. Once in the liver, FFAs are either oxidized by mitochondria to yield energy or together with glycerol form triglycerides as part of very low density lipoproteins (VLDL) synthesis. Excess lipids form triglyceride-containing lipid droplets in the cytoplasm of the hepatocytes, a condition named *fatty liver* if the fat content exceeds 5 % of the liver volume.

1.2 LIVER REGENERATION

Liver regeneration is a process where lost or damaged liver tissue is replaced to restore the functional liver mass. Since the liver is the major organ in the body with detoxifying features, the regenerative capability is most likely a result of an evolutionary adaptation to a toxic environment on earth. Liver regeneration occurs as a response to cell damage due to toxic injury, necroinflammation or after cell loss (e.g. resection) and the first cells to respond are the hepatocytes.

What triggers regeneration and what are the underlying mechanisms? Hepatocyte cell proliferation may be induced by chronic repair in an inflammatory environment, or when the liver is exposed to toxins (such as drugs or alcohol). The liver can increase its capacity in detoxification by increasing its functional mass via cell renewal –hyperplasia - or by an increased size of existing cells –hypertrophy. Hypertrophy may occur transiently after resection or after major cell death as a functional compensation before the existing

hepatocytes or progenitor cells have been able to proliferate [7]. Hyperplasia on the other hand, is the fundamental feature during liver regeneration, and an effect of enhanced cell proliferation. The changes in the tissue homeostasis leading to liver regeneration are of crucial significance for the organism. However, in all tissues with a high cell proliferation there is an increased risk for genetic changes and mutations to occur. Also, the liver is exposed to additional oxidative stress in a toxic environment, increasing the risk of cellular damage. Agents that may cause liver damage and trigger increased cell proliferation are chronic exposure to alcohol, hepatitis virus, iron overload and aflatoxin. Combinations of some of these agents can further aggravate the cell damage, such as hepatitis C plus alcohol [8] or hepatitis B plus aflatoxin.

Another major event inducing liver regeneration is surgery, in particular liver resection. Higgins and Anderson described the regeneration after partial hepatectomy (PH) already in 1931[9]. In the rodent PH-model two-thirds of the liver is surgically removed and the remnant liver enlarges until the initial mass is reached [10]. The liver mass is reconstituted in approximately 5-7 days in rats, and a peak of regenerative activity is seen during the first 3 days after which most hepatocytes become quiescent again [11]. Twelve hours after hepatectomy the majority of the hepatocytes enters the S-phase and reaches a peak at 24 hours. A complete restoration of liver mass requires 1,6 cycles of DNA in all cells, including non-parenchymal cells in which DNA synthesis occurs later compared to that seen in hepatocytes [12].

Basically the same mechanisms of regeneration take place during recovery in a liver exposed to toxic cell damage or hepatitis, and after experimentally triggered regeneration following PH, except for one major difference. In the rodent PH model, the two biggest lobes are removed by ligation of separate liver lobes, leaving the three small remnant lobes to regenerate without any damaged tissue and thus no necrosis or apoptosis. In a human liver under toxic stress there will be both necrosis and apoptosis and subsequently regeneration to compensate for that loss. A new term has appeared to describe the phenomenon of liver regeneration in liver tissue homeostasis: “hepatostat”, meaning to explain the vital functions of the liver in relation to the needs of the body [13].

The expression “liver regeneration” is somewhat misleading since the liver mass and functional capacity but not shape is restored. A more correct term would be compensatory hyperplasia, since the tissue is replaced by the vast cell proliferation of the remnant lobes in which virtually all of the cells will replicate. The regeneration stops when the organism´s demands are fulfilled.

Liver regeneration can be described as different processes which do not exclude one another. They may occur in different proportions depending on the extent of the loss of tissue and to what extent liver proliferation is impaired.

The different models described include 1) replacement of tissue loss where each cell type replicates to replace its own cellular compartment, 2) replacement of liver tissue by activation of transdifferentiation pathways originating from facultative stem cells, 3) an increase in liver size known as augmentative hepatomegaly, where an increase in size above baseline is seen [15].

Liver regeneration is complex and is orchestrated by several different pathways involving both intrinsic and extrinsic signals, growth factors and cytokines.

1.2.1 Initiation of cell proliferation

Studies of signaling events after PH have been going on for more than two decades and experiments intended to completely eliminate single pathways have not revealed any explicit pathway to be totally vital for regeneration to take place. It is rather a series of events and combinations of intrinsic and extrinsic signaling pathways that together orchestrate the initiation of liver regeneration.
It is important to keep in mind that after a 2/3 PH the remnant liver lobes are subjected to most of the portal blood flow that previously supported the whole liver, meaning that the remnant liver lobes are exposed to an increase load of nutrients and other factors, including growth factors. It has been seen that when the portal circulation was partly diverted from the liver, the regeneration was delayed and the levels of hepatocyte growth factors were decreased [16]. In a canine-model of PH where the whole portal blood flow was bypassed using a portacaval shunt, liver atrophy occurred, but once the portal flow was re-established the size was completely restored [17].

1.2.2 Intracellular signals

One of the intrinsic signaling pathways involves the activity of the urokinase-type plasminogen activator (u-PA), an enzyme produced by hepatocytes. A protease cascade starts where all steps contribute to remodeling of the extracellular matrix. Hepatocyte growth factor (HGF) which is a complete mitogen (see below) is also activated by u-PA and activated HGF further increases the expression of u-PA. Both of these above mentioned mechanisms functions as mitogenic signals for hepatocytes in liver and the increase in u-PA activity is the earliest biochemical change documented in the regenerating liver [18-20].
Beta-catenin, another protein in the intrinsic signaling pathways of early liver regeneration, migrates into the nucleus very short after PH. In the nucleus it dimerizes with proteins belonging to the Tcf family of transcription factors thus initiating transcription of cell cycle associated genes [21].The presence of beta-catenin is increased in the nuclei of hepatocytes already 5 minutes after PH and remains there for more than 24 hours [22]. Mice lacking beta-catenin show insufficient liver regeneration [23].
Yet another rapid actor is Notch, a transmembrane protein that after binding to its ligand and the intracellular domain of Notch is cleaved off will migrate into the nucleus. This occurs about 15 minutes after PH and induces transcription of Notch-dependent genes. Deficient rat liver regeneration has been observed when Notch or its ligand has been inhibited [24].

1.2.3 Extracellular signals

Extrinsic pathways (also called extra-cellular signals) can be divided in two main groups, based on their mode of actions. First, there are the complete mitogens that involve HGF and its receptor MET and EGFR with ligands (EGF, transforming growth factor alpha (TGFα), amphiregulin and HB-EGF)). The complete mitogens causes enlargement of the liver in intact non-resected animals and are mitogenic for primary hepatocytes cultured in chemically defined and serum-free media. Second, there are so called auxiliary mitogens that involve molecules that control the accurate timing of important transcription factors involved in events essential for initiation of liver regeneration, which include IL6, tumour necrosis factor

(TNF), norepinephrine, bile acids, leptin and more. Without any of the auxiliary mitogens the regeneration will be delayed but not totally impaired. Many of the signaling molecules belonging to both the complete and auxiliary mitogens rise in blood shortly after PH. There are studies showing synergistic effects between the complete and auxiliary mitogens.
In rat cell primary cultures norepinephrine reinforces the mitogenic effects of EGF and HGF and also offsets the negative effect on mitosis of TGF-beta [25]. The role of TGF-beta during liver regeneration is intriguing and was shown to inhibit proliferation in cell culture but enhances at the same time the motility of the hepatocytes. TGF-beta is expressed by stellate cells. There is no evidence that hepatocytes express it, but hepatocytes have receptors on its surface responding to it and most of hepatocarcinomas produces it [26].

1.2.4 Interleukin 6

The IL6 is a well-known cytokine that belongs to the innate immune system and is one of the acute-phase proteins. It is produced by macrophages, endothelial cells and T-cells in response to infection and tissue injury [27]. The level of IL6 is rapidly increased in the liver after PH and can be detected in plasma just a few hours after surgery. This pro-inflammatory cytokine stimulates secretion of other acute-phase proteins produced by the liver, it stimulates B-lymphocytes to produce antibodies and in concert with IL1, IL6 activates T-cells. Circulating IL6 binds to a soluble receptor and forms a complex. The IL6 –receptor complex binds to gp130 which is a receptor on the plasma membrane of cells including hepatocytes [28]. When the complex binds to the gp130 receptor it will lead to an activation of the JAK/STAT signaling pathway [29]. STAT3 will be phosphorylated by JAK, translocate to the nucleus and there function as a transcription activator and a mediator of expression for several genes involved in cell growth. When IL6 is knocked out in mice, liver regeneration is delayed because of a decrease in expression of STAT3 which is a key molecule in hepatocyte proliferation [30].

The involvement of TNFα in liver regeneration was first described 1992 when neutralizing antibodies was used against the molecule and liver regeneration was delayed [31]. The plasma levels of TNFα are raised shortly after PH and by assisting in NFκB activation with an unknown mechanism will promote cell proliferation [32]. An overview of the induction of the signaling machinery after 2/3 PH in rats is outlined in figure 2.

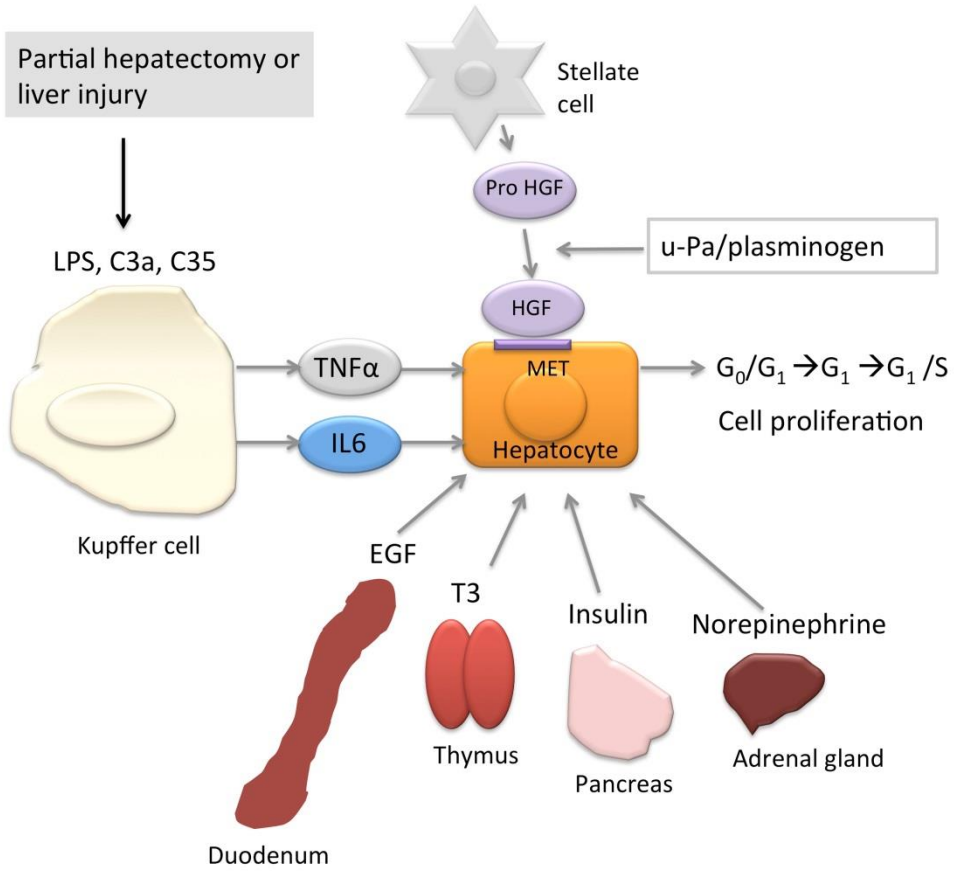


Figure 2. A schematic and simplified view of the complex signaling cascade that follows a partial hepatectomy. A complex orchestration of several signaling molecules and their respective pathways will simultaneously drive the hepatocytes towards proliferation quickly after partial hepatectomy in order to compensate for the lost liver tissue and to restore the functional liver mass.

1.2.5 Termination of liver regeneration

The regeneration stops when the liver has reached 100% of its original size and can fulfil the demands of the organism. The mechanisms lying behind termination are not as extensively studied as the initiating mechanisms, but the processes seem to be as complex. There is evidence of an increased number of hepatocytes at the end of regeneration and that a small wave of apoptosis occurs to adjust the number [33].

1.2.6 Role of the facultative stem cells in regeneration

A stem cell is a cell that has the ability to continuously divide and differentiate into various other kinds of cells. In the liver, hepatic progenitor cells (HPCs), also named oval cells, are proposed to reside within the Canals of Hering into which the bile produced by the hepatocytes is drained. The normal liver regenerates through proliferation of mature

hepatocytes, but in chronic liver injury this capacity may become lost or impaired. As an alternative for restoring lost liver mass the bipotential HPCs can give rise to both hepatocytes and biliary epithelium [7]. However, the HPCs may also be a potential source of liver cancer [8]. The underlying molecular mechanisms for activation of HPCs are not yet known. In transgenic mice an overexpression of a member of the TNF-family of cytokines was found to stimulate oval cell proliferation [9] and also a hepatocyte-specific deletion in a tumour suppressor gene was found to result in a progressive expansion of oval cells [10]. However, if these factors contribute to a direct stimulation of oval cells is still unclear and the exact mechanism remains to be further elucidated.

1.3 HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and accounts for around 6 % of all human cancers worldwide. It is the fifth most common cancer worldwide and the third most common cause of cancer-related death after lung and stomach cancer [11]. The prevalence is highest in Asia and sub-Saharan Africa which can be explained by the linkage to the endemic hepatotrophic viruses like hepatitis B and C viruses, which both are important factors in the development of HCC. However, vaccination programs against HBV are currently going on in many parts of the world, which will reduce the risk and incidence of HCC in the future [12]. In the western world the yearly incidence is lower but rising where excessive alcohol ingestion and obesity are plausible factors to the increasing number of HCCs [13]. Synergistic effects have been reported between obesity and alcohol abuse and the development of HCC [14]. Also interactions between alcohol ingestion and hepatitis C have been observed to aggravate the liver disease [15].
Other risk factors that are linked to HCC are aflatoxin-contaminated food, and inherited metabolic disorders like hemochromatosis and glycogen storage disease. In 80% of all cases HCC has developed from an underlying liver disease that has led to liver cirrhosis or chronic inflammation. In patients with HCV and excessive alcohol intake the risk is potentiated. The risk of getting HCC is 2-4 times higher for men compared with women.

