Prevention of liver cancer in chronic liver disease

An experimental study of sodium selenite and rat hepatocarcinogenesis

Suvd Erkhembayar

Stockholm 2011
To my family with endless love
ABSTRACT

Selenium treatment in supranutritional but subtoxic doses has previously been shown to inhibit cell proliferation in preneoplastic lesions in a rat liver carcinogenesis model. The mechanisms are not known, but thioredoxin reductase (TrxR1), a seleno-enzyme essential for maintaining intracellular redox status and cellular defence against oxidative stress might be involved. The aim of this work was to study whether selenium affected regenerative liver growth after partial hepatectomy as it did neoplastic growth and to study the effect of selenium on TrxR1 expression and regulation in regenerative and normal rat liver. To address these questions, we have compared the effect of sodium selenite on tumour growth in an experimental rat liver model for the development of hepatocellular carcinoma with the effect of sodium selenite on regeneration of liver mass after 2/3 partial hepatectomy and the effect of 10 weeks sodium selenite treatment on normal rats.

Sodium selenite administered in the drinking water (5µg/ml) reduced the rate of tumour growth during progression up to 12 months after initiation, but it did not affect gain of liver mass or rate of cell proliferation up to three weeks after 2/3 partial hepatectomy. Sodium selenite supplementation at dose levels of 1µg/ml and 5µg/ml showed an initial dose dependent increase of blood and liver levels of selenium. At 6 and 8 weeks, respectively, the selenium concentrations equilibrated regardless of dose and with no further accumulation. Sodium selenite did not affect body weight or relative liver mass at given doses.

We also explored whether TrxR1 was a marker of the liver tumour phenotype or if the TrxR1 over expression found in liver preneoplasia and neoplasia could be explained only by TrxR1 induction in growing cells. We found that from 6 months after initiation in the tumour model TrxR1 was only expressed in neoplastic liver lesions that showed signs of cell proliferation (bromodeoxyuridine (BrdU) incorporation), while remodelling preneoplastic liver nodules that were BrdU negative were immunohistochemically negative for TrxR1. Both types of lesions were, however, expressing the classical marker of preneoplastic and neoplastic nodules, glutathione S-transferase π (GST-π). During liver cell regeneration TrxR1 was induced at the time of cell proliferation, but was back to background activity 72 hours after partial hepatectomy. Sodium selenite potentiated this increase in enzyme activity. When sodium selenite was given to normal rats over long time TrxR1 activity was induced in the liver to an extent reflecting the liver selenium levels. TrxR1 mRNA was, however, only increased over background at the time when the selenium level was increasing. We found that, although TrxR1 did increase to a certain extent during cell proliferation, the neoplastic over expression of TrxR1 was not correlated to the rate of cell growth in the tumours. Our interpretation of the finding that TrxR1 was selectivity over expressed in the growing preneoplastic and neoplastic liver lesions is that TrxR1 is a tumour marker with a higher specificity to neoplasia with an increased risk for malignant transformation.
Based on the facts that long-term treatment of selenite did not cause accumulation of selenium or selenium toxicity and the fact that selenium inhibited only neoplastic growth but not regenerative growth we suggest that selenium is a suitable candidate for tumour prevention in patients with chronic liver disease dependent on sustained ability of liver regeneration. Furthermore TrxR1 is a histological marker in chronic liver disease for liver cancer and liver cancer prestages with a potential to be a marker for liver cancer risk.
LIST OF PUBLICATIONS

I. Erkhembayar S, Mollbrink A, and Eriksson LC (2011)

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

II. Erkhembayar S, Mollbrink A, Eriksson M, Larsen EH and Eriksson LC (2011)

   Selenium homeostasis and Induction of Thioredoxin Reductase during long term Selenite Supplementation in the rat

   Accepted for publication in JTEMB 2011
CONTENTS

1  Liver cancer as a preventable disease................................. 1
2  Liver cancer ...................................................................... 3
   2.1  Risk factors .................................................................. 4
   2.2  Diagnosis and Clinical course ........................................ 5
   2.3  Liver cancer treatment ................................................... 6
       2.3.1  Surgical radical resection ........................................ 6
       2.3.2  Liver transplantation .............................................. 6
       2.3.3  Locoregional therapy .............................................. 7
       2.3.4  Chemotherapy ........................................................ 7
       2.3.5  Molecular targeted therapy ..................................... 7
3  Tumour development in the liver........................................... 9
4  Liver cancer prevention ........................................................ 12
5  Clinical relevant markers for preneoplasia and neoplasia .......... 16
   5.1  Thioredoxin reductase 1 as a potential marker .................... 17
6  Selenium ............................................................................ 18
   6.1  Selenium metabolism .................................................... 18
   6.2  Selenium and health ..................................................... 20
   6.3  Selenium prevention of cancer and the suggested mechanism of actions.................................................... 20
   6.4  Selenium in chronic liver damage and liver cancer ............ 22
   6.5  Selenium prevention after surgical resection in HCC ........... 24
   6.6  Tumour prevention in chronic liver diseases with cell proliferation inhibitors ........................................... 25
7  Aim.................................................................................... 26
8  Results ............................................................................... 27
9  Comments on methodologies ................................................. 29
   9.1  Animal Experimental studies .......................................... 29
       9.1.1  The resistant hepatocyte model for hepatocarcinogenesis in the rat.................................................... 29
       9.1.2  The liver regeneration model, 2/3 partial hepatectomy .... 30
   9.2  Immunohistochemistry and labeling index.......................... 30
   9.3  Enzyme activity measurement ......................................... 31
   9.4  Inductively Coupled Plasma Mass Spectrometry ................. 31
10 General conclusion .............................................................. 32
11 General discussion and future perspectives .............................. 34
12 Acknowledgements .............................................................. 37
13 References ......................................................................... 38
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AAF</td>
<td>2-acetylamino-5-fluorone</td>
</tr>
<tr>
<td>ADH2</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>ALDH2</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>DEN</td>
<td>Diethylnitrosamine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HH</td>
<td>Hemochromatosis</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>Grx</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GST-π</td>
<td>Glutathione S-transferase π</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>MRD</td>
<td>Minimal residual disease</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>PBS</td>
<td>Primary biliary cirrhosis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Percutaneous ethanol injection</td>
</tr>
<tr>
<td>PH</td>
<td>Partial hepatectomy</td>
</tr>
<tr>
<td>PSC</td>
<td>Primary sclerosing cholangitis</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>SeCys</td>
<td>Selenocysteine</td>
</tr>
<tr>
<td>SeMet</td>
<td>Selenomethionine</td>
</tr>
<tr>
<td>SeMSC</td>
<td>Se-methylselenocysteine</td>
</tr>
<tr>
<td>TACE</td>
<td>Transarterial chemoembolization</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>TMSe</td>
<td>Thrimethylselenonium ion</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1 LIVER CANCER AS A PREVENTABLE DISEASE.

It is generally agreed that cancer is a preventable disease. Using the knowledge we already have the majority of cancer deaths seen today can be avoided. This is particularly true for the cancers where we know the major risk factors and causes of disease, like liver cancer. The most important tumour preventive action is to avoid exposure to known risk factors and to avoid risk behavior. However, this is more easily said than done in many countries with a low social-economic status and a poor population. In developed countries, on the other hand, this is possible for most people. For people already exposed to liver cancer risk factors, as chronic viral liver infection diseases, the risk of developing liver cancer can be reduced by active treatment of the viral disease. This is expensive and all patients do not tolerate the treatment or are not responding to therapy. There is today very little to do for patients with multi viral liver infections caused by several viruses or where a combination of risk factors exists. Thus there is a demand to obtain a cost-effective tumour preventive strategy for HCC.

The HCC incidence is very high among the population in sub-Saharan Africa and part of Asia, particularly Mongolia and China. Lately the occurrence of HCC has been shown to increase also in USA and part of Europe, including United Kingdom and France. In my home country, Mongolia, the incidence of HCC has been increasing tremendously and reaches now 99 cases of primary HCC per 100,000 inhabitants and represents the worst HCC threatened part of the world. Most patients will die within a year after established diagnosis. HCC is now also affecting patients at younger ages.

In this thesis I am addressing those patients that have a chronic liver disease and an increased risk of liver cancer development. The most prominent risk factors in my home country are hepatotropic viruses of type B, C, D and E. In different combinations these viruses causes chronic liver damage and ultimately liver cancer in patients who fail to clear the viruses after the acute infection and get a persistent infection. Today only a minority of these patients can be successfully treated for their chronic liver disease, but for the non-responders and the patients that cannot stand or afford the tough and long lasting therapy there is very little else to do, than to wait for the cancer to appear. For these people there is a need for markers for early detection of liver cancer in stages that can be treated and for methods to reduce the risk for cancer development and to reduce tumour growth once it has appeared.

There is a controversy connected to liver tumour prevention and treatment in patients with chronic liver diseases. First of all the protocol has to reduce tumour growth and tumour cell proliferation to be tumour preventive. This effect has to be specific for the tumour cells and not affect the growth of the non-neoplastic hepatocytes. In patients with chronic liver disease the normal hepatocytes are injured and some of them are dying reducing the liver function. For the survival of the patients the reduced functional mass of the liver has to be compensated by liver cell regeneration,
so-called compensatory liver hyperplasia. If this is inhibited the liver function will be compromised even further threatening the life of the patient.

This thesis work is focusing on the clinical dilemma of the patient with chronic liver disease using experimental liver models in the rat to study the development of liver cancer and the effect of sodium selenite to reduce the appearance or growth of liver neoplasia without inhibiting the growth of the normal hepatocytes. I have also addressed the search for a biomarker for liver cancer and liver cancer prestages that could be helpful in early detection of liver cancer in patients with chronic liver disease. Such a marker could be used to find those patients with chronic liver disease that have an increased risk of getting liver cancer and select them for a lifelong cancer prevention program.
2 LIVER CANCER

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and is the fifth most frequent cancer form and the third most common cause of cancer related deaths worldwide [1-2]. Annually about 500,000-1,000,000 new cases of HCC occur and 600,000 persons die due to HCC [3]. The incidence of liver cancer and liver cancer related death varies greatly around the world, depending mostly on geographic and socio-economic prerequisites in different areas of the world. Regions that have higher incidences of viral hepatitis of different types have higher incidence of HCC and the incidence varies with the type of virus hepatitis that is dominating in the area. Over 80% of the endemic viral infection related HCC occur in sub-Saharan Africa and Southeast Asia [4]. In these countries, the incidence is very high, generally over 20 cases per 100,000 individuals in the population. The highest incidence is in Mongolia with 99 cases per 100,000 individuals.

