

Diploma thesis

**Establishing a hepatocellular carcinoma model in
pigs**

submitted by

Josef Dürager

Mat.Nr.: 0433681

attaining the academic degree

Doktor der gesamten Heilkunde

(Dr. med. univ.)

at the

Medical University of Graz

conducted at the

Department of Transplantation Surgery

supervised by

Ass. Dr. Philipp Stiegler

Prof. Dr. Florian Iberer

Graz, May 2010

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Graz, May 2010

Acknowledgements

First of all I would like to thank my supervisor, Ass. Dr. Philipp Stiegler, for giving me the chance to join his research group, the interesting topic, his support, advice, ideas and patience during the development of this thesis.

Furthermore I would like to mention the Stiegler research group and the staff members of the Biomedical Research facility.

I would like to express my gratitude to my parents for giving me the opportunity to study medicine and for their support during all these years.

Last but not least I want to give credit to my girlfriend Natalie and all my friends for their support and understanding during the whole study.

I dedicate this diploma thesis to my family

Abstract:

BACKGROUND: Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and is the most frequently seen primary cancer of the liver. It has a very poor prognosis and has an incidence up to 120 per 100 000 depending on the geographical location. In order to gain more information about this widespread disease and in order to test new therapy strategies animal models represent a valuable research instrument.

AIM OF THE STUDY: Guided by the literature, this study attempted to create and establish a chemically induced HCC animal model in conventional domestic pigs.

METHODS: Once a week, six domestic pigs were treated with an intraperitoneal injection of 15 mg/kg diethylnitrosamin. Every second week, blood samples were taken to monitor the general health conditions and liver functions in terms of a possible HCC development. Either blood was sampled by direct puncture of the jugular vein or by puncture of a previously installed Port – a – Cath system. Computed tomography scans of the liver were performed on the pigs every 2 – 3 months to detect any signs of carcinogenesis. After scarification the liver was removed and the hepatic tissue evaluated histologically and photodocumented.

RESULTS: Pigs underwent the poisoning treatment with diethylnitrosamin for an average time of 30 weeks. 2 animals died from an inexplicable cause after 20 weeks and the remaining 4 pigs were euthanized as their health conditions became worse. Blood parameters did not change significantly during the first 9 months. Then, in the course of an acute decompensation of the liver aspartate – aminotransferase and bilirubin levels increased dramatically. The histological investigation revealed that there were no signs of a clear cut HCC. We found different stages of cirrhosis as pigs were euthanized at different phases of the experiment. Furthermore, various tumours were found including angiosarcomas and leiomyomas.

DISCUSSION: Due to the fact that the experiment was not carried out until the predicted end as the health conditions of the animals did not tolerate further treatments, several possible sources of error were evaluated. Either the dosage of the poison administered was too high and the duration of the poisoning treatment was too long or the weekly sedations, required for diethylnitrosamin application and blood sampling, caused intolerable side effects.

Zusammenfassung:

HINTERGRUND: Das hepatozelluläre Karzinom (HCC) zählt zu den am weitest verbreiteten Krebsarten weltweit und ist zudem der häufigste bösartige Primärtumor der Leber. Das HCC hat eine schlechte Prognose und man zählt bis zu 120 Neuerkrankungen pro 100 000 Einwohner pro Jahr. Um Informationen über diese weit verbreitete Krankheit zu sammeln und um neue Therapiekonzepte zu erarbeiten sind Tiermodelle von größter Bedeutung.

ZIELSETZUNG DIESER STUDIE: Diese Studie versuchte auf Grundlage vorhandener Literatur und mit Hilfe von chemischen Substanzen ein HCC Tiermodell in konventionellen Hausschweinen zu etablieren.