1.3.1 Pathogenesis

Carcinogenesis is a multistep process
Generally, carcinogenesis is described as a multistep process that starts in a single cell and that will ultimately lead to a malignant tumor. The molecular steps of hepatocarcinogenesis are not yet fully understood, and no dominant pathway of the development has been revealed. Evidence suggest that chronic liver damage and inflammation will lead to an oxidative environment mediated by free radicals and inflammatory cytokines that together with chronic repair processes may be of importance for the carcinogenic process. In experimental hepatocarcinogenesis on rat liver this multistep process has been defined. The first step, initiation, starts with formation of DNA-adducts changing the molecular structure of certain genes, (genetic alterations), the majority of such alterations are eliminated through the DNA-repair machinery within the cell within the first 24 h. If cell division in a cell occurs before DNA repair is complete, the molecular alteration of the DNA-base can cause a false transcription

giving rise to genetic alterations in the daughter cell, which now carries a mutation. In the next step, promotion, the initiated cells will by their growth advantage over normal cells in the presence of a toxic promoter proliferate and by clonal expansion form pre-neoplastic foci. The preneoplastic cell is dependent on a promoter for further development and represents a reversible stage of the development providing that the promoting agent or other circumstances that selects for growth of initiated cells are removed, for example clearance of hepatitis virus or avoidance of alcohol. A promoter is any agent creating a selective pressure that favors the cells that have acquired a resistance to the mitoinhibitory effect of the promoter. The preneoplastic cells cannot yet be defined as tumour cells but rather as cells adapted to a toxic environment possessing a growth advantage that will allow cells to grow, when normal cells do not. This adaptation has been crucial for survival in toxic environments and is frequently seen in cancer development and classified as dysplasia or intraepithelial neoplasia. Eventually the pre-neoplastic cells will by further mutations lose their ability to reverse to normal tissue and therefore become neoplastic. Finally, in the progression step, multiple alterations at the genetic level will occur in the growing cells and further clonal expansions and selection of cells with growth advantage will give rise to cell populations with the malignant growth pattern and phenotype. The phenotypic alterations described in the preneoplastic cells are multi-genetic and complex and referred to as the resistant phenotype [16]. In this thesis we used the Solt and Farber model for chemically induced carcinogenesis in rat liver (paper II). In this model initiation and promotion are achieved using chemical carcinogens in combination with mitoinhibitory agents and regenerative stimuli [17]. See methods.

Studies have shown that defective DNA mismatch repair, telomerase activation and induction of angiogenic and growth factors can contribute to the transformation of liver cells [18-20]. The knowledge of growth factor activation is incomplete but some are thought to be involved in one or more steps in hepatocarcinogenesis. Expression of insulin growth factor II -a factor that is active in the fetal liver and then silenced – has been noted in both animal models of liver cancer and human HCCs and was also suggested as an early marker [21-24]. During liver regeneration transforming growth factor alpha (TGF-α) is proposed to have a direct stimulating effect of DNA synthesis, and in a clinical study, 65 % of the patients was shown to have elevated levels of tumour TGF-α in the urine and the growth factor has in addition to that also been found to be overexpressed in the majority of HCCs [25-27]. In a rat study, the expression of TGF-α was increased and was suggested to have a promotive role in the liver carcinogenesis [28] . Oncogene activation of particularly the ones belonging to the *ras*-family has been found in animal hepatocarcinogenesis models whereas in humans no uniform pattern of activated proto-oncogenes has been seen [29-33].
The importance to understand the growth-regulation on a molecular level is of great value for understanding cellular events involved in transformation of hepatocytes and may be helpful to improve preventive strategies and novel therapies for hepatocellular carcinoma.

1.3.2 Drug metabolism

The metabolism of drugs in the hepatocytes involves three major steps. In the first step, called phase 1, harmful substances are detoxified by some of the cytochrome p450 supergene family of proteins. This step generates reactive intermediates since CYP 450 adds a reactive group, often a hydroxyl radical to the compound being bio-transformed. To protect the cell from the

now activated compound, a phase II conjugating reaction occurs. This step makes the intermediate compound water-soluble to facilitate excretion from the body. Several types of conjugation molecules are present in the body and the reactions include glucuronidation, sulfation, and glutathione and amino acid conjugation. Excretion of drugs often referred to as phase III of drug metabolism are mediated by membrane drug pumps, in the hepatocytes often associated with the bile canalicular plasma membrane.
In the resistant phenotype these drug metabolizing systems are altered, making the cells more resistant to toxicity and oxidative stress. The phase I, CYP450 reactions, are slowed down while the phase II conjugating processes are increased. As a consequence less toxic intermediates will form and the excretion of water soluble compounds will increase. In addition, levels of several antioxidants are increased such as the lipid-soluble membrane associated ubiquinols, the water soluble glutathione, SOD, catalase and thioredoxin to mention some. Another early alteration is the inactivation of p53 preventing it to enter and act in the nucleus [34].
P-glycoprotein also known as multidrug resistant 1 (MDR1) is an ATP-driven drug efflux pump with a broad substrate specificity for xenobiotics. This pump decreases drug accumulation in multi-drug resistant cells and contributes further to the development of resistance to anticancer drugs. [35]. Expression of the multidrug resistant genes MDR1 has been found in many cancers including liver cancer. A clinical study showed that MDR1 was overexpressed in patients with untreated liver cancer [36].
Since the cancer cells have acquired efficient ways to detoxify harmful substances there is a challenge to develop new regimens for the treatment of HCC.

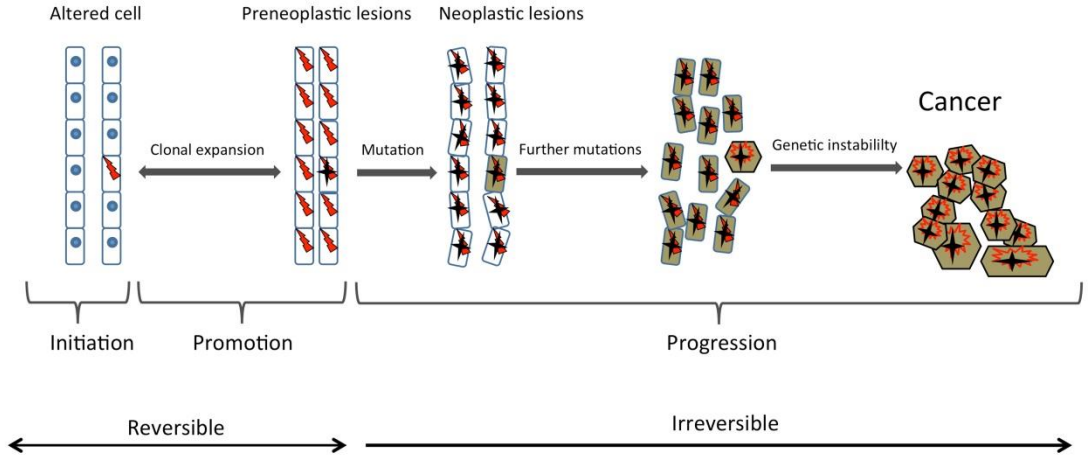


Figure 3. Schematic picture of cancer development. Not surprisingly the picture of what is known today about HCC development displays a large heterogeneity on the basis of the genetic instability and its mixed etiology [37]. However, in patients with alcohol abuse, hepatitis infection or certain metabolic diseases hepatic transformation occurs in the context of chronic liver injury, regeneration and cirrhosis, conditions which may lead to oxidative DNA damage and subsequently genetic alterations such as chromosomal rearrangements leading to loss of heterogeneity and inactivation of tumour suppressor genes, for example p53 [38].

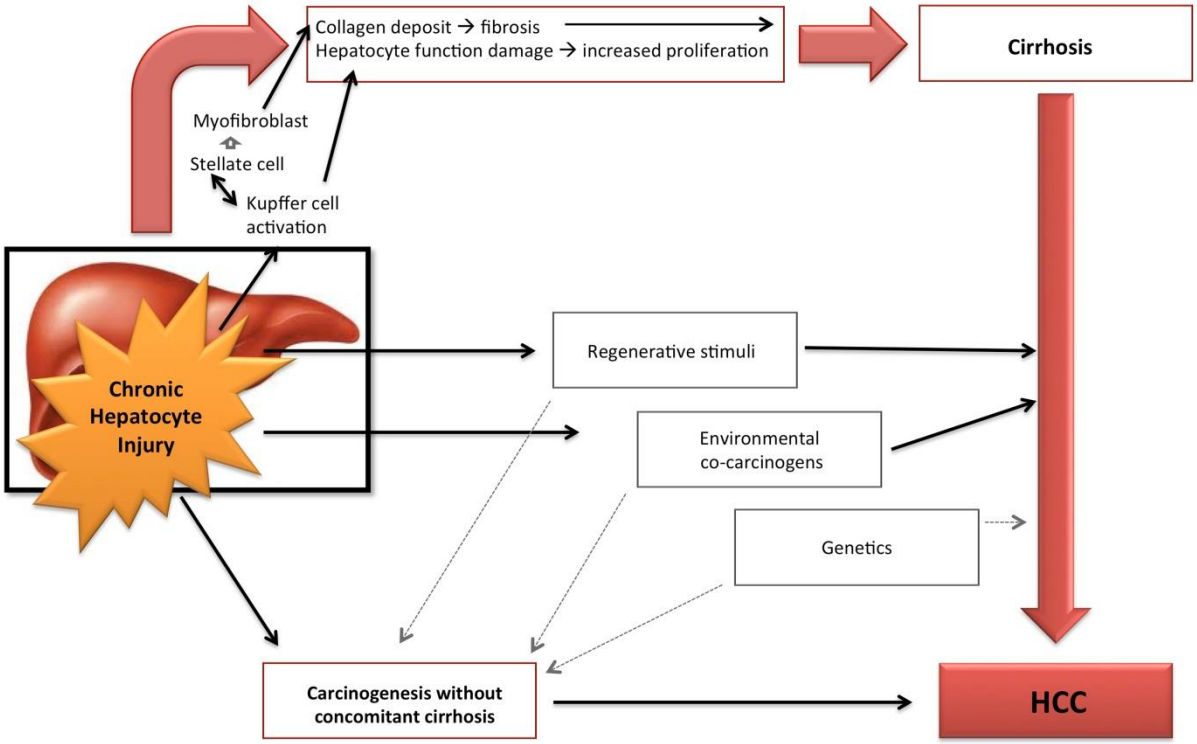


Figure 4. Chronic liver injury and chronic repair may lead to tumour development.
The regenerative growth factors expressed during chronic liver repair, together with the complex environment of oxidative components and co-existing cell proliferation will together support liver tumour development [39]. This whole series of events may take decades from the initial infection or initial exposure to harmful agents until manifestations of the tumour disease. The majority of human liver cancers develop in a cirrhotic liver, and if an underlying viral hepatitis exists, interactions between the virus and the host immune reactions may lead to chronic necroinflammation.

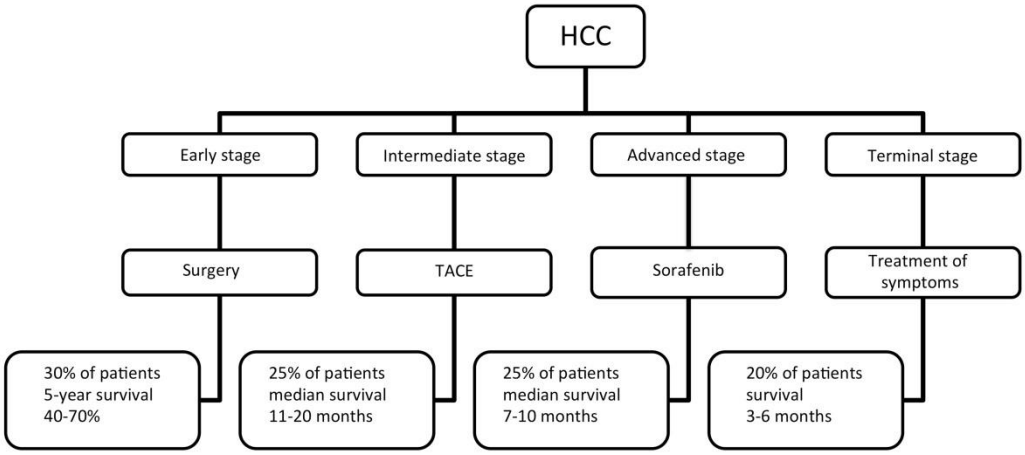


Figure 5. Staging of HCC as suggested of BCLC (Barcelona Clinic Liver Cancer). Adopted from Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet, 2003, 6;362(9399):1907-1917).

1.3.3 Diagnosis and treatment

One important problem with HCC is its asymptomatic progression; patients can go for a long period of time with a silent malignant disease and eventually when the tumour burden is so high that symptoms arise, the disease has progressed to an advanced stage. The liver has a profound capacity to compensate for loss of function and early symptoms are sparse and un-specific [40]. If a tumour eventually interferes with bile ducts, serum bilirubin will increase and jaundice (yellowing of the eyes and skin) arise. Patients with a known chronic liver disease and cirrhosis are often included in surveillance programs with repeated ultrasound investigations to detect tumors at an early stage and possibly offer a curative treatment such as liver transplantation, resection or radiofrequency ablation.
Staging of tumours is of paramount importance in order to offer the most appropriate treatment available. This applies to all types of cancer, but in the case of HCC, this is particularly difficult given the degree of the underlying liver disease in combination with the patient's general condition. For this purpose several staging systems have been developed, the most widely used of which is the BCLC (Barcelona Clinic Liver Cancer) system.
Treatments of HCC can be divided in three groups: curative treatment, palliative treatments or best supportive care. The first group involves transplantation, resection and local ablative

treatment. The palliative treatments include transarterial chemo-embolization (TACE) and systemic treatment with sorafenib or internal or external radiation. The treatment in the latter group aims to alleviating the symptoms of the disease and increase survival.
Patients with early diagnosed HCC may thus be subjected to hepatic resection, transplantation or local ablation for potentially curative purposes. Even though patients with liver cirrhosis are surveyed with ultrasound, the majority of tumours are still diagnosed at an intermediate or advanced stage [41]. Tumours discovered at an intermediate stage can be treated with transarterial chemoembolization (TACE) [42], whereas to date the only treatment proven to prolong life for patients with advanced HCC is the multikinase inhibitor sorafenib [43].
Sorafenib is a low weight molecule that inhibits phosphorylation of serine/threonine kinases like Raf-kinases and receptor tyrosine kinases such as vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor beta receptor (PDGFR-β) thus preventing cell growth promoting pathways and angiogenesis by inhibition of genetic transcription. Sorafenib was approved by Food and Drug Administration (FDA), in 2005 for kidney cancer and 2007 for treatment for non-resectable HCC [44].