In countries, with moderate to intermediate risk, including Italy, Spain, France, United Kingdom, the incidence is estimated to be 5-11 cases per 100,000, while the lowest incidence is less than 5 cases per 100,000 population is found in United States, Canada and Scandinavia. There are still some areas in the world, where the incidence of HCC is not reported [3]. The clinical prognosis for liver cancer is poor, the 5 year fatality rate in individuals with established diagnosis of HCC is greater than 95% [5].

In a global perspective, the incidence of HCC is rising progressively. In United States over the last 25 years a 70% increase in HCC cases was reported [6]. Due to the spread of HCV and HBV the increase of HCC incidence is also seen in other countries including the United Kingdom and Western Europe [7-9]. The incidence of HCC increases with age, the highest prevalence occurs among individuals older than 65 years [10-11]. However during the last decades, an increasing incidence among younger adults has been observed[12]. Age specific incidence of HCC varies greatly in different parts of the world. Onset of HCC peaks at 40 among West African and Mongolian men while the peak age of onset in China is 70-80 [13-14]. Gender can also play a role in the risk and outcome of HCC and generally the incidence of cancer development among men are three to five fold that in women [15]. The peak age of HCC incidence is 5 year earlier in men compared to females [16]. The reason for the male predominance of HCC is likely to be multifactorial. The incidence of HBV induced HCC is higher among men than the HCV related. Innate factors are likely to be involved in the male predominance of HCC. Testosterone have been shown to be correlated with HCC development [17]. In addition male drink more alcohol and smoke more than women do in the areas where liver cancer is endemic. In experimental hepatocarcinogenesis there are also gender differences, some of them indicating the complexity of the mechanisms in initiation and promotion. In the rat liver model used in this thesis, with 2-acetylaminofluorene (2- AAF) as a promoter there is a gender difference in promotion but not in initiation. This difference is dependent on the lack of a growth hormone dependent sulfotransferase in the female
liver that is necessary for activation of the promoter [18-21]. In a study on a mice liver cancer model an increased expression of IL-6 in male mice in relation to female mice was suggested to explain the male dominance in liver cancer development in the model [22]. Interestingly, reduction of IL-6 in male mice prevented liver cancer development in 90% of the male mice, while ovariectomy enhanced IL-6 production and induced HCC development in female mice.

2.1 RISK FACTORS

Chronic liver injury of different origin constitutes a potential risk for liver cancer development. In contrast to many other cancers, the main risk factors for HCC are well identified [23-24]. Geographical differences in liver cancer incidence usually reflect the main causal factor variations [23]. It has been estimated that approximately one third of the world population has been infected by hepatitis B virus (HBV) and that about 350 million people are persistent carriers of HBV [25]. HBV is the most prominent risk factor for HCC particularly among the population in Asia and Africa. High exposure to aflotoxin B1 and its synergistic action with HBV is making it one of the potential risk factors in these areas [26]. About 170 million individuals have been infected with hepatitis C virus (HCV) worldwide [25,27]. HCV and other cirrhosis inducing factors such as alcohol abuse and heamochromatosis are main causes of HCC in western countries and Japan [28]. 20% of individuals with chronic HCV will develop cirrhosis and 2.5% develop liver cancer [29]. The patients with the highest risk of liver cancer are those with chronic HBV infection and liver cirrhosis with 1,000 fold increased risk compared to healthy individuals [30]. HDV in association with HBV will further increase the potential for liver cancer. Coexistence of HBV and aflotoxin B1 comprises 5-10 folds higher risk for HCC development compared with exposure to only one of these factors [31]. HCC in chronic HCV infected patients is increased 20-fold compared to non-infected individuals [32]. Individuals with functional polymorphisms of alcohol metabolizing enzymes, alcohol dehydrogenase (ADH2) and aldehyde dehydrogenase (ALDH2) are more vulnerable to alcohol abuse and alcohol dependence. Consequently, heavy alcohol consumption, ≥80mL daily will increase the risk 5 fold and in combination with viral infection and related cirrhosis the risk for HCC will be increased even more. Moreover, HCC develops in the setting of several metabolic liver diseases, including heamochromatosis (HH), Wilson’s disease and nonalcoholic steatohepatitis (NASH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) [33]. Diabetes mellitus, (insulin or non-insulin dependent) is another metabolic disease which increases 4 fold the risk for HCC [34]. Practically, all liver cirrhosis inducing conditions belong to the important risk factors for HCC development as in about 80% of the liver cancer patients, HCC arises in a cirrhotic liver as an ultimate complication of the chronic liver disease [35]. The lifetime risk of getting HCC in patients with HH is increased up to 200 fold, particularly if it is complicated by cirrhosis [36-38]. The overall risk for patients with primary biliary cirrhosis (PBC)
Non-alcoholic fatty liver disease (NAFLD) has higher prevalence in developed countries and is related to obesity with an increased cancer risk in the patients developing NASH and cirrhosis. Up to 1/3 of individuals with NASH develop cirrhosis within 10 years [40]. NAFLD accounts for 30-40% of all cases of HCC among patients with established diagnosis of cryptogenic cirrhosis [41]. Thus, the increasing incidence of obesity, particularly in USA and lately in Western Europe will lead to an increase of HCC incidence due to fatty liver disease.

### 2.2 Diagnosis and Clinical Course

The patients diagnosed with liver cancer are sometimes known by the health care system because of their chronic liver disease but sometimes the tumour is found at medical examinations for other purposes. The symptoms of the cancer itself are very sparse and non-specific. The liver has a huge capability to compensate for the presence of the lump, unless it is not interfering with the central bile ducts causing jaundice. Ultrasound and CT-examinations showing hypervascularized mass lesion of >2 cm in diameter usually in cirrhotic liver with an increased serum AFP level of >400 mg/L is considered to be diagnostic for HCC [42].

The clinical course and outcome of liver cancer is dependent on different factors, such as age, gender, time point when initial infection had occurred and duration and activity of underlying disease. Presence of co-existing diseases and immune status are other important factors that have impact on the outcome. Due to lack of specific clinical symptoms and reliable markers for early stages and prestages of the disease HCC is usually detected in advanced stages when patients become symptomatic. At such late stages when liver functions are impaired and complications have appeared it is usually little chance to achieve prolonged survival and a successful outcome with available treatment options. The overall worldwide liver cancer survival rates indicate that only 5% of liver cancer patients will survive 5 year after established diagnosis [5]. Patients usually die within 1 year after established HCC and the vast majority of these patients are resided in that part of world, where HBV and HCV infections are endemic.

However, nowadays in wealthy societies, an increasing number of patients with chronic liver disease are followed up and are diagnosed with an early stage HCC when the patients are lacking cancer related symptoms and the liver function is not impaired to a critical extent [43]. Consequently, the final outcome will be improved from early detection and management. In cases where the cancer can be surgically removed 75% of the patients will survive for 1 year and 60-70% for 5 year with recurrence of 50-70% of patients at 5 years [44]. In selected materials 5-year survival up to 70% and 10-year survival up to 30% have been published [45-46]. Liver transplantation results have been improving due to better immunosuppressive therapy.
and development of better diagnostic tools. The overall recurrence rate after transplantation is still 40% within 5 years mainly due to remaining cancer cells after surgery and the underlying chronic liver disease especially in patients with HCV caused primary HCC [47-48].

However, better results can be achieved by improved early detection and pre and post surgical management. With improved early term management, patient survival rate at 10 years after liver transplantation exceeded 70% in a recent study [49]. The presence of HBeAg and its seroconversion is one of most decisive survival factors and significantly correlate to overall survival after curative resection for HCC.

2.3 LIVER CANCER TREATMENT
Treatment of HCC depends on extent of tumour burden and severity of underlying diseases. There are several potentially effective treatment options available for HCC, including liver resection and liver transplantation. However due to the lack of donor organs the latter is restricted. Overall liver cancer treatment can be divided into curative and non-curative treatment protocols.

2.3.1 Surgical radical resection
Surgical resection is the most commonly used curative therapeutic option. Immediately after the surgical resection the liver regeneration takes place in the remaining liver and the functional liver mass is reestablished. Meanwhile, the remaining liver mass should suffice to support adequate liver function under circumstances of an increased workload. Thus well-selected patients in accordance to stages of cirrhosis, tumour size, vascular invasion and intrahepatic dissemination are the decisive factors for outcome. Patients with one large solid tumour without or with early cirrhosis and preserved liver function usually benefit from surgical radical resection.

2.3.2 Liver transplantation
Liver transplantation (LT) is an effective therapeutic option in patients with advanced HCC. In addition patients with early HCC associated with severe cirrhosis are one of the most suitable groups of candidates for LT. According to the Milan criteria, the most used selection criteria, patients with liver cirrhosis who have single tumour nodule <5cm or a maximum of three tumour nodules each < 3cm without gross vascular invasion is acceptable for LT [50]. Other selection criteria try to cover more patients outside of Milan criteria and allow patients with bigger tumours for transplantation. The UCSF criteria allows LT for more advanced tumours but with similar results as with the Milan criteria, namely single lesion <6,5cm or up to 3
lesions, each <3cm with total of <8cm [51-52]. Moreover, LT is restricted due to a number of factors, including limited access to graft and graft rejection [53].

2.3.3 Locoregional therapy
The third most commonly used treatment option in HCC is locoregional therapy. Locoregional therapy comprises several treatment approaches focused on the tumour site, including ablation and transarterial chemoembolization (TACE). In non-resectable HCC, local ablation is considered to be the best suitable medical option [54]. The ablation can be performed by modification of the temperature in neoplastic lesions by using radio-frequency ablation (RFA), microwave or laser ablation, or cryoablation or by using chemical substances, e.g. alcohol. The main ablation now used is RFA which have been shown to be more effective than percutaneous ethanol injection (PEI), particularly in tumours <4cm [55]. In PEI, ethanol will be introduced intra tumourally and it causes necrosis in tumour cells and thrombosis in tumour vessels. In TACE, chemotherapeutic agents such as doxorubicin and cisplatin are injected via the hepatic artery or blood supplying arteries directly into the tumour.