1.3.4 Sorafenib

1.3.4.1 Modes of action

The signaling pathway Raf/MEK/ERK is a positive proliferative signaling cascade that results in the activation of ERK 1/2 and is activated by growth factors like EGF, PDGF, VGF and cytokines like IL6 and TNF-α. Sorafenib inhibits this cascade at the level of the serine/threonine kinase activity of Raf and also the receptor tyrosine kinase activity of VGFR-2 and PDGFR-b (fig 5). Sorafenib binds to the ATP binding site on its targets thus preventing the receptor kinases from binding ATP and to phosphorylate their respective substrates. There are other substances today that also prevent the above mentioned targets, while sorafenib is the only drug that also targets the Raf/Mek/Erk pathway.
Sorafenib can function as inhibitor, antagonist and/or substrate and over 20 molecules have been revealed as targets for the actions of sorafenib [45] . It inhibits Raf proto-oncogenes serine/threonine kinases and several isoenzymes in the CYP450 family of proteins as well as transporter proteins like multidrug resistance-associated protein 4 and MDR1 [46, 47]. Moreover sorafenib is a substrate for some CYP450 and UDP glucuronosyltransferases and an antagonist for VEGFR2 and 3, FL cytokine receptor, beta PDGFR and Mast/stem cell growth factor receptor (KIT).

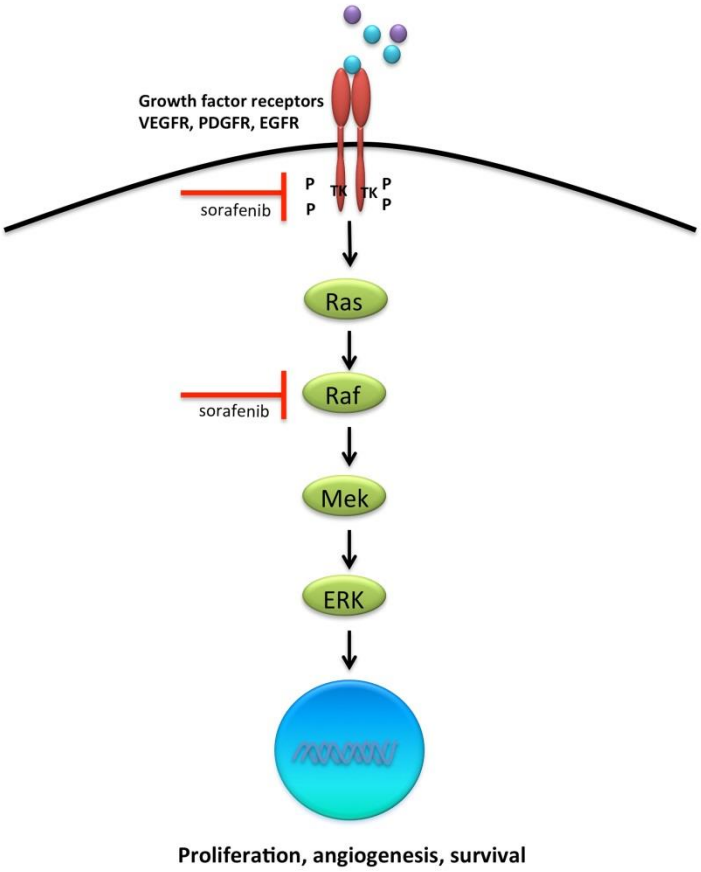


Figure 6. Schematic picture of mechanisms of the multi-kinase inhibitor sorafenib. Phosphorylation of tyrosine kinases of growth factor receptors are inhibited as well as inhibition of Ras in the Ras/Raf/Mek/ERK pathway. Inhibition of these pathways will slow down cell proliferation and angiogenesis.

1.3.4.2 Biotransformation and elimination

The clearance of sorafenib is primarily performed by the liver; the drug is oxidized by CYP3A4 and glucuronidized by UGT1A9 and the elimination half-life is 25-48 hours. Sorafenib conjugates may be degraded in the gastrointestinal tract by bacterial glucuronidase activity which allows reabsorption of the unconjugated drug. The UGT1A9 is a gene that encodes for UDP- glucuronosyltransferase, which is an enzyme that converts lipophilic molecules to water soluble excretable metabolites. Five of eight identified metabolites have been found in plasma. Of these metabolites, the pyridine N-oxide comprises of 9-16% of the circulating analytes and show a potency similar to that of sorafenib *in vitro.* Steady state of plasma concentrations of sorafenib is reached within 7 days [48]. Common side effects of sorafenib are diarrhea, hand and foot syndrome, weight loss, fatigue and hypertension.

1.4 IRON

1.4.1 The neoplastic phenotype and iron

In many neoplastic cells iron metabolism is altered [49]. These alterations in iron regulation often coincide with increased proliferation and may thus contribute to disease progression [50]. One feature of pre-neoplastic liver cells is that despite their higher uptake, they are depleted in ferritin- bound iron and appear iron-free on histological sections. The turnover in tumours is high and they require a lot of iron but have very low levels of iron stored. The tumour cells increase their expression of TfR1, thus facilitating a high iron uptake to meet the high demands of iron in a malignant fast and autonomously growing cell.
Iron free dysplastic foci have been described in several studies in patients with hemochromatosis and in animal models of iron overload [51, 52].
One of the alterations seen in neoplastic cells are elevated levels of TfR1 indicating a higher demand for iron for tumour cell survival [53]. Another alteration was seen in a breast cancer study, where tumour cells had lower levels of ferroportin (FPN), the cellular iron exporter, and higher levels of hepcidin, the iron hormone, thus blocking release of iron from the cells. In this study there was a correlation between low levels of FPN and a bad prognosis [54].
Ferritin is not only an intracellular molecule, but also exist extracellularly and is believed to deliver iron to some extent, and the levels of ferritin have been reported elevated in some tumour tissues and may promote angiogenesis [55, 56].

1.4.2 Iron and iron metabolism

As an essential trace element, iron is crucial for life and as a component of different metalloproteins it participates in important cellular activities. These proteins include hemoglobin, myoglobin, catalase and cytochrome P450 [57]. Iron can also act as a cofactor of metalloproteins and is required in enzymatic processes involved in metabolism, cell respiration and DNA-synthesis [58]. The iron needed to meet demands from cells and tissues is transported to the cells as transferrin-bound iron in the plasma. The level of iron in plasma mirrors the absorption of dietary iron by duodenum and also the release of iron mainly from macrophages that stores iron. The macrophages in the spleen break down red blood cells and hence iron can be recycled. The total amount of iron that is lost and has to be added is 1 mg/day for men and 1.5-2mg/day for women. Most of the 4-5 g iron bound to cellular enzymes or in iron stores is recycled. There is no excretion pathway more than a small amount that is lost through shedding of cells of the skin and the mucosal cells of the gastro-intestinal tract.
The uptake of iron starts on the brush border of the enterocytes in duodenum where inorganic Fe^{3+} iron is reduced to Fe^{2+}. The ferrous iron is then transported into the enterocyte via the divalent metal transporter 1 (DMT1). Once inside the cell the iron can bind loosely to iron chelators like citrate or more tightly to ferritin. Iron is reconverted into the ferric form, by the ferroxidase property of ferritin [59]. For transportation to the bloodstream ferrous iron is transported via the transmembrane transporter ferroportin (FPN) and is directly converted to the ferric form by heaphestin outside of the basolateral membrane of the enterocyte. After oxidation the ferric iron is bound to transferrin for further transportation [60].
The uptake of iron from blood to hepatocytes can occur in several different ways [61]. The most important iron-binders are transferrin and citrate and the uptake of iron from transferrin

is well understood involving TfR1 and 2. However, the mechanisms of non-transferrin (NTBI) bound iron is still poorly understood [62].

1) Receptor-mediated endocytosis of transferrin via the high affinity receptor transferrin receptor 1 (Tfr1). First, diferric transferrin binds to the receptor at the cell surface of the hepatocytes [63]. Clusters of bound receptors are formed and the cell membrane is invaginated and pinched off and results in clathrin-coated vesicles that will fuse with intracellular vesicles. At this point endosomes with low pH (5,5) have been formed and the iron can be released from its carrier [64]. The iron is reduced to the ferrous form, enters the cytosol and is bound to ferritin for storage. The receptor-apotransferrin complex is recycled to the cell surface and the apotransferrin is released.

2) The second way most likely involves Tfr2 and is a low-affinity process with endocytosis of diferric transferrin, delivery of the iron to the cell and then a recycling event of transferrin back to the plasma membrane similar to Tfr1 [65]. TfR2 seem to have an iron-sensing and hepcidin-modulating role rather than iron uptake [66].

3) Fluid-phase endocytosis. Transferrin-bound iron can also be picked up by a non-endosomal pathway, something that was seen in isolated hepatocytes [67]. This pathway involves a NADH-dependent oxidoreductase for reduction of ferric iron. The electron flow across the membrane is leading to a lowered pH locally at the cell-surface, reducing the affinity of iron to transferrin and ferrous iron crosses the cell membrane by a mechanism that is still unknown.

4) The current model for the cellular uptake of NTBI involves a ferrireduction by trans membrane ascorbate (Asc) cycling. Extra cellular Asc reacts with NTBI resulting in Fe^{2+} and dehydroascorbate. The divalent iron is then taken up by the cell via DMT1 or another ferrous-selective transporter called Zip14 [68-70].

5) Heme iron is taken up by the enterocytes via the heme carrier protein (HCP1). Within the cell iron is released from heme via the action of the enzyme heme oxygenase [71].

6) Hepatocytes can also be provided with iron from circulating ferritin molecules by specific receptors [72].

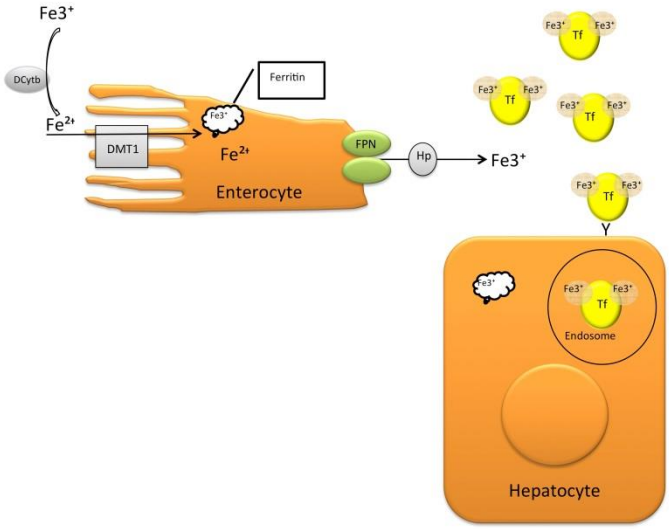


Figure 7. Uptake of iron in duodenum and transferrin-bound uptake of iron in hepatocytes. First ferric iron is reduced by DCytb on the surface of the enterocytes of the duodenum to ferrous iron to permit transport over the cell membrane via DMT1. In the enterocyte iron is either converted to the ferric form and stored in ferritin molecules or transported to the bloodstream via ferroportin. Iron is directly oxidized by heaphestin and bound to transferrin for further transport to the cells in the body.
Each transferrin molecule can carry two ferric iron ions. The diferric transferrin binds to Tfr1 on the cell surface of the hepatocytes and is invaginated via endocytosis; the pH in the endosome is lowered by an ATP driven membrane proton pump which facilitates the release of iron from its transporter. The iron is reduced and passes through the membrane into the cytosol where it is stored in ferritin or utilized for cellular processes.

1.4.3 Hepcidin, the master iron-regulatory hormone

Since there is no way to excrete and get rid of iron from the body, the uptake must be strictly regulated. In 2004 the peptide hormone hepcidin was discovered and is today recognized as the major regulator of iron homeostasis and metabolism. Hepcidin is synthesized by the liver and is circulating in the plasma after its secretion. The peptide reduces both uptake and release of iron by binding to the iron efflux protein ferroportin (FPN) on enterocytes and macrophages leading to its internalization and blocking iron export. Expression of hepcidin is induced by high iron levels or as a response to inflammatory cytokines such as IL6 [73, 74].
The iron-sensing pathway of hepcidin induction is complex and involves Tfr1 and 2, bone morphogenetic proteins (BMPs), the hemochromatosis gene (HFE), hemojuvelin (HJV) and Smad4. A central regulator in this pathway is HJV which is an iron-specific ligand of the BMP receptor leading to sensitization of BMPs [75]. Transcription of hepcidin is regulated via BMPs induction of Sma- and Mad related protein 4 (SMAD4) which in turn regulates a cascade of several Smad proteins [75].
The HJV/BMP/SMAD4 pathway interacts with Tfr1, Tfr2 and HFE. In collaboration with Tfr1 and 2, HFE detects the levels of holotransferrin in plasma and when iron levels are high iron will bind with high affinity to Tfr1, where upon HFE can interact with Tfr2. This interaction on the cell surface of the hepatocyte will increase the BMP/SMAD4 signaling and lead to activation of transcription of hepcidin [76].
The inflammatory pathway of hepcidin transcription induction involves interleukin 6 (IL6) and transcription factor of activation 3 (STAT3). There are evidence from several studies of at least one inflammatory cytokine to induce activation of transcription of hepcidin in both cells and animals [73].

During the acute-phase response of inflammation IL6 is released from macrophages and Kupffer cells, it binds to its receptor on hepatocytes and a signal goes via JAK/STAT to STAT 3 and an activation of transcription of hepcidin is induced [77]. Cross talking between the JAK/STAT and the SMAD pathways have been suggested in fetal brain, and may be a possible process in the liver as well [78].

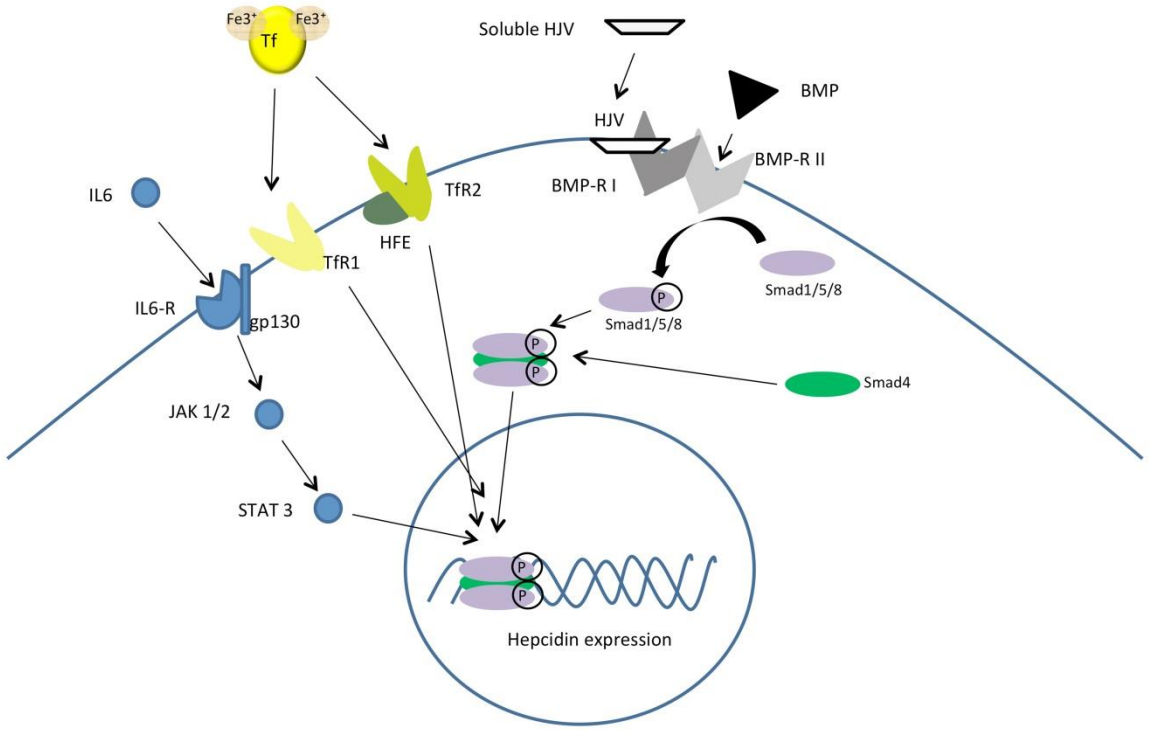


Figure 8. Simplified picture demonstrating the induction of hepcidin transcription via the 1) inflammatory pathway involving IL6 and JAK/STAT and 2) the iron sensing pathway that involves diferric transferrin, Tfr1 and TfR2, HFE, BMP/HJV and SMAD4. Under normal conditions the interplay between these pathways are fine-tuned and strict.

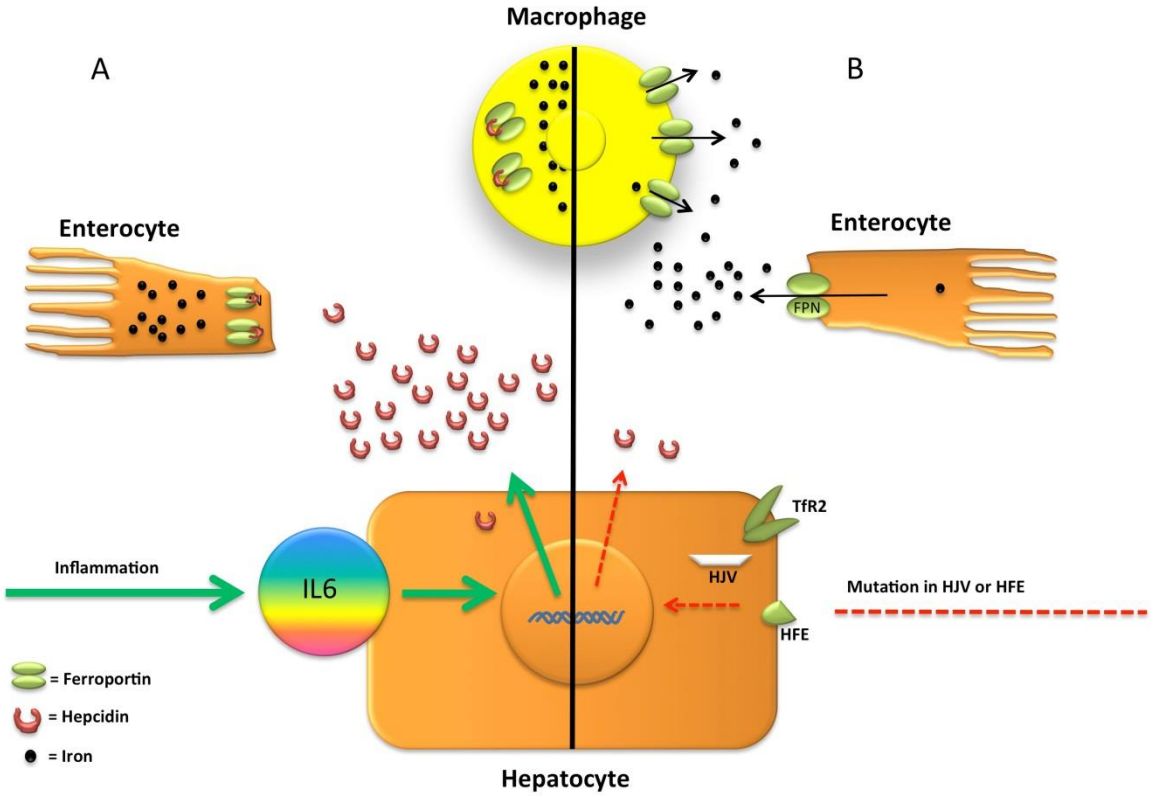


Figure 9. A) Inflammatory induction of hepcidin via IL6 will lead to internalization of the iron exporter ferroportin (FPN). Hepcidin binds to FPN and degrade the protein leading to a block of release of iron from the enterocytes to the bloodstream and block of release of iron from macrophages. The levels of iron in plasma will decrease. B) Deactivation of induction of transcription of hepcidin. The right side in the picture shows an impaired expression of hepcidin due to mutations in HJV or HFE, as in hereditary hemochromatosis. The iron sensing pathway of transcription of hepcidin will be disturbed and the decreased expression of hepcidin will lead to iron overload.

1.4.4 Iron storage and regulation

Although essential for life iron possess toxic properties in its free form and therefore organisms are equipped with a complex and carefully regulated machinery for storage of iron and regulation of iron homeostasis. Free iron is highly toxic to the cells and therefore the uptake as well as the storage is tightly regulated to avoid the fact that free iron can act as a catalyst in the formation of free radicals from reactive oxygen species (ROS). In the so-called Fenton reaction a hydroxyl radical is formed from hydrogen peroxide catalyzed by ferrous iron [79, 80].
The Fenton reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}\mathbf{OH}$

Ferritin, a protein synthesized by the liver is the major iron storage molecule within the cell, predominantly found in the cytoplasm and in lysosomes. Apo-ferritin consists of 24 iron binding subunits. Each ferritin molecule can therefore harbor thousands of ions of ferric iron [81]. The size of the ferritin is around 450 kDa and it is composed like a shell that surrounds a ferrihydrate moiety [82]. In addition ferritin has a ferroxidase activity and can accordingly convert ferrous iron to its ferric form.
The iron can also be bound to iron-regulatory-proteins (IRP) which becomes activated in iron deficiency and when iron demands are high will bind to iron-responsive elements (IRE) of mRNAs. This is a post-transcriptional regulation of iron uptake and storage. When intracellular iron stores are scarce, activated IRP binds to iron-regulatory elements (IREs) which stabilizes the mRNAs of Tfr1 and DMT1 but blocks the translation of mRNAs of ferritin and FPN [83].

1.4.5 The role of iron in cancer development

Despite its necessity for all cells iron in excessive amounts may lead to fatal toxicity. Since the liver is the major site of iron storage it is also in particular affected to the toxic effects of excessive iron. The catalytic process by which the toxic effects of iron occurs involves the enhanced formation of hydroxyl radicals via the Fenton reaction and may lead to peroxidation of membrane lipids of the lysosomes, the endoplasmic reticulum and the mitochondria and leading to organelle dysfunction and cell death [84-87]. In addition studies suggest that free iron has a direct hepatocarcinogenetic effect by the formation of ROS and oxidative injuries that are both mutagenic and carcinogenetic [88]. See more about free radicals under that section on page 24.
In disorders, genetic or acquired, with disturbed iron regulatory machinery, patients are at risk for iron overload of parenchymatous organs like the liver. This is seen in patients with hereditary hemochromatosis (HH) which is the most common cause of iron overload in individuals of Northern European origin. Excessive amounts of iron are absorbed from the gut and are primarily stored in the hepatocytes [89-91]. Several mutations of the HFE have been revealed and the most common in hereditary hemochromatosis in Sweden and Northern Europe is the Cys282Tyr mutation [92, 93]. This mutation renders an altered protein prevented to reach the cell surface. Iron regulation is hereby disrupted and results in altered iron-sensing signaling, hepcidin deficiency and iron overload [94]. Moreover, mutations in the HJV gene

leading to an ineffective protein have been shown to decrease hepcidin synthesis and cause a severe iron overload (juvenile hemochromatosis) [95].
Secondary iron overload implicates hepatic iron overload as a consequence of repeated transfusions or as a result of increased iron absorption in patients with thalassemia major or sideroblastic anaemias. In addition secondary iron overload is also seen in disorders in porphyrin metabolism like porphyria cutanea tarda [96].

1.5 OXIDATIVE STRESS AND FREE RADICALS

Atoms or molecules with one or more unpaired electrons are called free radicals. Molecules with free electrons are reactive and can participate in reactions to form new molecules, a prerequisite for metabolism and life. A reactive compound can bind to cellular molecules and compromise the structure or function of cellular organelles. Free radicals are naturally formed as by-products in the cellular metabolism of oxygen. Some cells can use free radicals in their mission to degrade foreign compounds and constitute a part of the immune system for the destruction of invading microorganisms. In biological systems, functions and mechanisms are double-edged swords, as in the case with free radicals, whilst being a part of the immune system against invading microorganisms; they may cause serious cellular damage to the body. They are highly reactive and by their capability to damage DNA, proteins, lipids or carbohydrates, free radicals by causing cell damage or cell death may contribute to the development of chronic disease like cancer and heart disease [97, 98].
In addition to the internally formed radicals exogenous compounds in our environment can be activated and reactive as in cigarette smoke, drugs, solvents , food additives, industrial pollution. In the process of cellular elimination of the exogenous compounds reactive intermediates will be formed. Also exposure to UV-light and radiation form reactive compounds of natural compounds in the cell. The most important free radicals in biology are the superoxide (O_2^-) and hydroxyl radical ($OH^\bullet$.)

The hydroxyl radical is formed in the Fenton reaction together with Fe^{3+}+ Or Cu^{2+} but can also be formed from superoxide and hydrogen peroxide via the iron-catalyzed Haber Weiss reaction:

1) Iron salt will be reduced by O_2^- : $Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$
2) A hydroxyl radical forms via the Fenton reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^\bullet\mathbf{OH}$
3) Net reaction = the Haber-Weiss reaction $O_2^- + H_2O_2 \rightarrow O_2 + OH^- + {}^\bullet\mathbf{OH}$

1.5.1 Anti-oxidant defense

There are several ways in which unpaired electrons can be scavenged and these have been divided into three different levels of defense. The first level involves prevention of radical formation and to this group a number of proteins, enzymes and enzyme systems belong, such

as superoxide dismutase (SOD), glutathione peroxidase (gpx), catalase, glutathione, members of the thioredoxin systems and metal-binding proteins. In the second level we find molecules that act to prevent and restrict chain formation and propagation; carotenoids, glutathione, ubiquinols, vitamin A, C, E and uric acid. And in the third and last level of defense there are proteins that perform excision and repair of damaged parts of molecules; lipases, peptidases, proteases, transferases, and DNA-repair enzymes among others.
Examples of mechanisms to scavenge free radicals:
Superoxide which is the most common free radical is converted to peroxide and oxygen by SOD through the following reaction:
$2O^{*}_{2} + 2H^{+} \rightarrow H_2O_2 + O_2$

Hydrogen peroxide formed is further detoxified by GSH-px which reduces it to water as follows:
$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$

The hydroxyl radical ($^{\bullet}OH$**)** is highly reactive and is stopped by scavenging of endogenous antioxidants such as glutathione and melatonin or via dietary sources of anti-oxidants like vitamin E or mannitol [99].
Thiols are a class of organic compounds containing a sulfhydryl group (SH) composed of a sulfur and a hydrogen atom bound to a carbon. This group is also known as the thiol group and as the functional group of the amino acid cysteine it has a very important role in biology. When thiol groups of two cysteine residues meet they may form a cystin unit with a disulfide bond via an oxidation reaction. If the two residues belong to the peptide chain, the disulfide bond will contribute to the tertiary structure of that protein. If they belong to different proteins these bond can form quaternary structures and multi-unit proteins. Thiol groups in the active site of enzymes can contribute to their catalytic activity by forming non-covalent bonds with substrates. On the cell surface and in extracellular compartments many proteins that are rich in disulfide bonds reside. Redox cycling in the cell is of fundamental significance for the function of the cell but at the same time it is important to keep the intra cellular environment reduced.
$R\text{-}S\text{-}S\text{-}R + 2H^{+} + 2e^{-} \rightarrow RSH + RSH$

1.5.2 The thioredoxin and glutaredoxin systems

As disulfide bonds between proteins tend to increase during oxidative stress the thioredoxin and glutaredoxin systems function as electron donors in order to reduce these bonds.
Both systems belong to the enzymatic defense against oxidative stress and are well studied and characterized in biology and their activity and expression are proven to be induced during oxidative stress [100]. They are ubiquitously expressed proteins belonging to the thioredoxin superfamily of proteins. One of the features this family shares is the three dimensional fold; the thioredoxin fold with a structural conserved active site motif containing Cys-X-X-Cys [101-103].

1.5.2.1 Thioredoxin

Thioredoxin is a fairly small redox protein and forms together with NADPH and thioredoxin reductase the thioredoxin system which is a fast thiol reductant and is expressed in all living organisms. It´s efficacy is built on the fast reaction between reduced thioredoxin and disulfide bridges in substrates. This reaction gives oxidized thioredoxin which in turn is reduced by NADPH and thioredoxin reductase (TrxR).

$$\text{Trx-(SH)}_2 + \text{protein-S}_2 \longrightarrow \text{Trx-S}_2 + \text{protein-(SH)}_2$$
$$\text{NADPH} + \text{H}^+ + \text{Trx-S}_2 \xrightarrow{\text{TrxR}} \text{NADP}^+ + \text{Trx-(SH)}_2$$

In addition to the reducing feature of the thioredoxin system, it is also involved in a variety of biological functions like cell proliferation, DNA-synthesis, cell signaling and apoptosis [104-106].
In humans two isoforms of Trxs have been found, the cytosolic form Trx1 that can translocate to the nucleus and the mitochondrial form Trx2 (18kDa) and they are both essential proteins. Knock-out experiments in mice led to embryonic lethality [107, 108]. The two forms are encoded by different genes. Thioredoxin 1 is compact and globular shaped with a mass of 12 kDa, and was isolated from Escherichia coli in 1964 [105]. It is composed of a twisted β-sheet with five strands and has on the surface four α helices and the three dimensional shape have been shown with both nuclear magnetic resonance and x-ray crystallography [109].
Thioredoxin reductases are homo-dimeric selenoproteins and belong to the pyridine nucleotide-disulfide oxidoreductase family. The size of the dimer is 112 kDa and they are the only enzymes capable of reducing the active sites of Trxs. Their N-terminal active site is a conserved sequence: Cys-Val-Asn-Val-Gly-Cys and in addition they also have a C-terminal selenium containing active site where thioredoxin as well as other substrates are reduced.