2.3.4 Chemotherapy
The effect of the systemic chemotherapy in HCC is limited. Both response rate and duration is short. Nowadays, no single or combined chemotherapy regimen has been found yet to be effective in HCC [56]. The main obstacles for systemic chemotherapy in HCC include chemoresistance of HCC cells, and intolerance to the cytotoxic drugs in cirrhotic patients [57]. Doxorubicin is the most used single effective agent in HCC [58].

2.3.5 Molecular targeted therapy
Molecular targeted therapies directed on distinct signal pathways involved in development and progressions of HCC have been investigated in several phase II and III studies. Phase II studies have recently been performed on drugs blocking epidermal growth factor receptor (EGFR), such as erlotinib and lapatinib, vascular endothelial growth factor (VEGF) inhibitor, bevacizumab, and multiple kinase inhibitor, sunitinib.

HCC is one of most vascularised solid tumours and the vascular endothelial growth factor (VEGF) is a target for anti-angiogenic drugs such as bevacizumab and sorafenib have been used with promising effects. Sorafenib, a multikinase inhibitor, is directed against several targets, including VEGF and Ras to inhibit tumour-cell proliferation and tumour angiogenesis.

Llovet’s phase III clinical trial and the Asia-Pacific phase III sorafenib study have shown that sorafenib adds three months to the lifespan of late stage HCC patients
with well preserved liver [59-60]. Based on these results, sorafenib has so far been approved by FDA as the only molecular targeted drug for treatment of late HCC.

Regardless, the type of available treatment choices, one of the most important factors for final outcome is the proper selection of patients. The 5-year survival rate exceeds 70% in well selected patients upon radical resection and this figure is markedly reduced in case of improper selection. In addition, in more than 80% of the patients recurrence will appear during follow-up [46,61].

Due to liver tumour heterogeneity and drug resistant liver tumour cells, the prevention of recurrences has been enduring difficulties. Previous attempts to diminish recurrence rate after resection by preoperative chemoembolization and chemotherapy had no or little effect, whereas radiation therapy and immunotherapy have shown some promising effect.

As mentioned above encouraging results of LT by Milan criteria were >70% 5 year survival rates and recurrence rates <15% [52,62]. Moreover, in patients who already had been resected due to solidatory HCC, LT is possible in cases with high risk of recurrence [63]. A recent study with split LT (SLT= the donor liver is split to two recipients) in adults and children has shown a promising result regarding the patient long-term survival. In this study, the 1 and 5 years overall survival in adults and children were 75%/66% and 84% /72% respectively [64]. Thus, SLT might enable to overcome the restriction due to the organ shortage and reduce the waiting list and mortality rate. In addition, the combination of TACE and PEI resulted in better survival rates than single treatment with TACE or PEI in non-resectable patients [65-66]. Furthermore, TACE can be used in order to stabilize the tumour during the time when patient is waiting for LT and/or to diminish the tumour burden with a purpose of making the patient eligible for transplantation later on. TACE causes tumour necrosis in more than 50% of cases and is generally well tolerated [67].

Finally, the recent advances in pre- and intra-operative patient management and improved post-operative care and prevention of infection and rejection have been providing a better outcome.
3 TUMOUR DEVELOPMENT IN THE LIVER

Carcinogenesis is generally described as a multistep process that can be divided into separate processes of initiation, promotion and progression. These processes have been defined and described in experimental models but can also be recognized in human cancer development in the liver, although overlapping processes and the underlying chronic liver disease that is usually seen make the steps less well distinguishable.

In this thesis we have worked with a synchronized rat liver model where initiation, promotion and progression can be separated and defined [68]. (See fig 5)

Briefly the multistep hepatocarcinogenesis process is described in the following (See fig 1). In a situation with a chronic liver disease creating an oxidative, necrogenic environment rare cells might be initiated in a process involving DNA alterations that by DNA replication results in a mutation or mutation like event. This mutation renders these rare cells or some of these cells capabilities to grow under circumstances where normal cells are not growing or allowed to grow by cellular defense mechanisms. Promotion of the growth of these initiated cells is a selection of cells that are able to grow in an environment that is toxic/mitoinhibitory for most hepatocytes. The hepatocytes selected to grow in the liver will by clonal expansion form a population that in the model is defined as a preneoplastic liver nodule. This clonal adaptation is beneficial for the organism and helps the animal to survive in the acute toxic situation.

The preneoplastic growth is dependent on the presence of the promoter or a promotive environment creating the selective pressure that favors the cells that have acquired a resistance to the mitoinhibitory effect of the promoter. These cells are therefore not defined as tumour cells but rather as cells adapted to a harsh environment by clonal adaptation. The growth advantage acquired by these cells however has a price. Being able to grow under circumstances that are growth-inhibitory for normal hepatocytes and oxidative, exposes these cells to further risks for genetic alterations and mutations. The cost is a reduced cellular defense and an unstable genome. The probability for the second mutational event dependent on environmental stress, the size of the preneoplastic population and the growth rate therefore increases. These factors are related and not independent from each other.

In the model the second mutation represents the events necessary for the preneoplastic cell to acquire a neoplastic phenotype and express the neoplastic growth advantage in the absence of the selective pressure from the promoter. This autonomously growing cell will by clonal expansion form the tumour that by further alterations in the unstable genome progress to an invasive malignant tumour with metastatic properties.

The phenotypic alterations acquired by the preneoplastic and neoplastic hepatocytes are multigenetic and very complex rendering them able to grow in a toxic environment [69]. The drug metabolizing systems are changed in a direction making the cells more resistant to toxicity and oxidative stress. The phase I, cytochrome P450 catalyzed drug activation process, is slowed down, while the phase II conjugating processes are increased. As a consequence of these alterations fewer reactive intermediates will form and bind to cellular macromolecules. In addition, water-soluble and lipid-soluble antioxidants are heavily increased as are certain antioxidant...
regenerating enzymes, like thioredoxin reductase, regenerating a large number of antioxidants, including the active lipid-soluble, membrane associated antioxidant ubiquinol from the oxidized form ubiquinone. Another important early alteration is inactivation of the tumour suppressor P53, not by mutation, but by an inability to enter the nucleus [70]. The inactivation of P53 will permit cell proliferation although the DNA is compromised.

![Fig 1. Schematic presentation of the multistep process of hepatocarcinogenesis.](image)

The key factors in the carcinogenic process are genetic alterations and growth. The genetic alterations are dependent on the oxidative environment and the genetic instability caused by ineffective processes defending the genome. In the clinical situation regenerative growth is triggered by hepatocellular malfunction reducing the functional liver mass. In human liver cancer development usually the cause of disease lead to chronic liver damage with hepatocyte injury, activation of Kupffer cells, which affect the stellate cells so they transform into myofibroblasts and deposit collagen. The fenestration of the endothelial cells disappear and the basal membrane of the sinusoids appear as a continuous, capillary basal membrane reducing the exchange between hepatocytes and blood. This functional insufficiency will be compensated by increased cell proliferation. (See fig 2)
Chronic liver injury – chronic repair

**Fig 2. Schematic presentation of cellular changes in chronic liver cell injury causing liver cirrhosis, sinusoidal capillarisation and compensatory cell proliferation and its relation to tumour development.**

Chronic liver injury with compromised liver function creates a need for chronic repair processes with chronic regeneration. In this complex environment the oxidative components as well as cell proliferation coexists and fulfil the prerequisites for liver tumour development. In fact, also in human hepatocarcinogenesis liver lesions comparable to preneoplastic foci/nodules, neoplastic nodules/adenoma and hepatocellular carcinoma are seen. The whole series of events, from the initial infection or initial exposure to etiological agents until development of clinically detectable malignant tumour usually takes 5-20 years [71].

Most human liver cancers are developed in a cirrhotic liver. Due to failed viral clearance and consequent interaction of viral and host immune reactions a chronic necroinflammatory hepatitis appears. The carcinogenic process progresses in these livers in parallel with the progression of the chronic hepatitis. The risk to develop liver cancer increases with the increasing activity of viral replication and grade of hepatitis.

Understanding the causes of liver cancer as well as mechanisms and stages of cancer development is important for the design of tumour preventive strategies.
Liver cancer development has a long duration and the risk factors are often known as the liver injury itself. Preventive strategies can be applied in all stages of the disease from the early stages of development of tumour prestages including initiation and promotion through progression and malignant transformation to further progression to an invasive tumour that spreads and metastasizes. The overall prevention can be divided into three parts, primary, secondary and tertiary. The primary prevention is based on strategies to avoid exposure to risk factors and to correct risk behaviour. In secondary prevention of liver cancer the chronic disease is already established and the focus is on reducing the risk of tumour development interfering with the mechanisms of the necro-inflammatory liver cell damage compromising the functional liver mass. In tertiary prevention the neoplastic disease of the liver is diagnosed and the aim will be to eliminate the tumour and to reduce the growth rate of the residual neoplasia after treatment and to prevent recurrence of disease in the liver or elsewhere in the body. A reduced growth rate will considerably impair progression of disease and significantly prolong survival.

Primary prevention eliminates or reduces the exposure to the causal factors for HCC and includes reduction of exposure to hepatitis virus, vaccination against hepatitis virus disease, refrain from alcohol, avoidance of exposure to aflatoxin, body weight control etc. Hepatitis of importance for the development of liver cancer is usually spread as a blood-borne infection via the skin, mucus membranes or directly into the blood. It is therefore important to reduce the exposure to the viruses that can be spread through infected syringes and blood transfusion, but also unprotected sexual intercourse with infected persons etc. Screening of blood donors for blood transfusions for hepatitis viruses and the use of disposable syringes in the health care system are important contributions for reduction of virus spread. Screening of blood donors for HCV was introduced in 1990. The first vaccine against HBV was introduced on the market 1981. Universal vaccination against HBV was started during 1984-1986 in countries with endemic disease, including Taiwan and Mongolia. In those countries and in other countries where hepatitis is an endemic disease and where HBsAg positive carriers are usually infected by the vertical transmission route during birth or in early childhood HCC usually develops at the age of 40 or younger and is usually detected late when treatment is not effective. The protective efficiency of HBV vaccination has been in the range of 90% to 95% from HBV vaccination programs in Korea, China and Taiwan, which is very promising for HCC prevention. Moreover infant mortality in fulminant hepatitis and childhood HCC incidence decreased in Taiwan. As a result of the nationwide vaccination of infants, the prevalence of HBV carriers in childhood is almost eradicated, decreasing from 15% to 1%. Consequently the HCC incidence in Taiwan decreased by 60% [72]. Nowadays vaccination against HBV is available for all infants. Even HBV negative adults have now the possibility to get vaccine. In spite of the success of HBV vaccination and the noticeable reduction of the global burden of
HCC among vaccinated children and younger adults, an increasing rate of HCC is seen in individuals over 30 years of age.