1.5.2.2 Glutaredoxin

The glutaredoxin system consists of glutathione (GSH), glutathione reductase (GR), glutaredoxin (Grx) and NADPH. Four different glutaredoxins have been found in humans so far, they are small enzymes with oxidoreductase activity and possess several important biological functions in the redox cell signaling. It catalyzes the reduction of disulfide bonds in proteins and mixed disulfide bonds with GSH by glutathionylation and de- glutathionylation. They are oxidized by different substrates and reduced via the oxidation of glutathione which is regenerated by glutaredoxin reductase.
Depending on how their motives are constructed, Grxs can be divided to the dithiol (Cys-XX-Cys) and the monothiol (Cys-XX-Ser) Grxs. The dithiol functions by using both the cysteines in the active site in order to reduce disulfides whilst the monothiol variant uses the cysteine in the N-terminal active site for the reduction of GSH mixed disulfides [110].

The first discovered was Grx1 which is localized to the cytoplasm and is involved in DNA synthesis by its reduction of ribonucleotide reductase (RNR). Its mass is 12 kDa and the conserved N-terminal active site consists of two redox active cysteines (Cys-Pro-Tyr-Cys). As Trx1, Grx1 has been shown to be secreted and is suggested to possess extracellular functions [111].

Grx2 was the first Grx identified as an iron-sulphur containing member of the Trx-fold super family. The role of the iron-sulphur cluster is to coordinate the regulation of the activity of Grx2 in complex with glutathione. As a homodimer Grx2 is inactive, and in presence of one-electron oxidants or reductants the 2Fe-2S cluster will be disrupted leading to monomerization and activation of the enzyme [112, 113]. Unlike the other Grxs, human Grx2 is a substrate for TrxR and NADPH and can therefore use electrons provided by both systems [114].
Three splice variants of Grx2 have been discovered so far: Grx2a residing in the mitochondria, Grx2b was localized to the nucleus and the third form Grx2c has been found in several cell lines and in testis. [115, 116]. It is not clear whether or not the splice variants are present in human livers. The antibodies used for IHC in this thesis do not distinguish between the three splice variants.
Grx3 PICOT is a monothiol iron-sulphur-binding protein localized just beneath the plasma membrane and has a role in the regulation of signal transduction in the activation of immune cells and may also exhibit a response to ROS and RNS [117]. This redox protein was shown to be induced during oxidative stress in mice and a deletion resulted in early embryonic death suggesting a defective cell cycle progression during late mitosis [118].
The last Grx found is Grx5 which is located in the mitochondria and is a member of the Fe/S cluster assembly machinery. Simplified this machinery is a step wise process that starts with the formation of a 2Fe-2S cluster on a scaffold protein via an electron transfer chain. The cluster is released from this scaffold protein and is then transferred to apo-proteins with the help of chaperones and the Grx5. Subsequently a 4Fe-4S cluster is generated and inserted in target apo-proteins. Defects in this assembly system lead to increased iron levels and mitochondrial iron accumulation [119].

1.5.2.3 The role of thioredoxin and glutaredoxin systems in neoplasia and cancer

The thioredoxin system is involved in control and regulation of activity of several transcription factors, and has been proposed to exhibit a role in cell growth and overexpression of Trx has been observed in a variety of cancers, including lung cancer, pancreatic cancer, stomach cancer, gallbladder cancer colorectal cancer and squamous cell carcinoma of the tongue [120-126].
Moreover, in cell culture, increased expression of Trx1 has been shown to be associated with increased VEGF expression and enhanced tumor angiogenesis [127].
Studies of the role of glutaredoxins in cancer have revealed an association with Grx1 and pancreatic cancer and basal cell carcinoma [122, 128]. In lung and colon-cancer over-expression of Grx3 have been seen [129]. The enzyme TrxR1 was overexpressed in a rat study of neoplastic lesions [130].

1.6 SELENIUM

Selenium is an essential trace mineral incorporated into several proteins of which biological features is important for several physiological functions. In humans, 25 selenoproteins have been identified so far being involved in cell growth, differentiation and cell death [131, 132]. They also possess antioxidative properties preventing the cells from oxidative damage of free radicals. Moreover, selenoproteins are important in the immune system and assist in the regulation of thyroid function [133, 134].
Examples of selenoproteins are glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases.
Selenium was discovered by the Swedish chemist Jöns Jacob Berzelius in 1818 [135]. It was found to have similar properties with sulfur, and is placed at position 34 in the periodic system and can exist in five different Valente states: -2, 0, +2, +4 and +6. The inorganic forms of selenium includes selenite (SeO_3^{2-}), selenate (SeO_4^{2-}) and selenide (Se^{2-}) which differs in the oxidation state. Inorganic selenium is found in the soil and is taken up via plants which incorporate selenium into organic forms like selenomethionine (SeMet), selenocystein (SeCys) and selenomethyselenocystein (SeMSC) which are subsequently transferred to grazing animals [136]. Because of geographical variations of selenium content in the soil over the globe there are big variations of the levels of bioavailable selenium in food. Even though selenium is essential for living organisms and deficiency will lead to pathological conditions it is also highly toxic in high levels.
Dietary main sources of selenium are: nuts, cereals, meat, kidney, mushrooms, fish (tuna), eggs and seafood (crabs and lobster) [137].

1.6.1 Selenium metabolism

The metabolism of selenium compounds and formation of selenoproteins has been thoroughly investigated. The uptake of organic Se is preferentially in the SeMet form which is converted into SeCys within the body and used for the synthesis of selenoproteins after formation of a Secis-element [138, 139]. Selenocystein is the main form of organic Se in our bodies. The inorganic and the organic Se are metabolized via two different pathways with hydrogen selenide (Hse-) as the common key metabolite used for selenoprotein synthesis or excretion [140] *(Fig. 10).*
Elimination of selenium at normal selenium levels is usually via feces or urine after methylation, but volatile dimethylselenide is exhaled via the lungs. The exhalation route of eliminating Se-levels is considered to be the major pathway when Se is ingested in toxic doses. Urine excretion is usually as selenosugar that is synthesized directly from selenide or in case of high selenium levels, from trimethylselenonium ion [140].

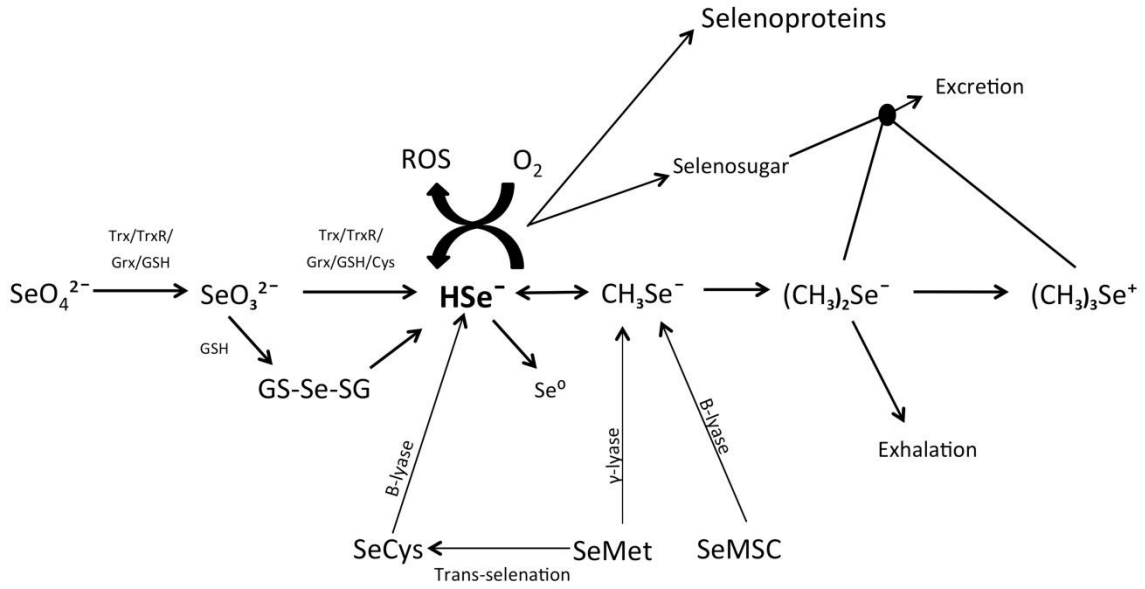


Figure 10. A schematic presentation of the selenium metabolism. Hydrogen selenide, the provider of Se to all selenoproteins, will together with ATP form monoselenophosphate via the activation of selenophosphate synthetase. The metabolism of inorganic selenium starts by reduction of selenate to selenite which is performed by GSH mediated by the Trx or Grx systems. Selenite is then further reduced to selenide of these systems and of thiols [141]. Selenite can also be reduced by a non-enzymatic mechanism affected by free cysteines [142]. In addition, selenite can be reduced to HSe- via the intermediate glutathione conjugate GSSeSG also in the presence of GSH [143]. The both organic compounds SeCys and SeMSC will be cleaved by the enzyme β-lyase to selenide and methylselenol. Methylselenol is converted to selenide via the action of demethylation and note that selenide can be methylated back to methylselenol in order to release selenium from the organism either through exhalation or via excretion in urine or feces to protect the organism from surplus of selenium [144, 145].

The recommended daily intake of selenium is based on the selenium concentration sufficient to saturate the selenoprotein glutathione peroxidase (GPx) and is set to 55 µg for healthy adults [146]. However, during pregnancy and breastfeeding the daily requirement rises to 65-75 µg. In Sweden the selenium content in the soil is low so here the daily dose recommended is suggested to 100-200 µg. The distribution of selenium between the tissues after blood transportation occurs in a hierarchical manner, the brain tissue being the organ with the highest demands followed by skeleton muscle and thirdly liver tissue. As a consequence of this

hierarchy, the brain will conserve selenium most efficiently in situations when the selenium levels are insufficient [147, 148].
Intoxication of selenium involves hair and nail loss, garlic smelling breath, tachycardia and respiratory disorders because of pulmonary edema and death [149].
Symptoms of deficiency involve severe cardiomyopathy seen in children and young women with Keshan disease in certain areas in China where the selenium content in soil is extremely low. Another disorder is the Kaschin-Beck disease which is a degenerative joint-disease. In recent years a whole spectrum of disorders related to inherited disturbances in the selenium utilization, transport, and metabolism have been done and this field is expanding [150].
Deficiency of Se, may also lead to serious disturbances in human health and more than 40 Se-deficiency related conditions have been reported so far, wherein cancer is one of them. Studies have shown an association with lower serum contents in cancer patients compared to healthy controls [151, 152].

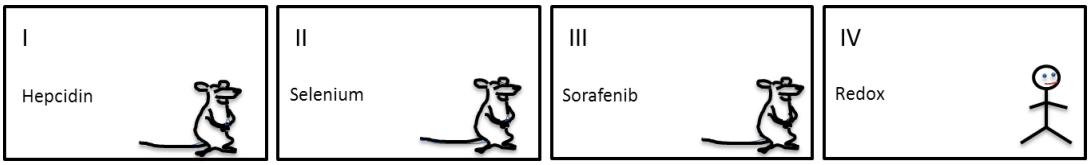
1.6.2 Selenium and prevention of cancer

Multiple epidemiological human and prospective animal studies have investigated the relationship between selenium and cancer. Associations between low serum contents in cancer patients have been noted compared to healthy controls and a relationship between low levels of Se content in food and risk for cancer and related mortality rate have been observed [151, 152]. There are more than 100 animal studies in which a preventive effect of selenium on different cancer types has been observed and moreover, there are several human studies that support these observations.

Selenium in the prevention of human cancers is discussed in a review by Fernandes and Björnstedt from 2010 [153]. The underlying molecular mechanisms of the tumor preventive effects of selenium are not fully understood but several mechanisms have been proposed so far. These include anti-oxidative effects mediated by selenoproteins, stimulation of DNA-repair and induction of apoptosis in preneoplastic cells. The role of selenoproteins in tumour prevention is not known but some redox active selenoenzymes such as thioredoxin peroxidase and glutathione peroxidase have been extensively studied [132]. The antioxdative effect is proposed to be mainly preventive, thereby affecting the very early stages of carcinogenesis [154]. Selenoproteins play a central role in maintaining the redox homeostasis of the cell and are involved in all three levels of antioxidant protection against both ROS and RNS. It has been found that selenoproteins enhance DNA repair and cell integrity via protection against ROS, and that SeMet can induce DNA repair in human fibroblasts *in vitro* and also regulate DNA chain break control, and reduce aberrant crypt foci in colorectal tissue in rats.

2 PRESENT INVESTIGATION

2.1 AIM OF THE STUDY



This thesis was based on four separate projects. Three projects involve rat liver studies on various aspects of regenerative and neoplastic cell proliferation and the last study was performed on human liver from patients with hepatocellular carcinoma or liver metastases from colorectal cancer where the expression of redox proteins in tumour and surrounding liver tissue was studied.

The overall aim of the thesis was to characterize the role of iron during liver regeneration, to study the effects of two anti-cancer compounds (sodium selenite and sorafenib) on regeneration following liver resection in rats, and to evaluate redox enzyme expression in regenerative and neoplastic growth, as well as their expressions in human liver cancer.

The specific aims of the study were:

- To characterize gene expressions of the different pathways involved in **hepcidin** regulation after partial hepatectomy, until liver regeneration is complete (paper I)

- To study the effects of **sodium selenite** on regenerative vs. neoplastic liver cell proliferation in rat (paper II)

- To investigate if **TrxR1** is a constitutive tumour marker or an unspecific marker for cell growth in rat liver (paper II)

- To study the effects of the anticancer agent **sorafenib**, a multikinase inhibitor, on normal liver regeneration after partial hepatectomy in rat (paper III)

- To evaluate if redox protein (**thioredoxins and glutaredoxins**) expressions correlate to clinical features in human HCC, and if they can be used as prognostic markers after liver surgery for HCC (paper IV)

2.2 REMARKS OF THE METHODOLOGY

2.2.1 Animal models

2.2.1.1 Partial hepatectomy (used in paper I, II and III).

In the first three papers in this thesis, we have used a rat model for 2/3 partial hepatectomy. This is a reliable and standardized procedure as far as timing and amount of the liver removed is concerned, and the animals recovered quickly after the procedure.
Male Fischer-344 rats, purchased from Charles River, Germany, were first allowed to acclimatize to their new environment one week prior to the experiments. They were fed a standard chow diet and housed in the animal facility in a 12 hour light-dark cycle where temperature, humidity and ventilation were controlled according to international standards. Partial hepatectomy was performed under general anesthesia with isoflurane. An incision was made in the abdominal wall and the two main liver lobes were made to pop out by a slight pressure on either side of the abdomen. After placing a ligature around the blood vessels, the two lobes were removed. The peritoneum and abdominal wall were sutured, and Marcain was administered as a pain killer before the skin was closed using clamps. In the sham-operated animals the abdomen was opened, and was gently pressed without any removal of the liver lobes.
In project I the PH model was used without any other treatment and three animals was sacrificed from the PH group and the sham operated control group at the following time points: 0 h, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, 72 h, and 1 w, 2 w and finally 3 weeks after surgery.

2.2.1.2 The resistant hepatocyte model for hepatocarcinogenesis

This model called the “Resistant Hepatocyte model” (RH-model), was originally described by Solt and Farber was used in paper II, with slight modifications [17, 155]. In short: The RH-model is a synchronized model which makes it possible to study the three consecutive processes in the liver tissue during carcinogenesis, initiation, promotion and progression. The initiation and promotion steps consist of administration of a necrogenic dose (200 µg) of diethyl nitrosamine (DEN) followed after two weeks by administration of 2-acetylaminofluorene (2-AAF) and PH. 2-AAF is a strong mitoinhibitor but initiated cells have acquired resistance to its mitoinhibitory effects. During promotion only the hepatocytes resistant to the mitoinhibitory effect of 2-AAF will be able to respond to the mitogenic signals induced by the hepatectomy in the presence of the promoter. Through this procedure, the resistant cells have a growth advantage over the non-resistant cells and grow in a clonal way to form preneoplastic foci and nodules. During progression when administration of the promoter is stopped, most lesions re-model to normal liver phenotype over time. In a small subfraction of the nodules (less than 1%), clones of cells will appear that are able to grow in the absence of the promoter. In these persistent lesions changes will occur that during the progression phase, giving rise to malignant tumors.

2.2.1.3 Treatment of the animals

Selenium and PH:

In paper II two different experimental rat liver models were used, the resistant hepatocyte model for liver cancer development, and the model for rat liver regeneration after partial hepatectomy. In each experiment one group of animals were treated with sodium selenite in the drinking water and compared to one group drinking tap water. The selenium treated groups were administered 5 µL/mL sodium selenite in the drinking water as illustrated in Fig. 11. At time points indicated in the figure three animals per group were sacrificed and livers and blood collected. The dose of sodium selenite was chosen according to earlier studies where a tumour preventive dose of sodium selenite was titrated [156].

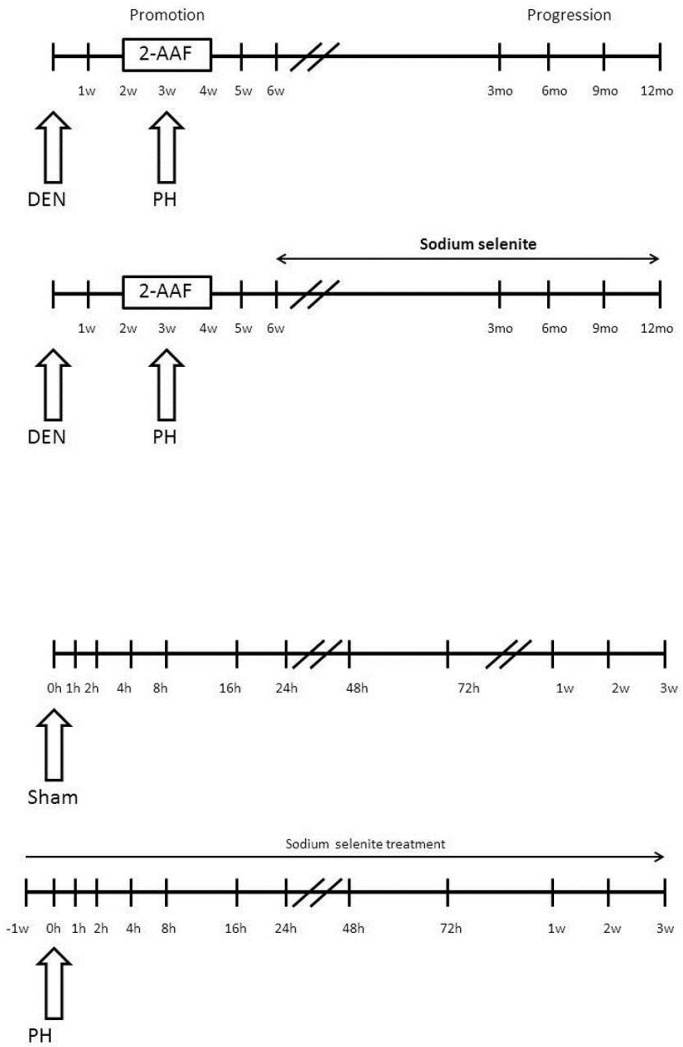


Figure 11. This protocol shows how the treatments in the studies were performed.
A) In the cancer model animals were treated with sodium selenite during the progression phase and animals were sacrificed at 3, 6, 9 and 12 months.

B) In the PH model without any neoplastic nodules animals were given sodium selenite one week prior to PH and until the liver regeneration was completed. Animals were sacrificed at indicated time points.

Sorafenib and PH
For assessment of toxicity, the animals were subjected to pH and were divided into three dose groups (5, 10 and 15 mg/kg body weight) and were then treated daily with sorafenib for one week before surgery. Four animals per group were sacrificed at 16, 20 and 24 hours after surgery. Four animals per group were sacrificed after one week of treatment and without PH. The dose 5 mg/kg body weight was chosen for the second part of the sorafenib study where the animals were treated daily one week before PH and were then divided in three groups. The first group received the vehicle Cremophor-ethanol solution after PH the second received sorafenib in Cremophor ethanol until 3 days post-surgery and the last group received sorafenib in Cremophor ethanol continuously until 14 days after surgery. Four animals from each group were sacrificed at the time for PH and then at the following time points: 16, 20, 24, 48 and 72 hours and at 5, 7, 10 and 14 days post PH. The time-points were chosen to capture the peak of proliferation that occurs at 18-72 hours and also to investigate the effects of sorafenib in the later stages of liver regeneration and until it is completed at 14 days. Sorafenib tosylate (BAY 54-9085, Bayer Healthcare Pharmaceuticals, Montville, NJ) was dissolved in a 50% Cremophor EL (Sigma Cat. No 5135) /50% ethanol mixture diluted with water in the distribution 12,5% Cremophor EL/12,5% ethanol/75% water. The mixture was administered daily in a volume of 250 µL/100 g body weight by oral gavage.

Administration of BrdU
The administration of the synthetic nucleoside BrdU was done in two ways, in project II the animals got an osmotic mini-pump inserted right under the skin three days before sacrifice and in paper III BrdU was injected two hours before sacrifice. The shorter time between administration and sacrifice in the latter case was because we wanted to capture a small window for the proliferation analysis.
BrdU was incorporated to DNA in replicating cells replacing thymidine. By using specific antibodies against BrdU quantification of the number of proliferative cells in liver tissue sections could be done with immunohistochemistry.

2.2.2 Tissue sampling

The rat livers were sliced immediately after sacrifice and either snap-frozen in liquid nitrogen for gene and protein analysis, or put in 4% formaldehyde, routinely processed and embedded in paraffin for immunohistochemical stainings. The sampling of the human liver was done in the same way except from a little longer processing time.

2.2.3 Quantitative Polymerase Chain Reaction

For analysis of gene expression real time-QPCR was used in paper I, III and IV. Gene-specific primers were designed and where possible these were exon-spanning for high specificity. For detailed information on primer sequences and individual steps during the amplification process, please see the methods section in each project.
In paper I we used a system that is built on gene specific primers and a TaqMan probe and in paper III and IV we used the system based on gene-specific primers and SYBR-green.
The TaqMan system is based on a fluorogenic sequence-specific probe that will detect a specific PCR-product as it accumulates during the cycles while the SYBR green system involves

a dye that intercalates with any double stranded DNA. Post PCR steps are required when the SYBR green method is used involving sequencing of the PCR product for detection of false positives, a step that is unnecessary using the TaqMan method. However, the SYBR- green method involves higher sensitivity and is also a cheaper method to set up the lab in comparison to the TaqMan-method.

2.2.4 Immunohistochemistry

For detailed information on the different protocols used, see each individual project in this thesis. For a brief summary may be mentioned that in the three rat projects we stained for MIB 5 (Ki67) and BrdU both of which are nucleus stainings for cell proliferation analysis. The human counterpart to MIB5, MIB1 was stained for in the fourth project. In paper II stainings for GSTπ and TrxR1 was performed and in the fourth paper stainings for Trx1, Trx2, Grx1, Grx2, Grx3 and Grx5 was done.
BrdU and MIB5 indices were calculated in a light microscope (Microscope Nikon Eclipse E1000M) as the ratio of the total number of nuclei seen in one field to the number of positive stained nuclei. For the mitotic index the total number of nuclei was divided with the number of mitotic figures seen. The method was modified slightly between the different projects; in paper I and II, tissue sections were evaluated in 200X magnification and at least 1000 cells were counted until a cumulative mean value was reached.
In paper III the cumulative mean from 15 randomly counted fields of the tissue slides was calculated in a magnification of 400X, and in the fourth paper the labeling index of MIB1 was counted in randomly fields in a magnification of 200X until a cumulative mean value was reached.

2.2.5 Imaging analysis of IHC

For quantification analysis of the staining level of thioredoxin and glutaredoxins an imaging analysis was set up using the software photoshop and NIS-Elements. The pictures of the slides were photographed under the light microscopy with standardized settings, blue color elimination was done in photoshop and analysis of the saturation was done using NIS-Elements Br. For detailed information see project IV.

2.2.6 Nodule and tumour density

The number as well as the volume fraction of liver tumours in the rat cancer model was quantified using morphometric densitometry on tissue slides stained with Gst Yp. The number of focus profiles in the slice was divided by the area. The nodular volume density was expressed as % of the cellular volume. The number of foci/mm^2 and the volume density of nodules were calculated in a computerized morphometric analyzer (Minimop, Kontron, Munich, Germany).

2.2.7 Preparation of liver tissue homogenate

In order to make a 20% homogenate of rat liver tissue, frozen samples was transferred to pre-weighed tubes containing 1 mM EDTA (pH 7,5) and 50 mM tris-HCl in a total volume of 3 mL and weighed. The samples were minced with a pair of scissors and homogenized in a Potter-

Elvehjelm glass-teflon homogenizer connected to a pillar drill. With four gentle up and down strokes at a speed of 440 rev/min the liver went completely homogenized, the homogenate was transferred to centrifuge tubes and were subsequently spun in an ultra-centrifuge at 105,000 x gAV for 90 minutes at 4°C. The samples were kept cold through the whole process. Protein determination on the supernatant was made according to Lowry et al [157].

2.2.8 Specific activity of Trx1

The specific activity of TrxR1 was measured in rat liver homogenate by an enzyme assay according to A. Holmgren and M. Björnstedt [158]. Briefly, A sample containing 100 µg of protein was incubated with 80 mM Hepes, (pH 7,5), 0,9 mg/mL NADPH, 6 mM EDTA, 2 mg/mL insulin and 10 µM *Escherichia coli* thioredoxin at 37°C for 20 minutes in a final volume of 120 µL. For termination of the enzymatic reaction 0,4 mg/mL dithiobis 2-nitrobenzoic acid (DNTB) and 6M guanidine hydrochloride in 0,2 M Tris-HCl (pH 8,0) was added to the solution. Within 20 minutes the absorbance was measured at 412 nm and the value of a corresponding blank sample without added Trx was subtracted. A standard curve was prepared using purified thioredoxin reductase from rat liver (Sigma).

2.2.9 Protein detection

For quantification of IL6 in rat serum the ELISA kit "Quantine Rat IL-6 Immunoassay" (R&D systems, Minneapolis, USA) was used.
Western blot technique was used to quantify the protein expression of STAT3, Smad4, ppERK, pJNK and pp38 in rat liver tissue. For details please see the respective method section in study I and III.

2.2.10 Statistical analysis

Statistica software 9.0 and 10.0 has been used for statistical analysis. For analysis of normal distribution the Shapiro-Wilks test was used. One way analysis of variance (ANOVA), student's t-test or Mann-Whitney U test was used for comparison of mRNA expression, enzyme activity, body weights, relative liver weights, labeling indices of BrdU and MIB5 and mitotic indices between animal groups and for comparison of immunohistochemical saturation in HCC versus CRC. For anthropometric and laboratory data, t-test was used for continuous data and Chi2 test or Fischer's exact test when appropriate, for categorical data. The statistical significance of the values of saturation in tumors and its corresponding surrounding non-tumorous tissue was determined with paired t-test and Wilcoxon matched t-test. For correlation tests linear regression was used. P value <0.5 were considered statistically significant. Error bars represent ±SD. Figures were created using GraphPadPrism5 (GraphPad Software, Inc., La Jolla, CA, USA).

2.2.11 Ethical approval

The studies in this thesis were approved by the Stockholm South local Committee for Ethical Review of Animal Experiments and by Stockholm Regional Ethics Committee.

2.3 RESULTS

2.3.1 Paper I

Iron-regulatory genes during liver regeneration.

Background
Liver regeneration is characterized by an acute-phase reaction during the first 18-24 h after a partial hepatectomy. Others have shown that during this phase IL6 is released which in turn stimulates hepcidin gene expression. Hepcidin however, decreases iron availability since it decreases iron uptake from the gut and iron release from macrophages, and therefore hepcidin-induced iron deficiency may impair liver regeneration.

Aim
We asked if other hepcidin-regulatory pathways would down-regulate hepcidin expression during the later phases of liver regeneration after surgery

Methods
We used a rat model comparing partial hepatectomy with sham operation. Liver tissue was sampled at different time points after surgery. Gene expression of hepcidin-regulatory molecules were quantified with real-time PCR and correlated to proliferation index, IL6 levels in plasma and STAT3 protein levels in liver tissue.