Due to the viral genetic heterogeneity of hepatitis C virus HCV vaccination has been much more complicated [73], but new strategies are developing. In contrast to HBV, HCV is rather variable and prone to mutation, which makes it similar to HIV. A DNA vaccine made of the more conserved part of the HCV genome, NS3, is under investigation. By a new delivery technique the DNA vaccination improved its efficiency of the immune based attack to combat HCV [74].

In many parts of the world aflatoxin is a frequently contaminant in the food. Aflatoxin by itself but also because of its synergistic action with HBV for induction of HCC makes it an important target to prevent liver cancer development. Aflatoxin is a fungal toxin which can be produced in any foodstuff. Contamination of basic food staples such as corn, nuts and rice is a major problem in developing countries. Moisture content in the crop, air humidity and temperature are main factors for fungal growth and aflatoxin production. The most important actions to reduce aflatoxin exposure in the population is to invest in proper facilities for food storing and processing in the entire chain of events from the fields, warehouses, stores and all the way to the plates. Regular test for contamination have to be performed and contaminated food should not be distributed and consumed.

Alcohol consumption is a well known risk factor for liver cancer in all societies. Drinking excessive amount of alcohol and alcohol abuse is linked to HCC, particularly in combination with other liver risk factors or diseases. Among many studies all over the world a recent Japanese study on association of alcohol consumption and HCC development concluded that the avoidance of heavy alcohol drinking (≥69.0 g alcohol/day) in men and moderate drinking (≥23.0 g alcohol/day) in women reduced the risk of HCC [75]. Policymakers and health care providers should be aware of the importance of making public health recommendations on alcohol drinking and the consequences of heavy drinking habits. In Sweden, alcohol purchases and trade is prohibited for people under age of 21 and alcohol is provided only by Systembolaget. In addition it is forbidden to advertise beverages with an alcohol content exceeding 15 volume percent. It is becoming increasingly difficult to monitor and control alcohol consumption in a modern and open society.

Insulin resistance and obesity, particularly among children is increasing in USA and recently in all Western countries. Obesity and diabetes mellitus are often associated with NAFLD and NASH and ultimately increase the risk for HCC development. Observational studies have shown that food rich in fruits and vegetables protects against cancer since they contain essential micronutrients with antioxidant potential, including
selenium, but the specific cancer protective elements in this diet rich in fruit and vegetables are still not identified [76]. Study on relationship between different antioxidant supplementations and tumour prevention has not been consistent and in the case of beta-caroten showed an inverse effect [77-79]. In one Chinese study including selenium a cancer preventive effect was seen [80]. In a recently published meta analysis of 67 studies performed on more than 230,000 individuals supplemented with Vitamin A, C, E, betacaroten and selenium the authors concluded that antioxidants increased the cancer risk, especially Vitamin A, betacaroten and Vitamin E but not selenium [81-82].

In diets rich in fruits the fructose consumption is increased and in contrast to glucose, fructose is entirely entering into the liver and causes de-novo lipogenesis and is thereby implicated in NASH development [83]. In addition, daily fructose consumption has shown to prompt fibrosis and increase liver inflammation in patients with NAFLD [84].

Secondary prevention is focused on the interference with the pathogenetic mechanisms of HCC development. During secondary prevention it is important to treat the patients with chronic liver disease for their specific disorder. In addition to alcohol restriction and diet adjustments it is important for patients with viral hepatitis to reduce the viral load and the necro-inflammatory reaction in the liver using antiviral and anti-inflammatory therapy. To achieve a sustained virological response on antiviral treatment and keep the viral replication at a low level over time is the goal for these patients. The inflammatory response in the virus infected liver can also be treated by a combination of antiviral therapy and interferon. There are seven FDA-approved medications in use for the management of HBV infections: two formulations of interferon (IFN), conventional IFN and PEG-INF and five nucleoside analogues (lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir disoproxil) [85].

This treatment strategy has proved successful reducing the viral load as well as the inflammation and surprisingly also reducing the amount of fibros tissue in liver fibrosis and cirrhosis [86]. Even when tumour prestages and tumours have developed the progression to cancer can be stopped or slowed down. Antiviral treatment to reduce the risk of developing liver cancer in patients with HCV chronic viral infections has also been improved. Sustained viral response up to 75% was achieved by a combined antiviral treatment. Antiviral treatment to reduce the recurrence of HCC after diagnosis and initial therapy of HCC can also be included as a strategy for secondary prevention.

Patients with hemochromatosis (HH), a hereditary disease in iron metabolism, causes chronic liver disease with 200 fold increased risk for liver cancer. If HH is diagnosed early and treated soon after confirmed diagnosis the increased risk for liver cirrhosis and liver cancer can be eliminated by simply reducing the iron load by venesection on a regular basis.

The aim of tertiary prevention is to prolong the life of patients with an established and diagnosed cancer by removal of the cancer or by other treatments reduce the growth
rate of the already established tumour. For the principles and tools for tertiary prevention of liver cancer I refer to the chapter of liver cancer treatment above.

A successful tumour prevention strategy has to take all factors into consideration and include knowledge of epidemiology, mechanisms of tumour development, early tumour diagnosis and tumour treatment. In this perspective the knowledge of the mechanisms of tumour development and the appearance and detection of prestages and early stage cancers are very important.
5 CLINICAL RELEVANT MARKERS FOR PRENEOPLASIA AND NEOPLASIA

There is an urgent need for clinically relevant markers for liver cancer and for liver cancer risk. An optimal marker will provide us with a non-invasive tool not only for early diagnosis but also for estimation of cancer risk on an individual basis. The marker or marker panel should provide information on the activity of the underlying chronic liver disease, the existence of liver cancer prestages as well as early liver cancers. With the help of such a tool the patients at risk could be found and subject to intense follow up, prevention strategies and early treatment. Moreover, with an easily available and relevant marker it will be easier to estimate tumour burden, to follow up on treatment response and to detect relapse and minimal residual disease (MRD).

The activity of the liver injury is playing a decisive role for liver cancer development. Nowadays, there are no good, reliable markers to identify and follow the activity of the liver disease, the grade of liver injury and its’ progression. Blood levels of enzymes and compounds reflecting liver function and liver damage are helpful but need to be complemented with markers for the inflammation and the deposit of fibrous tissue in the liver. Core liver biopsies provide the clinician with information of the liver damage and the activity of the disease and serve as a basis for estimation of prognosis, but the risk for complications when a sick liver is punctured with a 1.2 mm needle limits its value as a marker for repeated follow up of disease. The histological examination of core biopsies is used for estimation of the extent of liver cell necrosis, the activity of inflammation and the stage of fibrosis. Core needle biopsies are considered to be the golden standard to determine the liver disease as well as to diagnose liver cancer, but the method is not patient friendly. The risk for profuse bleeding and bile leakage and tumour dissemination along the biopsy tracts are most prominent risks for this method and explain why it is used with restriction in clinical centres for diseases in the liver. Fine needle aspiration biopsies have been suggested for studies of the inflammatory infiltrate in hepatitis patients, but as of today it is not used in the clinic, although the intrahepatic inflammatory cells could provide clinically relevant information on the activity of a necro-inflammatory disease in the liver [87-88].

The currently recommended and the only clinically used predictor in the blood of HCC is serum α-fetoprotein (AFP). An increased serum level of AFP, > 20 μg/L, initiates further investigation in order to exclude malignancy. However, AFP is neither a sensitive nor a specific marker for liver malignancy as it can be negative in up to 40% of small HCC and is increased during pregnancy and in benign liver diseases as well. AFP is also increased in other malignancies, including stomach and pancreatic cancers. In addition the tissue AFP is uninformative. Seromarkers for HBV and HCV is not very informative as a clinical marker for a patient with a known chronic liver disease.
5.1 THIOREDOXIN REDUCTASE 1 AS A POTENTIAL MARKER

TrxR1 is a selenoenzyme essential for maintaining the intracellular reducing state and is also participating in the cellular defense against oxidative stress. TrxR has a conserved penultimate C-terminal selenocystein (Sec) residue which plays an essential role in selenium metabolism. In the liver two main forms are expressed, one cytosolic form, TrxR1, and one mitochondrial form, TrxR2 [89-90]. Moreover, TrxR1 is involved in regeneration of important antioxidants such as ubiquinone, lipoic acid and ascorbic acid [91-93]. TrxR1 is also a key protein in cell proliferation and participates in DNA-synthesis [94].

The role of TrxR1 in cancer was illustrated by the fact that TrxR1 removal by si-RNA mediated knockdown in lung cancer cells resulted in reversal of the neoplastic phenotype and inhibition of malignant characteristics. Tumour growth and metastasis was also drastically reduced in mice injected with TrxR1 knockdown tumour cells and a TrxR1 deficient cancer cell line has been shown to lose self-sufficiency in growth by S-phase progression defect and inhibition of DNA polymerase expression, which is essential for DNA replication [95]. Being a selenoenzyme, TrxR1 is dependent on sufficient supply of selenium for its own synthesis and functional status and for the synthesis of all other selenoenzymes. TrxR1 has been implicated in the pathogenesis of a multitude of diseases with special emphasis on cancer development and drug resistance [96-97]. TrxR1 has been suggested to be constitutively over expressed in many human cancer forms including liver cancer. TrxR1 was also shown to be 3,5 fold over expressed in isolated liver nodules from a rat liver cancer model compared to the activity in normal liver tissue, but with the same sub cellular distribution pattern [98].