Results
During the acute-phase response after PH high serum levels of IL6 was seen inducing STAT3 protein expression and mRNA of hepcidin. The gene expressions of proteins involved in the iron-sensing pathway (HFE, HJV and TfR2) were instead decreased while the mRNA levels of proteins involved in iron uptake (TfR1 and DMT1) were increased and transcripts of proteins involved in cellular iron export were unchanged (FPN and ceruloplasmin). In the regenerative phase after PH namely >24h post PH, gene expression of HFE and HJV belonging to the iron-sensing pathway were continuously suppressed and mRNA levels of hepcidin declined.

Conclusion
Hepcidin gene expression peaks during the acute-phase response, but a sustained down-regulation of the iron-sensing pathway of hepcidin regulation gradually reduces hepcidin gene expression until regeneration is complete, thereby promoting iron mobilization to the regenerating liver.

2.3.2 Paper II

The effect of sodium selenite on liver growth and thioredoxin reductase expression in regenerative and neoplastic liver cell proliferation.

Background
A previous study performed in our lab showed that administration of sodium selenite reduced the volume fraction of preneoplastic nodules during the promotion as well as the volume fraction of neoplastic nodules during the progression 12 months after initiation. Selenium did not inhibit initiation in the DEN/2AAF + PH rat model [159].

Aim
The aim of this project was to investigate the effect of administration of sodium selenite on the neoplastic growth during progression in tumour development and compare that with the effect of selenite on the regenerative cell proliferation after PH. In addition we wanted to compare the expression of TrxR1 in regenerative liver to that in liver neoplasia to evaluate if TrxR1 could be considered as a constitutive tumour marker or only an unspecific marker for cell growth.

Methods
We performed two studies on rats, the first of which included chemical induction of cancer and treatment with sodium selenite, and the second included treatment with sodium selenite after PH.

Results
No effect on body weight or gain of liver mass could be seen after administration of sodium selenite followed by PH, even though a slight delay in the S-phase peak and mitosis could be seen. However, the tumour volume in the DEN/2AAF/PH model was significantly decreased in animals treated with sodium selenite during progression compared to animals not treated with selenium during progression. The expression of TrxR1 mRNA was not affected by administration of sodium selenite in the non-neoplastic regenerating liver cells and the activity of the enzyme was only transiently increased during the peak of cell proliferation. In the neoplastic liver lesions TrxR1 was constantly overexpressed in a way that did not correlate to the growth rate of the individual lesions or within the individual lesions.

Conclusion
Administration of 5 μg/μL sodium selenite did not compromise liver cell proliferation after PH in an extent that affected liver regeneration and gain of liver mass. In contrast to this effect the neoplastic liver nodules grew significantly slower in the selenite treated rats in comparison to the lesions in rats drinking tap water. The consistent over expression of TrxR1 in liver neoplasia contrasted to the transient increase in regenerating hepatocytes that was not even clearly visible by IHC. This made us suggest that the neoplastic overexpression of TrxR1 seen could only partly be explained by the tumour cell growth rate.

2.3.3 Paper III

Sorafenib prolongs liver regeneration after hepatic resection in rats.

Background
Sorafenib is a multi-kinase inhibitor that targets the tyrosine kinase activity of growth factor receptors such as EGFR, PDGFR and VEGFR and also the threonine kinase activity of Raf in the Ras/Mek/ERK pathway. By binding to the ATP-binding pocket on these kinases sorafenib inhibits angiogenesis and cell proliferation. Sorafenib has been suggested as an adjuvant treatment in conjunction with hepatic resection for HCC, and therefore the effect of sorafenib on liver regeneration is of interest.

Aim
To investigate the effects of sorafenib treatment on liver enzymes, cell proliferation and liver weight gain during the entire length of liver regeneration after partial hepatectomy in rat.

Methods
Rats were administered sorafenib once daily for one week, and then underwent a 2/3 partial hepatectomy (PH). In the first study, sorafenib was given in three different doses (5, 10 and 15 mg/kg/day) and animals sacrificed 0-24 hours after PH. In the second study, sorafenib 5 mg/kg/day was given 0, 3 or 14 days after PH. The number of S-phase nuclei was calculated using immunohistochemistry, and pERK was analyzed with Western blot.

Results
Treatment with sorafenib increased the levels of serum ALT and serum AST in a dose-dependent manner at baseline. The number of S-phase positive nuclei was increased at baseline in animals treated with the highest dose (15 mg/kg). Liver weights and levels of pERK were decreased at 24 hours post PH.

At 48 hours after PH, the number of S-phase nuclei and mitotic indices were decreased in sorafenib-treated animals. At seven days after PH the number of S-phase positive nuclei was instead *increased* in animals treated with sorafenib 3 or 14 days after PH, as compared with controls. The relative liver weights were restored at day five in control rats, day seven in animals receiving sorafenib before surgery, day ten in rats where sorafenib treatment was ceased three days after surgery and at day 14 in rats were on continuous treatment with sorafenib.

Conclusion
In our rat model, sorafenib given in conjunction with a 2/3 PH decreased cell proliferation and prolonged the liver mass restoration in proportion to the length of sorafenib treatment. One week after PH, we found an adaptation to the inhibitory effects of sorafenib with increased cell proliferation in sorafenib-treated animals compared to that of the controls.

2.3.4 Paper IV

Characterization of redox proteins in human hepatocellular carcinoma.

Background
Redox proteins like thioredoxins and glutaredoxins are ubiquitously expressed proteins involved in several biological processes. The expression of Trx and Grx is induced in many neoplasms and was found to correlate to the prognosis in colorectal and gallbladder carcinoma

Aim
The aim was to characterize the expression of Trx1 and 2 and Grx1, 2, 3 and 5 in human HCCs and investigate their possible association to clinical parameters and their value as prognostic markers for recurrence after resection for HCC.

Methods
Immunohistochemical stainings with antibodies specific for the six different redox proteins were made on liver sections from paraffin embedded resected liver tumours. The stainings were analyzed with a computer-based imaging method described in detailed in project IV. As internal control surrounding non-tumorous tissue was used and the ratio of saturation in tumour to the saturation in surrounding non-tumorous tissue was calculated. Gene expression was analyzed from five of the patients from fresh frozen liver tissue using quantitative real-time PCR. Correlations of the IHC protein expression and clinical parameters were performed. As controls resected colorectal cancer (CRC) liver metastases were used.

Results
An up-regulation of Trx1, Trx2 and Grx5 was seen in HCCs compared to its respective surrounding non-tumorous tissue. The same was also observed in CRC metastases where also the saturation of Grx1 and Grx3 was significantly higher. In HCC, Trx1 correlated significantly to cell proliferation but not to tumour differentiation, micro-vascular invasion or tumour recurrence. A down regulation of Trx1 in tumours relative to the surrounding was seen in males compared to females and in smokers and in patients with high alcohol consumption.

Conclusion
Redox proteins investigated in the present study are more up-regulated in the *surrounding* livers of patients with HCC as compared with CRC liver metastases except for Grx2. The expressions in the tumours are also weaker in HCC compared to CRC. Our results suggest that the expression of Trx and Grx proteins reflects the grade of oxidative stress in chronic liver disease and mirrors the environmental state in the liver tissue that surrounds HCC. Our data indicates that redox proteins are ubiquitously expressed in livers exposed to oxidative stress and various malignancies and can therefore not be used as diagnostic markers for HCC. Even though a correlation between cell proliferation and the expression of Trx1 in tumours was found, patients with or without tumour recurrence were not significantly different in redox protein expressions, indicating that redox proteins lacks a prognostic significance in HCC.

3 DISCUSSION

The underlying mechanisms of liver regeneration have been thoroughly studied the last decades and a complex and fine-tuned orchestration have emerged involving several signaling pathways. The significance of understanding these features is evident since a majority of liver cancers are induced in a highly proliferative environment. With a deeper insight to the exact mechanisms of tumour development, better tools for early diagnosis, prognosis, prediction and development of new treatments could be accomplished.
The most studied phase of liver regeneration so far is the initiation, but new findings including the termination are arising.
Given that the same signaling machinery lies behind normal regeneration and the regeneration that occurs during chronic liver repair eventually leading to malignancies, the 2/3 PH rat-model permits the study of these pathways and may shed light on future targets for treatment of cancer or prevention of cancer development.
This thesis comprises of four papers where the three first is conducted on the 2/3 partial hepatectomy rat model. We studied the expression of iron regulatory genes, the effect on regeneration after administration of sodium selenite and how the multi kinase inhibitor sorafenib influences the liver regeneration. In the second paper we also investigated the usefulness of TrxR1 as a neoplastic marker and selenium as a preventative agent in carcinogenesis. In the fourth paper we examined the role of redox proteins in human hepatocellular carcinoma and investigated the use of redox proteins as diagnostic or prognostic markers.
In the study of the expression of iron-regulatory genes we used the 2/3 PH-model and sacrificed animals at different time points during the whole phase of liver regeneration. The aim was to elucidate the regulation not only during the acute-phase but also during the regenerative phase to see if the IL6 -induced acute-phase induction of hepcidin expression would persist or decline after the ending of the acute-phase reaction to facilitate iron mobilization to the growing cells. In earlier studies the expression of acute-phase proteins declined 18 hours after PH [160] but no studies have been performed to investigate the expression of the major key for iron homeostasis, hepcidin, during the later phases of liver regeneration.
Our results showed a significant elevation of hepcidin expression starting at 2 hours post PH and peaked at 8 hours and declined after 16 hours. The control animals also had a peak at 8 hours after the sham operation – although not as pronounced- and which is most likely an acute reaction to the opening of the abdomen. Elevated serum levels of IL6 however, could only be detected in the PH animals. The role of IL6 during the acute phase of liver regeneration is a well-established feature. After partial hepatectomy or other liver injuries this cytokine is released by Kupffer cells, and by binding to the hepatocytes starts the positive signal cascade JAK/STAT pathway that induce gene activation to promote proliferation. We found increased levels of STAT3 in the PH animals but not in the SO ones. Our findings confirm previous work demonstrating an IL6 induced hepcidin gene expression during the acute-phase response after liver surgery [161]. Following the completion of the acute-phase response, which is 2-14 days after the hepatectomy, we saw a significant reduction of hepcidin mRNA levels as compared with levels found after sham operation.

At 48 hours post PH a reduction of the expression of hepcidin was seen compared to sham operated animals and at 72h and 1 w, this decrease was significant in the PH rats. At two and three weeks after the surgery the levels were the same in PH and SO animals. As in other studies, the expression of HJV was decreased during the acute phase. Expression of the three hepcidin-regulatory molecules TfR2, HFE and HJV was down-regulated during the whole process of regeneration suppressing hepcidin gene expression and thereby facilitating iron uptake, even if the transient induction of IL6 overrules this decrease in the acute phase.
The expression of Transferrin receptor 1 was simultaneously over-expressed in the same pattern as hepcidin during the acute phase, indicating an increased iron uptake, although TfR1 is also post-translationally stabilized by IRP which activity is activated during oxidative stress. Also the expression of the metallotransporter DMT1-IRE was over-expressed in comparison to the SO-animals.
In a recent study conducted on mice, two important growth factors for regeneration, HGF and EGF, decreased the expression of hepcidin via PI3 kinase MEK/ERK pathway. This finding suggests a modulation of the BMP transcriptional regulators Smad1/5/8 and others and can serve as an explanation for high iron load in patients with chronic liver inflammation caused by hepatitis C virus or alcohol hepatitis [162].
In the first paper we thus concluded that a down-regulation of the iron-sensing pathway during the later phases of liver regeneration, when the effects of the acute-phase reaction had disappeared, leads to reduced hepcidin expression and enhanced iron mobilization to the growing liver.
In the second paper we turned our interest to the effects of another trace element, namely selenium, on liver regeneration and neoplastic cell proliferation. We focused on the selenium-containing enzyme thioredoxin-reductase1 (TrxR1) as a marker for neoplasia and/or proliferation, as well as the putative anti-tumoral effects of sodium selenite.
We could see that the TrxR1 was overexpressed in the initiated cells and through all the steps of the carcinogenesis until liver cancers were formed. This expression persisted during the progression phase only in tumors and not in the remodeling nodules which remained in the progression phase. TrxR1 was thus over-expressed only in tumors with a neoplastic growth advantage and the remodeling liver nodules positive for the classical liver nodule marker GST-π but negative for the S-phase marker BrdU, were also negative for TrxR1 during the late progression. The following observations from our immunohistochemical studies made us draw the conclusion that TrxR1 indeed is a constituent of the neoplastic phenotype and not only a proliferative marker: (1) the TrxR1 immunohistochemical signal in the nodules was strong and homogenous within the lesions, and (2) the intensity of the signal was not correlated to the rate of cell proliferation measured as BrdU-index, or to the distribution of growing cells in the nodules.
The increase in enzymatic activity of TrxR1 seen during the regenerative growth could not be detected by IHC, with one exception at 24 hours post PH where a weak signal in the periportal zone 1 could be detected in two rats of four in the group that was not treated with selenite. This induction of TrxR1 enzyme activity during regenerative cell proliferation was transient and noted only from 16 hours to 72 hours post PH. These data indicate that TrxR1 indeed is a constituent of the neoplastic phenotype and a marker for neoplasia, although the expression of Trx1 can partially be explained by increased proliferation. As a component of the resistant phenotype which is induced during clonal adaptation and dysplasia, the expression of TrxR1 could be regarded as a response to the toxicity of the carcinogenetic protocol used in this

model and thus be a part of the cell defense against free radicals and toxicity. The role of this selenoenzyme in cancer has been illustrated in an experiment on lung cancer cells where removal of TrxR1 by using siRNA-mediated knockdown, reverted the neoplastic phenotype and inhibited malignant characteristics. Moreover, a TrxR1 deficient cell line lost growth self-sufficiency in both S-phase progression and expression of DNA polymerase [163]. In contrast to these data, transgenic mice with a deficient TrxR1 phenotype was not affected in normal liver development or during regeneration, indicating a TrxR1-independent route to supply RNR with electrons to provide for a complete DNA replication and normal proliferative growth [164]. TrxR1 deficient cells have further been shown to up regulate glutathione and to increase the activity of glutaredoxins thus maintaining a redox steady state [165].

In the synchronized and sequential model of carcinogenesis used in this thesis we have shown that TrxR1 was over-expressed in the preneoplastic expanding clones as well as in growing neoplastic lesions during progression. Thus, the use of TrxR1 as an immunohistochemical marker for liver preneoplasia and/or neoplasia could be suggested. One drawback regarding the use of tissue markers are that liver biopsies are needed, a procedure that is not without risks for the patient, and also a costly method. We tried to measure the levels of TrxR1 in rat serum with a sensitive ELISA method but without success. A plasma or serum marker that is specific and sensitive for preneoplasia would be of great clinical value.