Our preliminary and unpublished studies of human chronic liver disease showed that the TrxR1 marker was not expressed in the hepatocytes in any of the activity stages of the inflammatory response. Neither was it expressed in fibrotic or cirrhotic livers with the exception of focal areas of proliferation, regarded as regenerative foci, but with a probable preneoplastic potential.

Consequently, in our experimental rat liver tumour model, we showed that TrxR1 was constitutively over expressed in preneoplastic liver nodules during promotion and in neoplastic nodules during progression [98-99]. TrxR1 therefore seemed to be a relevant marker for liver cancer and liver cancer prestages with a risk for malignant transformation. It was not clear if TrxR1 over expression in neoplasia was strictly related to growth rate or if it could be regarded a tumour specific marker.

Consequently, in our experimental rat liver tumour model, we showed that TrxR1 was constitutively over expressed in preneoplastic liver nodules during promotion and in neoplastic nodules during progression [98-99]. TrxR1 therefore seemed to be a relevant marker for liver cancer and liver cancer prestages with a risk for malignant transformation. It was not clear if TrxR1 over expression in neoplasia was strictly related to growth rate or if it could be regarded a tumour specific marker.
6 SELENIUM

Selenium is a most interesting and at the same time the most controversial trace element. Selenium is thought to maintain the equability of human health condition. The Swedish chemist Jöns Jakob Berzelius discovered selenium in 1817. He first thought it was Tellurium, another related element named after the earth, but eventually realized that it was a unique element that he named after the moon. Selenium has similar chemical properties to sulfur and exists in five different valence states, -2, 0, +2, +4 and +6. Inorganic selenium can exist in the forms of selenite, selenate and selenide differing in their oxidation states. Selenomethionine (SeMet) and selenocysteine (Sec) and selenomethylselenocysteine (SeMSC) are the examples of organic forms of selenium. The selenium content in the soil varies greatly depending on geographical location and mineral composition. Consequently, selenium content in the food can vary as selenium enters into food via plants. The plants absorb Se from the soil as inorganic Se, selenate(SeO$_4^{2-}$) and selenite (SeO$_3^{2-}$), and incorporate selenium in a number of organic forms, SeMet, Sec and SeMSC [100].

During the thirteenth century, Marco Polo observed that certain plants consumed by his horses caused their hoofs to drop off during his journey in Asia [101]. The plants were recognized later as the most selenium rich plants, Genus Astragalus. Astragalus transforms selenium mainly into the form of SeMSC [102].

The major intake of selenium in human comes from consumption of bread, cereal, fish, meat, poultry, seafood and grain products [103] and also through drinking water as inorganic selenate and selenite.

6.1 SELENIUM METABOLISM

The molecular metabolism of several selenium compounds and their relation to anticarcinogenic action has been extensively investigated. The main selenium form in the body is selenocysteine (Sec). Selenium is taken in via the food in an organic form, preferentially SeMet, which can be converted into Sec within the body. The inorganic and organic selenium is metabolized differently in the body, but with selenide as a common key metabolite, as it represents a branch point of two metabolic pathways, selenoprotein synthesis and excess selenium excretion [104]. (See fig3)

In the presence of glutathione (GSH), the inorganic, selenate is reduced by thioredoxin (Trx) or glutaredoxin (Grx) systems into selenite, which in turn is reduced by these systems and other thiols into selenide [105]. In addition, selenite can be reduced by free cysteine non-enzymatically. Moreover, selenite is reduced to HSe$^-$, via an intermediate glutathione conjugate GSSeSG in the presence of GSH [106-107]. The organic selenium compounds, Sec and SeMSC are cleaved by β-lyase to selenide and methylselenol respectively [108]. Methylselenol in turn is converted to selenide by demethylation [109]. The common key metabolite of all absorbed selenium, hydrogen
Selenide provides Se for synthesis of all selenoproteins via activation to selenophosphate [110].

Selenide can be methylated into excretory metabolites and eliminated via urine and feces but also exhaled as volatile compounds via the lungs [111-112]. Urinary excretion of Se is the major route of excretion and the amount of excreted Se is closely related to dietary intake. The exhalation pathway is considered to be the route of excretion when Se is ingested in high, toxic doses. Selenium is excreted in the urine mainly in the form of selenosugar and during excess selenium supplementation as trimethylated Se, trimethylselenonium ion (TMSe) [113], while, dimethylated Se, dimethylselenide is exhaled via the lungs. In addition demethylation may take place and convert the organic selenium back into inorganic forms.

Selenium is usually absorbed effectively, but depending on the route of administration selenium uptake varies as in oral administration, a certain amount of selenium has already reacted with constituents of food in contrast to intravenous administration. Selenium is distributed in the body via the blood, but the affinity for selenium differs in different tissues. In this selenium hierarchy liver tissue represents the tissue with the third highest affinity after skeletal muscles [114], whereas brain tissue is the tissue with the highest demand for selenium. In situations with selenium insufficiency the brain will conserve selenium most effectively [115-116].

![Schematic presentation of selenium metabolism.](image)

Inorganic sodium selenite metabolic pathways highlighted with thick arrows.
6.2 **Selenium and Health**

Selenium is a trace element and dietary micronutrient essential for life. The physiological effects of selenium can vary from essentiality to toxicity, depending on the used chemical forms and doses.

In human, 25 selenium containing proteins have been identified with important roles for various physiological functions. The recommended daily intake of selenium based on the selenium concentration sufficient for saturation of the selenoprotein glutathione peroxidase (GPx) is 55µg Se per day for healthy adults [117]. During pregnancy and breastfeeding, the dose should be increased by 10-20 µg/day. Both excess and deficiency of selenium leads to serious disturbances in human health.

More than 40 selenium deficiency related conditions, diseases and disorders have been reported, including aging, arthritis, cardiovascular disease, immunodeficiency, diabetes, stroke, muscular dystrophy, neurodegeneration, reproductive disorders and cancer. The serum selenium contents in cancer patients were shown to be lower than in cancer free individuals and selenium in serum could function as an indicator of increased risk to develop cancer [118-119].

Absolute selenium deficiency is involved in two major known diseases, the Keshan disease and the Kaschin-Beck disease. These disorders are common among the population in areas with extremely low selenium soil contents, like parts of China and some other countries, with a daily intake of selenium of 11µg or less [120]. The Keshan disease is a severe cardiomyopathy that affects children and women in childbearing ages. The Kaschin-Beck disease is a degenerative joint disease caused by oxidative damage to cartilage with deformation of bone structure and osteoarthropathy. Selenium deficiency can be caused by long term parenteral nutrition in immunosuppressed patients as well. Recently, selenium and selenoproteins have been shown to be involved in inherited defects. The diseases related to inborn defects of selenium utilization, transport, and metabolism is expanding, including inherited muscle disorders, growth retardation and neurodegenerative disorders [121].

At the same time, in high doses, selenium is very toxic and can cause serious physiological disturbances and death. The maximal safe multiple oral dose of selenium in human is suggested to be 5µg/kg body weight and the maximal single safety dose is 50µg/kg body weight [122]. The symptoms of acute selenium intoxication is garlic odour of breath, tachycardia, and respiratory disorders due to pulmonary oedema and ultimately death. Chronic selenium toxicity is manifested by morphological changes in fingernails and loss of hair [123].

6.3 **Selenium Prevention of Cancer and the Suggested Mechanism of Actions**

The association between selenium and cancer have been studied in many epidemiological and prospective studies since a long time. An inverse relationship
between selenium content in food and blood and risk for cancer and related mortality rate is observed in many studies. The selenium serum level in cancer patients is lower than in control cancer free patients [118]. In more than 100 animal studies selenium preventive effects are reported and several human studies supported the results as well [124-126].

Nevertheless, the molecular mechanism of selenium tumour prevention is not yet fully elucidated. Several mechanisms so far have been proposed as possible selenium tumour preventive actions including anti-oxidant effects, mediated by selenoproteins, stimulation of DNA repair and induction of apoptosis in precancerous cells. Selenium in low concentrations is essential for growth but in moderate to high concentrations it might inhibit cell growth (See fig 4).

In our previous studies we have been able to show that the effective tumour preventive doses of selenium are high, in a supranutritional dose range [99]. The metabolism of supranutritional and subtoxic levels of selenium in long term studies is therefore important to elucidate.

The exact function of the 25 selenoproteins found in the body is not known but groups of important redox active selenoproteins, including the thioredoxin reductase and glutathione peroxidase families are well studied [127-128].

Depending on the doses used and the chemical forms of selenium, selenium can affect cancer development in different ways and at different stages. The antioxidant role is suggested to be mainly preventive and affect the process also at very early precancerous stages. Selenoproteins play a central role in upholding redox homeostasis and are involved in all three levels of antioxidant defence against ROS and reactive nitrogen species (RNS). Affecting DNA integrity and repair organic selenium protects against oxidative stress. Seo et al 2002 showed that SeMet induces DNA repair in normal human fibroblasts in-vitro. Furthermore, Mukherjee et al 2001 showed that SeMet was most effective in regulating of DNA chain break control and reduces aberrant crypt foci in the colorectal tissues of rats.

In addition to detoxifying harmful ROS and RNS, selenium acts as a detoxifying agent by chelating metals like Au, Pt, Cd, Co and Hg [129-130]. Several of these metals can react and inhibit important proteins like thioredoxin reductase. Moreover, selenium have been reported to prevent cadmium induced peroxidative damage and cancer development in prostate and breast [131].

Furthermore, it became evident that selenium combat cancer progression by regulating several genes implicated in tumour progression, including phase II detoxifying enzymes, tumour suppressor genes and certain caspases [132-133]. Selenium have also been shown to regulate genes involved in cell cycle regulation in an inhibitory manner [134].

The effect of selenium in late stages of cancer development and treatment is believed to depend on the prooxidative effects of selenium in high doses and on ROS production [135]. ROS, mainly superoxide produce oxygen radicals and lead to cell death due to an intracellular imbalanced oxidative status [136]. ROS, will interfere with the activity
of several important cell signalling molecules, enzymes, tumour suppressors and transcription factors like, p53, AP-1, Sp-1, Nf-kB, ASK-1 and JNK [137-139]. The function of these proteins, in turn, is regulated by thioredoxin [140].