We also administered sodium selenite to rats during liver regeneration and hepatocarcinogenesis. We found that this selenium compound reduced the nodule density during the progression phase of hepatocarcinogenesis but sodium selenite in the dose of 5 µg/mL did not impair the normal liver regeneration.

When we treated rats with 5 µg/mL a reduction of the expression of TrxR1 mRNA was observed in advanced liver cell carcinomas (data not published). This may indicate a different response to selenite in neoplastic cells to that of normal cells since a transiently increase in the mRNA expression was seen in normal regenerating hepatocytes.

In a study done in our lab, long term administration of sodium selenite did neither cause toxic effects nor accumulation of selenium in liver tissue or serum, although the levels of selenium was initially increased in a dose dependent way [166].

There are several reports about the tumour preventive effects of selenium but in a recent review these effects are questioned [167]. Based on over hundred studies of the selenium effect on different cancer types the authors of this review concluded that regular intake of selenium supplements for cancer prevention cannot be recommended to either the selenium-replete or deficient populations, and they also suggested new and better studies in order to evaluate selenium as a preventive agent in cancer [167]. Thus, the anti-neoplastic effects of selenium compounds seen in the rat model of hepatocarcinogenesis used in the present study needs to be confirmed in clinical studies on humans. It is also important to consider that some organic selenium compounds, like selenomethionine seems to differ from inorganic, like sodium selenite, or other organic compounds like selenomethyselenocystein.

So far, the only compound for systemic use which increases survival in patients with non-resectable HCC is sorafenib. Sorafenib was registered in 2007 after the results of the SHARP trial demonstrated a survival benefit compared with placebo on advanced HCC [43]. Sorafenib is a multikinase inhibitor, inhibiting the tyrosine kinases of vascular endothelial growth factor receptor (VEGF-R), and also on kinases downstream this receptor such as threonine kinase activity of Raf in the Ras/Mek/ERK pathway, leading to decreased levels of phosphorylated ERK

and inhibition of cell division. Target cells are both hepatocytes and endothelial cells, making sorafenib a combined anti-angiogenic and anti-proliferative compound.
The discovery of sorafenib was a big progress in the treatment of advanced liver cancer. However, sorafenib has side effects which must be taken into considerations when evaluating patients´ quality of life. Since sorafenib has been suggested as an adjuvant treatment after liver resection for HCC, the effects of sorafenib on liver regeneration is of interest. We therefore performed a study on the effects of sorafenib on liver regeneration using the same rat model as in paper I and II, namely the 2/3 PH rat model.
In our first and short-term study we evaluated three doses of sorafenib, 5 mg, 10 mg and 15 mg/kg bw, that was administered daily for one week before PH and then animals were sacrificed in groups of four at 16, 20 and 24 hours after PH. An additional group was sacrificed without any PH (0h).
We could see a mild but non-significant effect on cell proliferation measured as MIB5 positive nuclei, while the relative liver weights were decreased in all doses at 24 hours. The levels of both ALT and AST were increased in a dose dependent manner at base-line (0h) and the highest dose gave an elevation of MIB5 positive nuclei indicating a toxic effect of the treatment with sorafenib inducing compensatory liver cell proliferation.
In the long-term study in paper II we choose the 5 mg dose that was less toxic but still had an effect on liver growth after surgery. Previous studies report the use of doses ranging from 5-10 mg/kg [168].
In other similar studies conducted in mice conflicting results upon liver regeneration were shown. Hora et al showed inhibition of regeneration when sorafenib was administered after surgery but not if it was given before surgery. These results are in line with our data except for one important difference [169]. They could not detect any inhibiting effect on liver cell proliferation when sorafenib was stopped 24 hours before surgery while we saw a prolongation of the liver regeneration also in the “sorafenib pre-PH-group” in which treatment with sorafenib was stopped before PH.
However, the last dose given in present study was two hours before PH. The half –life of sorafenib is 25-48 hours and the diverging results between their results and ours may be explained by the sorafenib dose given closer to the surgical event. The study by Hora et al. lasted until five days after surgery while we studied the whole regenerative process until 14 days after surgery. Another murine study by Kurniali et al. could not detect any inhibitory effects of liver regeneration at the time points they measured which was 48 hours, 4 days and 15 days after surgery [170]. However, in the study by Hora et al. the most marked effect on inhibition was seen at 72 hours post-surgery, and in the present study the most prominent inhibition was seen at 48 hours for BrdU positive nuclei and at 48 hours and 72 hours for inhibition of mitotic figures. Interestingly, at 7 days and 10 days we saw a compensatory increase in proliferation in the treated animals.
One may speculate that the time points chosen in the Kurniali study failed to capture the inhibitory effect reported in ours and Hora´s study and also that a compensatory increase in cell proliferation that occurred in later time points may have masked a transient inhibitory effect of sorafenib.
In a recent publication where rats received sorafenib for 14 days before PH in a dose of 15 mg/kg body weight and sacrificed at day 2, 4 and 8 post hepatectomy, they showed results in line with our study [171]. The increase in proliferation in the treated animals seen in our study at day 7 was not seen at day 8 in their study. One explanation for these discrepancies can be

the dose, their dose being 3 times that of ours. Also, the period of increased proliferation might be missed because they sacrificed their animals at three time points only.
In our study we can conclude that sorafenib prolonged the liver regeneration in proportion to the length of treatment. Interestingly, we saw that the liver cells adapted to the mitoinhibitory effect of sorafenib one week after surgery, and eventually reached a complete regeneration, but this was delayed until two weeks after surgery, which was significantly longer than the placebo animals, which completed their liver regeneration in 5 days. These findings indicate that sorafenib, if used in conjunction with surgery, prolongs regeneration but does not impair it completely.
Studies have shown that cell lines adapted to the treatment of sorafenib and developed resistance upon long term treatment [172]. Hypothetically, treatment with sorafenib could select for the most aggressive growing tumour cells which in time may develop resistance. No studies so far on long term treatment have been reported, but this hypothesis has to be taken into consideration when giving sorafenib as a long-term prophylactic treatment in conjunction with resection or transplantation for HCC.
Indeed, sorafenib has been tested as an adjuvant treatment preventing HCC recurrence after transplantation. The outcome was disappointing in one study [173], but other studies demonstrated better results [174, 175] with a delay of recurrence and metastases after liver transplantation [175]. In the post-transplantation setting, sorafenib is a safe and efficient therapy for recurrent HCC [176]. So far neither our data or data from others support a differentiated effect on surrounding and tumour tissue, respectively, that in theory could work as a selective force increasing the risk of relapse of sorafenib resistant HCC in long term treated patients.
In the fourth paper we examined the difference in expression of the redox proteins Trx and Grx in hepatocellular carcinomas versus their surrounding tissue, and also correlated the expression to clinical parameters and elucidated if the expression of redox proteins could be used as diagnostic or prognostic markers for HCC. As internal controls we used the adjacent surrounding non-tumorous tissue and the relation of the expression in terms of color saturation after immunohistochemical staining was determined as the ratio of saturation in tumour to the saturation in the surrounding. The same was also done in CRC liver metastases patients and we compared the results with those of the HCCs. We found that the surrounding tissue in the resected HCC livers up-regulated redox proteins, even though the expression was higher in the tumours than in the surroundings.
In HCC we found a significant higher ratio of the immunohistochemical saturation of Trx1, Trx2 and Grx5 compared to respective surrounding non-tumorous tissue. In CRC liver metastases an even stronger up-regulation was seen in the tumours compared to the surrounding, which was seen for all redox proteins except for Grx2. The ratios of saturation of Trx1 and Grx3 in the tumours were significantly higher in CRCs compared to HCCs. This indicates a more prominent up-regulation of redox-proteins in CRCs than in HCC. This raised the question whether the difference seen was due to a stronger expression in the CRC tumours compared to HCCs, or stronger expression in HCC surrounding compared to CRC surrounding, or maybe both?
To answer this question we compared the saturation of the immunohistochemical staining of Trx1 in all surroundings under the exact settings in the camera and microscope when shooting the photos and in the following processes in the computer when analyzing the saturation. We found that the saturations of Trx1 were significantly higher in HCC surroundings compared to the CRC surroundings. One explanation for these findings may be that since the HCCs resides

mostly in a liver with an underlying chronic liver disease with fibrosis and cirrhosis, there is an increases oxidative stress leading to an up-regulation of anti-oxidative defense systems like the thioredoxin and glutaredoxin systems. We draw the conclusion that redox proteins do not have a value as diagnostic markers for HCC since they are not specific for that tumour and also due to the up regulation seen in the surrounding tissue in chronic liver disease.

To evaluate possible correlations between the expression of the redox proteins in HCCs and life-style factors such as smoking and alcohol consumption, we did correlation analysis and found decreased saturation ratios of Trx1 in the tumours relative to surrounding in smokers and in patients who drank more than 30 grams of alcohol per day. We found that after performing separate analysis of the saturation in tumours and surroundings, this difference was due to a significantly increased Trx1 expression in the surrounding livers from smokers and drinkers.

The saturation of Trx1 in the tumours however did not differ compared with the non-smokers and those with low or moderate alcohol consumption. Accordingly, we concluded that drinking and smoking leads to an over-expression of Trx1 in the surrounding non-tumorous tissue while it does not affect the Trx1 level in the tumours themselves. A murine study demonstrated that Trx1 attenuated systemic inflammatory response in Trx1 overexpressing mice [177], indicating an important role of this protein in response to toxic induced oxidative stress.

We also found a correlation between the metabolic syndrome and an up regulation of Grx2 in the HCCs, an indication of a different modulation of the redox defense system in this condition as compared to over-consumption of alcohol. Others have reported increased levels of thioredoxin in serum in patients with non-alcoholic steatohepatitis (NASH), which is a condition associated with the metabolic syndrome, indicating increased oxidative stress in this condition [20]. In a Chinese study of HBV associated HCC, metabolic changes was reported with up regulated antioxidant systems in patients with high levels of free fatty acids (FFA) [178].

We could not find any correlation between tumour differentiation and the expression levels of the redox proteins in HCC, however a significant inverse correlation between the total tumour diameter and the saturation levels of Trx2 was found, demonstrating a higher expression in patients with smaller HCCs. There was also a non-significant trend towards increased Trx1 protein expression in HCC tumours with micro-vascular invasion.

Studies on redox proteins as prognostic markers have demonstrated that Trx1 expression in the invasive front was associated with a poor prognosis [124], and also in colorectal cancer liver metastases the expression of Trx1 was associated with bad prognosis [125]. All the CRCs were positive in our study which can be explained by the relatively low number of patients included.

Tumour recurrence in the HCC patients of our study was 36% after a mean observation time of 213 days, and we could not detect any differences in expression levels in this group compared to patients without recurrence. Possibly a longer observation time is needed to evaluate whether or not Trx and/or Grx protein expression differs between those with or without recurrence. Thus, from present data we cannot conclude that the expression of redox proteins can be of use as a prognostic marker in HCC.

No correlation analysis between survival and redox proteins could be done because the observation time was too short with only 6 deceased patients.

We did correlation analysis of the IHC protein expression and mRNA expression and found a significant correlation in Trx1 but not Grx1. However, we had only fresh frozen material from five patients, but due the low number of samples and to sampling variability, these results must to be taken with caution.
The cell proliferation varied both within and between tumours, but a correlation was found between high IHC ratios of Trx1 saturation in the tumours and the mean labeling index of the proliferation marker MIB5. We could also see a regional co expression of high proliferation and the expression of Trx1. The correlation between cell proliferation and Trx1 has been described earlier [179].
In conclusion, the present study demonstrates that redox proteins are up-regulated in the surrounding livers of patients with HCC as compared to those with CRC liver metastases, while the expression in the tumour itself is weaker in HCCs compared to CRC metastases. These data indicate that redox proteins are ubiquitously expressed in livers exposed to oxidative stress and various malignancies, and therefore cannot be used as diagnostic markers for HCC. Cell proliferation in HCCs correlated to the tumour expression of Trx1, but patients with tumour recurrence had similar redox expressions as those without, indicating that redox expressions lack a prognostic significance in HCC.

3.1 GENERAL CONCLUSIONS

- The iron-sensing pathway of hepcidin regulation is down-regulated during liver regeneration, which reduces hepcidin gene expression and promotes iron mobilization to the regenerating liver.

- Treatment with sodium selenite reduces tumour volume in a rat model of hepatocarcinogenesis, but does not significantly compromise proliferation of normal hepatocytes during regeneration.

- Overexpression of TrxR1 is a constituent of the neoplastic phenotype.

- In a rat model of partial hepatectomy, sorafenib decreases cell proliferation and prolongs liver regeneration in proportion to the length of treatment. There is a partial adaptation to the inhibitory effects of sorafenib one week after the surgical event.

- Thioredoxins and glutaredoxins are ubiquitously expressed in livers exposed to oxidative stress and various malignancies and can therefore not be used as diagnostic markers for HCC. Smoking and high alcohol consumption increase Trx1 expression in tissue surrounding the HCCs, whereas expression of Trx1 in the HCCs correlates to cell proliferation. Redox protein expression in HCCs cannot predict tumour recurrence after liver resection.

3.2 FUTURE PERSPECTIVES

It is well known that the cytokine IL6 is crucial for induction of liver regeneration after partial hepatectomy. In our study we found an IL6 induction followed by increased hepcidin gene expression after surgery. However, it is unknown if hepcidin itself is essential for liver regeneration. This could be investigated using by the gene-knocking out method siRNA in cell lines.

Given the result in paper II where we concluded that TrxR1 was a constituent of the neoplastic phenotype, it would be of interest to evaluate the diagnostic and prognostic significance of this enzyme in human HCCs. Another interesting protein involved in coordination of regulation of genes involved in the response to oxidative stress is nuclear factor erythroid 2 related factor 2 (Nrf2). This factor was reported to be persistent activated in liver cancer cell lines and in mice [180].

We showed that selenium had inhibitory effects on tumours in our rat model of hepatocarcinogenesis without impairing the growth of normal hepatocytes. We also showed that sorafenib prolonged the regeneration after PH in rat. One could speculate that selenium and sorafenib in combination could have beneficial effects in the treatment of human hepatocarcinogenesis. Thus, It would be interesting to evaluate if a combination treatment with sorafenib and selenium would enhance the inhibitory effect on liver tumours in rats and if so, the dose of sorafenib could be lowered to decrease side effects and still be an efficient mitoinhibitor.

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Jag vill rikta ett särskilt tack till:

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