A recent in-vitro study on selenium cancer specific toxicity revealed that the extracellular reducing environment established by the cancer cells enabled the specific selenium uptake by these cells [141]. Moreover, redox active metabolites of selenium have been demonstrated to induce apoptosis by different mechanisms of action. Selenide induces caspase independent apoptosis by activation of p38, p21 and p53 and generating DNA strand breaks. Monomethylselenol causes caspase dependent apoptosis by upregulating p21 and p16. In addition it is known that selenium compounds cause cell arrest at different phases of the cell cycle, for example selenite treatment leads to S-phase arrest, while monomethylselenol causes G1 arrest. Furthermore, these compounds also inhibit cell proliferation and growth by suppressing Erk, AR, Akt and cyclins and CDKs [142-143]. An inverse relationship between resistance to conventional chemotherapy and sensitivity to selenite cytotoxicity was also observed. In addition, selenium inhibits neoangiogenesis by down regulation of VEGF and counteracts metastasis by down regulating of osteopontin and collagen [144-145].

Fig 4. Selenium concentration and suggested biological effect.

6.4 SELENIUM IN CHRONIC LIVER DAMAGE AND LIVER CANCER

Selenium has been proposed to have a liver cancer preventive effect. Prevention of liver cancer was reported from a population based study performed in China, in an endemic
area of chronic viral hepatitis and hepatocellular carcinoma. In a 5 year long study on selenium supplementation, the persons in the selenium supplemented group were provided table salt fortified with sodium selenite which resulted in an additional 30-50µg selenium ingestion daily, while the control groups was provided by ordinary table salt. The incidence of HCC was reduced by 35% in the selenium supplemented group compared to the control group [124]. In a trial including exclusively HBsAg positive patients, 226 patients were provided either with 200µg selenized yeast or placebo tablets daily for four years. Seven out of 113 patients in the placebo group developed HCC compared to none of 113 subjects in the selenium group [125]. The interpretation of these results was that selenium inhibited the replication of hepatitis virus, which resulted in a sustained delay of HCC development. One proposed mechanism of selenium tumour prevention in chronic liver diseases is that selenium causes apoptosis of cells in pre stages of cancer while it is not affecting normal neighboring hepatocytes. Results from studies revealing selenium status in different liver diseases and their progression and severity of liver damage showed that the selenium level in serum and liver was reduced in all forms of chronic liver damages [146]. The same results was found in patientes with liver cirrhosis [147]. The reduction of serum selenium in cirrhotic patients was more pronounced than in patients with chronic hepatitis without cirrhosis. The results indicated an inverse relationship between the intensity of the hepatic injury and serum selenium levels. In children with liver cirrhosis, the serum level of selenium was also found to be significantly lower than in controls [148]. Selenium supplementation in experimental hepatic fibrosis induced by chronic carbon tetrachloride administration decreased the collagen fibers and the number of collagen producing stellate cells [149]. Serum selenium was also found to be decreased in alcoholic and nonalcoholic liver diseases [150].

Selenium in supranutritional and subtoxic doses was shown to have a cancer preventive effect in rats. In our previous study on the DEN-induced hepatocarcinogenesis rat model selenium inhibited liver cancer development during both promotion and progression phases. Numerous epidemiological studies have reported that the selenium level in cancer patients were lower than in cancer free subjects [151]. One of the suggested mechanism of selenium tumour prevention is that selenium has a direct regulating effect on the cell cycle progression and causes cell cycle arrest at G1, S-phase and G2 phases of the cell cycle [152]. If a direct acting effect on the cell cycle is of importance for liver tumour prevention in patients with chronic liver disease it is also of importance to consider the effect of selenium on the normal regenerating hepatocyte. Patients with chronic liver disease have an increased compensatory hyperplasia to compensate for the loss of hepatocytes in the disease. This hyperplasia is vital for the patients to maintain their functional liver mass. Consequently, it is important to compare the selenium effect on liver regeneration with that on dividing neoplastic cells. This question is addressed in this thesis.
6.5 **SELENIUM PREVENTION AFTER SURGICAL RESECTION IN HCC**

Due to the increased number of patients with HCV the incidence of liver cancer has been increased during the last decade in the United States and Europe. Improved surgical techniques and methods for early detection of liver cancer, including α-fetoprotein measurements has increased the number of patients that have been offered the option of surgical resection. However intra hepatic tumours reappear within the first few years after resection in 80% of cases [153]. Within 2 years after primary resection the cancer usually relapses with multifocal and more aggressive multidrug resistant tumours. The relapse in HCC can occur in two different ways. Recurrent tumours can arise from invisible microscopic spread of cells from the initial tumour or from a new focus independent of the resected cancer in patients with chronic active hepatitis complicated with cirrhosis. Early relapse within one year after resection is likely to be derived from the initial tumour whereas late relapses can be related to development of a new tumour in the cirrhotic liver [153]. Strategies are needed to diminish the post operative early and late recurrences that could be caused by different molecular mechanism acting at early carcinogenesis and late cancer spread and metastasis. Systemic chemotherapy is not effective in prolonging survival and is not well tolerated for its significant toxicities [154]. Micro vascular invasion and spread of tumour cells followed by formation of satellite tumour foci or nodules are the main predictors of tumour recurrence. The treatment strategy for recurrent disease is not well established and there is a need of novel approaches to improve the clinical outcome. Repeated hepatectomy may provide a 5-year survival of up to 50% but it is usually associated with high incidence of rerecurrence. The following second and third resections are considered to enable a better outcome compared to only a single resection strategy. Local ablative treatment of HCC recurrence show comparable results with reresection. Up to 60-70% of the recurrence after resection represent foci previously undetected in the liver after radical resection [46]. It is reasonable to suggest that treatment of the remaining tumour cells (Minimal Residual Disease, MRD) with tumour cell proliferation inhibitors will delay or inhibit relapse in patients after resection or transplantation. Many patients do not benefit from liver resection so therapies targeting the underlying liver disease must be used to improve the 5-year survival rate [155]. 5 year survival rate after liver resection is 65 % and the underlying liver disease is the main factor important for long-term outcome of HCC. Interferon alpha and Interferon beta can inhibit the recurrence in HCV related HCC.

Liver cancer is one of the most drug resistant tumours and the main reason for unsuccessful treatment with chemotherapeutic drugs in HCC is the chemo resistance of the tumour cells and the heterogeneity of the liver tumour. Based on experimental data, knowledge of the carcinogenic process and clinical experiences this resistance can be characterized as primary multi drug resistance (MDR). So far the attempts to fight MDR in liver cancer have been of limited success. Recent experimental *in vitro* data, however, suggest that sodium selenite in high dose levels sensitizes cancer cells to radiation therapy [156] and enhances the transition of drug resistance cells into drug sensitive. It is therefore intriguing to suggest that selenite could be used either alone or in combination with other drugs to treat drug resistant liver cancer, residual tumours
after therapy, MRD or disseminated tumour cells (DTC) and circulating tumour cells (CTC).

6.6 **TUMOUR PREVENTION IN CHRONIC LIVER DISEASES WITH CELL PROLIFERATION INHIBITORS**

Despite its enormous metabolic activity the liver is one of the most quiescent organs in entire organism with an only 1 per 10,000-100,000 cells that is in the cell cycle in any given time in an adult individual [157]. In chronic liver diseases, a sustained cycle of hepatocyte injury, cell death, inflammation and repair with coexisting fibrosis and cell proliferation take places, rendering an increased rate of cell proliferation, that in many cases are exceeding 1-2 %, i.e a 100-1000 fold the normal rate. In many acute or chronic and progressive liver diseases, ongoing liver cell damage leads to apoptosis and necrosis resulting in impaired liver function and associated morbidity. The compensatory cell proliferation is essential to maintain hepatic function in chronic liver disease and to life [158].

The regulatory mechanism of liver cell proliferation in chronic liver diseases is not fully explained. In management of liver cancer prevention it is important to reduce the activity of the underlying disease, particularly in chronic hepatitis caused by HBV, to prolong the expectancy of tumour free survival. One of the dilemmas in cancer therapy is that normal rapidly dividing cells usually are affected by the anticancer therapeutic drugs, since chemotherapeutic drugs frequently target mechanisms involved in cell division. To overcome these difficulties, the researchers are challenged to discover more tumour specific and individually adapted treatment strategies. Drugs interfering with small molecular targets and antibodies directed against certain receptors and molecules in cell signaling cascades have been developed and tested in clinical phase II and III studies. Sorafenib is one of them and today the only FDA approved drug for the indication of treatment of patients with advanced HCC. Sorafenib targets several cell cycle kinases, particularly it inhibits neovascularization which is usually activated in liver cancer and of importance for tumour nutrition and spread.
7 AIM

The aim of this experimental study concerns liver tumour prevention and uses animal models to form strategies based on the use of sodium selenite. The previous work on experimental hepatocarcinogenesis performed by the research group has shown that sodium selenite administered in the drinking water in a dose dependent way reduced the volume fraction of preneoplastic liver nodules in promotion and progression, but did not affect the initiation step. It was also clear in these studies that thioredoxin reductase, a selenium containing, selenium inducible enzyme was affected and induced in the process. The cytosolic thioredoxin 1, but not the mitochondrial thioredoxin 2, was heavily increased in liver nodules and cancers induced in the model. The doses of selenite that showed the best effect on tumour prevention were high and in the range where selenite was pro-oxidative and potentially toxic.

The following questions were raised:

- If selenite inhibits neoplastic growth, will it also inhibit regenerative growth in non-neoplastic cells?

- Is TrxR1 a constitutive tumour marker or an unspecific marker for cell growth?

- Is it feasible to expose the rats for sodium selenite in tumour preventive doses in long-term studies or will selenium eventually give rise to selenium toxicity, limiting its use in tumour prevention to patients with chronic liver disease?

- What is the homeostasis of selenium upon long-term distribution of sodium selenite in tumour preventive doses? Will selenium accumulate in the body to toxic levels when selenium is administered during long time?

- Is thioredoxin reductase 1 induced by tumour preventive doses of sodium selenite, and how is the kinetics of the induction. What is the relation between thioredoxin reductase activity and levels of sodium selenite?
8 RESULTS

Paper I

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

We have shown that selenium inhibit cell proliferation in a dose dependent manner in both preneoplastic and neoplastic liver lesions in a synchronized experimental model for hepatocarcinogenesis in the rat. In this study the effect of selenium in a tumour preventive dose on normal regenerative cell proliferation was compared to the effect on tumour progression in order to investigate selenium as a candidate for liver cancer prevention in chronic liver disease with chronic repair. We also studied the effect of selenium on the regulation of thioredoxin reductase in normal regenerating liver. Thioredoxin reductase is a redox active selenoenzyme that has been shown to be over expressed in preneoplastic and neoplastic liver lesions in the experimental rat model as well as in human hepatocellular carcinomas.

Sodium selenite (5µg/ml) administrated in the drinking water to rats that was either partially hepatectomiced or sham operated did not affect the body weight or the gain of liver mass after 2/3 partial hepatectomy, although a slight delay of S-phase and mitosis was seen. Tumour growth studied as increase of tumour volume or mass during tumour progression was however significantly reduced by selenium. In non-neoplastic regenerating liver cells thioredoxin reductase mRNA was not affected by selenium, while the activity of the cytosolic enzyme was only transiently increased over the time of cell proliferation. Another interesting observation was that thioredoxin reductase during tumour progression was over expressed in comparison to the surrounding tissue only in the growing, persistent, precancerous lesions but not in the remodeling lesions where there was no sign of liver cell proliferation.

We concluded that 5 µg/ml sodium selenite did not compromise liver cell proliferation after partial hepatectomy in an extent that affected liver regeneration and gain of mass. We also concluded that the neoplastic over expression of thioredoxin reductase could only partly be explained by the tumour growth rate. The discrepancies between TrxR1 expression and tumour growth heterogeneity made us suggest that TrxR1 in fact is a constituent of the neoplastic phenotype.
Selenium homeostasis and Induction of Thioredoxin Reductase in long term Selenium Supplementation in the rat.

From our previous study it became clear that sodium selenite in supranutritional but subtoxic doses exerted tumour preventive effects. Consequently, in this study we wanted to investigate the long term effect of sodium selenite supplementation in tumour preventive doses, 1µg/ml and 5µg/ml.

The kinetics of selenium uptake and accumulation and TrxR1 induction after treatment with sodium selenite in the drinking water in doses of 1µg/ml and 5µg/ml for 10 weeks were studied in young male Fisher rats. Long term selenite exposure via the drinking water caused a dose dependent increase of selenium levels in the blood and liver. This increase leveled out at 6 and 8 weeks, respectively, at the same level of selenium regardless of treatment and dose. Thus, there was no accumulation of selenium in blood and liver over time at chronic exposure. A selenium dependent increase of the activity of TrxR1 was also seen and was parallel with the selenium levels in the liver tissue, while an initial induction of TrxR1 mRNA was seen during the first two days of treatment, during which time the selenium levels in the liver was increasing. Discontinuation of selenite exposure did not result in significant reduction of neither selenium content nor TrxR1 expression levels during the following weeks and even at later time points. Sodium selenite at the dose levels of 1 and 5µg/ml did not affect body weight or relative liver mass. Thus we concluded that long term treatment of selenite did not cause accumulation of selenium or permanent changes of TrxR1 expression.
9 COMMENTS ON METHODOLOGIES
The details of the methods used are described in the individual papers included in this thesis. In this chapter I only comment on some methods used, particularly regarding the animal experimental models we used in this study.

9.1 ANIMAL EXPERIMENTAL STUDIES
In this thesis work we used two animal models, a rat liver model for hepatocarcinogenesis, the Resistance Hepatocyte model by Solt and Farber [68] and a rat model for liver regeneration after 2/3 partial hepatectomy, first described by Higgins GM and Andersson RM [159].

9.1.1 The resistant hepatocyte model for hepatocarcinogenesis in the rat
For the study of experimental liver cancer we used the Solt and Farber model [68] with slight modifications [160].

![Diagram of Solt and Farber model](image)

The resistant hepatocyte model allowed us to perform a consecutive study on tissues harvested from all three phases of liver cancer development, initiation, promotion and progression. In this model, initiation is performed using diethylnitroamine (DEN) in a necrogenic dose (200µg) and promotion using a combination of 2-acetylaminofluorene (2-AAF) and 2/3 partial hepatectomy. During promotion only the hepatocytes resistant to the mitoinhibitory effect of the promotor will be able to respond to the mitogenic signals after partial hepatectomy and grow. After termination of promotion, most of the lesions that appeared during promotion will stop growing and remodulate back to a normal looking liver phenotype.
In only 1% of the nodules changes will appear that gives rise to cells that expand clonally in the absence of the promoter and progress until the development of a malignant tumour. During this phase of carcinogenesis, the neoplastic growth is autonomous and not dependent on the selective pressure from a promoter inhibiting growth of the surrounding non-neoplastic hepatocytes.

9.1.2 The liver regeneration model, 2/3 partial hepatectomy

For the study of rat liver regeneration 2/3 partial hepatectomy was used. The method is reliable and easy to standardize as far as timing and relative amount of liver removed is concerned and the rats recover quickly from anesthesia and surgery. There are alternative methods described that use chemical hepatectomy, which are more difficult to reproduce. In this study we used 2/3 partial hepatectomy under inhalation anesthesia with Isopenthal in a nebulizer. After opening of the abdomen the median and the left lateral lobes were ligated with silk and removed. One group, drinking tap water, was compared to one group exposed to sodium selenite in the drinking water. Sham surgery was used as control. The sham surgery is described in paper I.

9.2 IMMUNOHISTOCHEMISTRY AND LABELING INDEX

Glutathione S transferase-π (GST-π) is usually considered as a marker for liver preneoplastic and neoplastic lesions in rat liver carcinogenesis as GST-π is not expressed in normal rat liver [161-162].

GST-π staining was performed to localize preneoplastic and neoplastic liver lesions at 3, 6, 9 and 12 months after DEN initiation. GST-π is expressed in preneoplastic and neoplastic liver nodules and is considered a marker for the neoplastic process in the rat. GST-π is very low in normal rat liver and not induced by drugs, cell proliferation or expressed in fetal liver [163]. The limitation of the enzyme as a marker is that it is does not differentiate between preneoplastic, remodeling preneoplastic and neoplastic lesions, which are a limitation in studies of tumour progression. The S-phase specific, cell proliferation marker bromodeoxyuridine (BrdU) was administrated 3 days before the rats were extinguished and livers harvested. BrdU-labeling index in GST-π, TrxR1 and BrdU positive neoplastic lesions in selenium treated and non-treated rats were calculated in randomly selected image fields. The number of fields calculated was decided by determination of accumulated mean. At least 1000 hepatocytes were counted in each slide. For TrxR1 immunohistochemistry we used a method described and verified before [99]. In this work we have used the term over expressed to indicate that the staining was more intense in for example a liver nodule to that in the surrounding tissue in the same slide. We have used immunohistochemistry as a way to visualize the relative presence of the epitopes on the proteins and not as an expression of increased enzymatic activity in absolute terms. The existence of isoenzymes cross reacting with the antibodies makes it hard to translate staining intensity into amount of active enzyme. Cell proliferation kinetics after PH in selenium treated and non-treated rats were detected by cell proliferation marker MIB-5, an endogenous marker that
covers all cell cycle phases. The number of mitotic figures was also counted in the slides. MIB-5 and mitotic figures were counted in randomly selected image fields as above.

9.3 **ENZYME ACTIVITY MEASUREMENT**

The TrxR1 activity was measured in liver tissue homogenates by an enzyme assay according to Holmgren and Björnstedt [164]. In this assay, we used Trx from E.coli and insulin as substrates. The substrate specificity of mammalian TrxR1 is broad and it reduces thioredoxin from different species, including E.coli. The advantage to use Trx from E.coli is that, due to lack of regulatory Cys residues, E.coli Trx is stable and does not cause protein aggregation and inactivation due to oxidation.

9.4 **INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY**

The measurement of selenium in blood or liver is the most accurate way to assess selenium status. For the measurement of selenium levels in serum and in the 20% particle-free liver cytosol samples, Inductively Coupled Plasma Mass Spectrometry (ICP-MS), an analytical method for the quantitative determination of total selenium, was used [165]. In brief, the samples were digested in nitric acid using microwave-assisted digestion at 70 bar and 220°C. Following dissolution, the solutions were diluted with water, and the selenium concentration was determined by ICP-MS using the $^{78}$Se isotope and by making use of the dynamic reaction cell of the ICP-MS for removal of the argon dimer interference based on the specific mass to charge (m/z) ratio.

ICP-MS is applied for detection and quantification of a wide range of sample types and the method is considered to be sensitive and reliable for detection of trace elements in biological samples. Moreover, the required sample size is small. The unique feature of ICP-MS is to decrease the polyatomic ions, by chemically removing them out of the ion beam before the analysis. The detection limit of ICP-MS for selenium is below 50-100 ppt (parts per trillion, $10^{12}$).
10 GENERAL CONCLUSION

In this thesis rat liver models were use to study the effect of sodium selenite on tumour growth and neoplastic cell proliferation and compare that to the effect on regenerative liver cell proliferation and gain of liver mass after partial hepatectomy. We also studied the selenium homeostasis and induction of thioredoxin reductase during long term supplementation to normal rats.

We concluded that sodium selenite reduced the rate of volume expansion of neoplastic lesions during progression up to 12 months after promotion and that the rate of cell proliferation in the lesions was lower in the rats treated with sodium selenite. On the contrary sodium selenite did not affect the rate of liver mass expansion after 2/3 partial hepatectomy and did not significantly reduce the rate of cell proliferation after partial hepatectomy.

Thioredoxin reductase (TrxR1) was over expressed in the neoplastic liver lesions in comparison to the surrounding tissue. TrxR1 did increase during cell proliferation after partial hepatectomy with a peak activity at the time when the MIB 5 marker and mitotic index showed that the cells were dividing. After that the enzyme activity declined to background activity, although gain of liver mass was still going on for several days. This increase in enzyme activity was further potentiated by sodium selenite. It is therefore possible that at least a part of the neoplastic over expression of TrxR1 could be explained by the fact that the tumour cells were growing. The fact that remodelling liver nodules, positive for the classical liver nodule marker GST-π, but negative for BrdU, were negative also for TrxR1 during late progression could support this conclusion. However, we believe that TrxR1 in addition to be increased during cell proliferation, was a constitutive tumour marker based on the following observations from our immunohistochemical studies. The TrxR1 immunohistochemical signal in the nodules was strong and homogeneous within the lesions and 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 of TrxR1 activity during regenerative growth could not be detected by immunohistochemistry, with the exception for a very brief period 24 h after partial hepatectomy, when a periportal, zone 1, weak immunohistochemical signal was seen in 2 rats of 4 in the group not treated with selenite. We have also noticed in a so far unpublished work that TrxR1 mRNA was reduced by 5 µM selenite in advanced rat liver cell carcinomas, which indicate a different response to selenite in neoplastic cells in comparison with normal and regenerating cells where TrxR1 mRNA is transiently increased.

Selenium did not accumulate in serum or liver over time upon long-term exposure to sodium selenite. After an initial increase of the selenium content in serum and liver a steady state equilibrium was established at a selenium level that was independent on the given dose of sodium selenite in the serum but slightly higher in the liver in the high dose group indicating a balance between selenium uptake and elimination. TrxR1 activity was following the selenium levels in the liver, but TrxR1 mRNA was only induced at the time when the selenium levels in serum and liver were increasing.
During a 10 week period of selenite supplementation at tumour preventing doses no signs of toxicity was observed. Nor did the rats drinking tap water with 5µg/ml sodium selenite subjected to hepatectomy or sham operation show signs of toxicity.

Based on our experimental results we propose that sodium selenite is a good candidate for tumour preventive treatment in patients with chronic liver disease and increased cancer risk under long time, without suppressing the life-essential compensatory liver regeneration.

We also suggest that the selenoenzyme, thioredoxin reductase 1 could be used as a histological marker for preneoplasia and neoplasia in chronic liver disease.
11 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The work that has been performed in the rat liver model on selenium prevention of hepatocarcinogenesis shows that sodium selenite influences the carcinogenetic process in a direction that reduces the cancer risk. Based on the data from the animal model sodium selenite can also be used for long-term treatment at supranutritional and pro-oxidative doses without accumulation of selenium and severe side effects. It is therefore reasonable to assume that sodium selenite has a liver tumour preventive effect. The differentiated effect of sodium selenite on neoplastic and regenerative liver cell proliferation allowing regenerative growth while the growth of tumour cells are reduced is also beneficial for tumour prevention in a situation where regenerative cell proliferation and repair is requested.

It is also an advantage that selenite is effective in reducing growth of preneoplastic liver lesions during promotion as well as of neoplastic lesions in early progression. Our interpretation is that a selenite preventive strategy could be designed for secondary prevention delaying malignant transformation and for tertiary prevention reducing growth of established HCC.

Although the experimental studies are promising we do not know if selenite has the same effect in humans and at what doses we could expect a clinical effect. However based on existing data from human prevention studies [124-126] we believe that selenium could be given to healthy humans in doses of 200 to 300µg per day for a long time without side effects. But the knowledge of the effect of selenite given to humans with a chronic liver disease is limited as is information on the interaction of selenite with other treatments interfering with the immunsystem or being cytotoxic. In collaboration with the infectious clinic at Karolinska University Hospital Huddinge we have designed a clinical intervention study on patients with chronic hepatitis B and hepatitis C that are resistant to conventional therapy or that has failed on conventional therapy due to side effects. Initially we want to evaluate the effect on the activity of the hepatitis after one year, but our aim is also to be able to evaluate the effect on cancer risk in a long-term study. We are also investigating the prerequisites for doing this study in Mongolia, where viral hepatitis and HCC is much more frequent, making it easier to find patients to include in the study. NASH is another diagnosis of interest for selenium tumour prevention.

Another very interesting clinical application for selenite in tertiary prevention is to give selenite to HCC patients before and after radical surgery with the aim to reduce activation, growth and establishment of manifest metastasis from remaining cancer cells. Early relapse of HCC in the liver or outside the liver is the major cause of death after surgery and most relapses from metastasis occurs within the first two years [166-168].
The selective growth inhibiting effect of selenite on the tumour cells but not on the normal cells makes selenite a suitable drug that could be given already during the postoperative phase of liver regeneration after surgery. At this time we have reasons to believe that the regenerative growth factors released vigorously could stimulate also the growth of the remaining tumour cells. Sodium selenite as an adjuvant treatment to patients with liver cancer that cannot be treated with radical surgery, with the purpose of reducing tumour growth and progression is an interesting option, but needs further clinical studies on doses and drug interactions. The potential of treating drug resistant tumors with toxic doses of selenium is certainly of interest and could have a potential to override the drug resistance of the tumour cells making them sensitive to selenite toxicity but also to other cytotoxic substances to which the tumour earlier has been resistant or to radiation. The use of toxic doses of selenite for treatment of resistant disease is supported by experimental studies on human cells [169-170] and in clinical trials [124-125].

In our work we have shown that the redox enzyme thioredoxin reductase 1 is affected by sodium selenite in tumour preventive doses and we do not exclude that TrxR1 might be a target in selenium tumour prevention. TrxR1 activity was induced by sodium selenite and reflected the tissue levels of selenium. TrxR1 mRNA was transiently increased over background only when the selenium levels were increasing but not during selenium steady state conditions even if the activity of the enzyme was increased. TrxR1 activity was also increased in S-, G2- and M phase in the cell cycle during liver cell regeneration and this activity was increased by selenite. TrxR1 was shown to be over expressed over surrounding in liver cancer in humans as well as in the rat liver tumour model. In the synchronized and sequential experimental model we could also show that TrxR1 was over expressed in the preneoplastic expanding clones during promotion as well as in growing neoplastic lesions, with a high risk of malignant transformation, during progression. We could therefore use TrxR1 as an immunohistochemical marker for liver preneoplasia, liver neoplasia and cancer risk. Such a marker is of potential clinical value for patients with chronic liver diseases accompanied with a high risk for liver cancer. Today, in the absence of a reliable marker, these patients can only wait for the cancer to appear. The limitation of TrxR1 as a marker for liver cancer and cancer risk is that it is only established as a tissue marker that needs liver biopsies for evaluation. A plasma or serum marker would be of much greater clinical value. We have made efforts to measure TrxR1 in serum in the rat model but so far we have failed to find the enzyme protein or to measure the activity in serum.

In so far not published work on human liver biopsies from patients with HBV and HCV viral hepatitis with different grades of necro-inflammatory activity as well as at different stages of liver fibrosis we have concluded by TrxR1 immunohistochemistry that the enzyme is neither over expressed in the hepatocytes in relation to the grade of inflammation nor the stage of fibrosis. Interestingly, however, foci of hepatocytes with aberrant organization, oriented in multi cellular cores or acini, were positive for the TrxR1 marker. This foci, or rosettes, were also shown by MIB-1 immunohistochemistry to be proliferative foci. It is intriguing to consider these lesions as
preneoplastic foci and indicators of the increased cancer risk in these livers. To address this question a more extensive clinical study of redox proteins in liver cancer using material from the liver sample collection at the pathology clinic is on its way.

Our work support the conclusion that sodium selenite is an interesting candidate for liver cancer prevention that should be explored in humans. It has a low toxicity at tumour preventing doses and can be given as a lifelong treatment to patients with chronic liver diseases. It is cheap, easily available and simple to administrate. It is therefore attractive to suggest that sodium selenite tumour prevention should be explored also in countries outside the western world, with a low socio-economic status and where chronic liver diseases and liver cancer is much more common and a dominating cause of death in the population, even at young age. A future perspective of this work for me is therefore to collaborate with the authorities and the health care in my home country, Mongolia, to establish a clinical research program for the use of sodium selenite in secondary and tertiary liver cancer prevention. Such a program could be performed at the university hospital in Ulaanbaatar in collaboration with Karolinska Institutet, supported by grants from the Mongolian and Swedish governments in a collaborative effort as well as by research grants and resources from the health care providers in Ulaanbaatar.
ACKNOWLEDGEMENTS

The investigation in this thesis was performed at Division of Pathology, Department of Laboratory Medicine, Karolinska Institutet and supported by grants from the Swedish Cancer Society and Karolinska Institutet.

First off all I’d like to express my great appreciation to all the co-workers at Pathology for accepting me and allowing me to join your warm family and supporting me during all these study years.

In particular, I would like to thank my supervisor Lennart Eriksson. From the beginning you allowed me to do my master thesis in your group and thereafter to continue with my current work. Your enormous knowledge on medical science and endless enthusiasm and positive encouraging attitude was a great support all these years. My co-supervisor Mikael Björnstedt, thank you for sharing your knowledge and valuable discussions and guidance.

I also want to thank my group lab engineer Ulla Britta Torndal for her support and encouragement and help on laboratory work. I have to tell that I got the most valuable present from you when I delivered my baby boy, Baltsar. It was a sweater with cute teddy bear buttons that you hand waved for my baby-boy and the beautiful flowers.

My group mate Annelie Mollbrink will get a warm great for her help with all laboratory experiments and for her open-minded talks about life. My officemate Vicki thank you for your encouragement and advices.

Our division head Göran Andersson, thank you for providing me with possibilities to accomplish my study and encourage me under the most critical period of my life.

I would like to thank Anna Klara Rundlöf and the former PhD students Erik Olm and Marcus Selenius for help to understand thioredoxin system and answering all my questions helping me in the lab and Kristin Gustafson for her help in the lab. I also would like to thank Aristi Fernandes for support and interesting journal clubs.

My cytology course teacher Gareth Morgan is acknowledged for awaking my interest into the interesting world of the cells.

The time at KI was an important and challenging phase of my life to grow not only in science but also to go through all important life events and adjust and integrate into my second home country, Sweden.

I would like to thank my kids Binderiya and Jamma for being such good kids and taking care of Baltsar and all the pickups from kinder garden. Even though you guys had a lot to do you always was there and cared for us. My son, Baltsar for being such a sweat boy and bringing me all happiness and making me the most proud mother in the world. My husband, Batbayar for being there for me and sharing all happiness and sadness throughout the life and being a lovely caring father for our kids.
REFERENCES