METHODEN: 6 Hausschweine erhielten einmal pro Woche eine intraperitoneale Injektion von 15 mg/kg Diethylnitrosamin. Um den Gesundheitszustand und die Leberfunktionen zu überwachen wurde jede 2. Woche eine Blutabnahme durchgeführt. Das Blut wurde entweder durch eine direkte Punktion der Vena jugularis oder durch die Punktion eines zuvor installierten Port – a – Cath Systems gewonnen. Um etwaige Anzeichen einer Karzinomentstehung zu erkennen, wurden alle 2 – 3 Monate Computertomographien der Leber durchgeführt. Nach der Sakrifizierung der Tiere wurde die Leber entfernt, das Lebergewebe histologisch untersucht und die Ergebnisse dokumentiert.

ERGEBNISSE: Die Schweine wurden für durchschnittlich 30 Wochen mit Diethylnitrosamin behandelt. 2 Tiere starben aus unerklärlichen Gründen nach 20 Wochen und die verbliebenen 4 Schweine wurden eingeschläfert nachdem sich ihr gesundheitlicher Zustand zusehends verschlechterte. Die Blutwerte veränderten sich in den ersten neun Monaten kaum. Danach stiegen die Aspartat – Aminotransferase und Bilirubin Werte im Zuge einer akuten Dekompensation der Leber drastisch an. Histopathologisch konnte kein eindeutiges HCC nachgewiesen werden. Da die Schweine nach unterschiedlicher Dauer der chemischen Behandlung eingeschläfert wurden fanden sich verschiedene Stadien der Leberzirrhose. Weiters wurden verschiedene tumorartige Veränderungen gefunden, bei welchen es sich unter anderem um Angiosarkome und Leiomyome handelte.

DISKUSSION: Da das Experiment wegen der gesundheitlichen Verschlechterung nicht bis zum vorgesehenen Ende durchgeführt wurde werden verschiedene Fehlerquellen diskutiert. Einerseits kann die Dosis zu stark und die Dauer der Behandlung zu lang gewesen sein, andererseits ist es möglich, dass die wöchentlichen Narkosen, welche für Verabreichung von Diethylnitrosamin und der Blutabnahmen gebraucht wurden, mit zu starken Nebenwirkungen verbunden waren.

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List of Abbreviations

%	percent
°C	degree celsius
µg/kg	microgram per kilogram
AAT	α – 1 – antitrypsin
AFP	alpha - fetoprotein
AFU	α -l-fucosidase
AJCC	american joint committe on cancer
ALC	alcohol
ALT	alanine - aminotransferase
AP	alkaline phospatase
AST	aspartate - aminotransferase
Bili	bilirubin
CHE	choline esterase
cm	centimeter
CP	child pugh
Crea	creatinine
CT	computed tomography
DCP	des- γ -carboxyprothrombin
DENA	diethylnitrosamine
DNA	desoxyribonuclein acid
EDTA	ethylenediaminetetraacetic acid
Ery	erythrocytes
Fig.	figure
g/L	gram per liter
Gd - DTPA	gadolinium – diethylenetriaminepentaacetic acid
GGT	γ -glutamyl transferase
Hb	haemoglobin
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCCs	hepatocellular carcinomas
HCV	hepatitis C virus
HDV	hepatitis D virus
HR	hepatic resection
ICG R15	indocyanine green retention rate at 15 minutes
IE/ml	international unit per milliliter
kDa	kilodalton
LDH	lactate dehydrogenase
Leuco	leucocytes
MELD	model of end stage liver disease
mg/kg	milligram per kilogram
mg/kg/h	milligram per kilogram per hour
min	minute/s
ml	milliliter
MRI	magnetic resonance imaging
NAFLD	non alcoholic fatty liver disease

OLT	orthotopic liver transplantation
PAC	port - a - cath
Phos	phosphate
PIVKA	protein induced by vitamin K absence or antagonist
RFA	radiofrequency ablation
RNA	ribonuclein acid
ROS	reactive oxygen species
Tab.	table
TACE	transcatheter arterial chemoembolisation
U.S.	united states
US	ultrasonography
WHV	woodchuck hepatitis virus

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1 INTRODUCTION

1.1 Hepatocellular Carcinoma (HCC)

1.1.1 General Consideration

Hepatocellular Carcinoma (HCC) represents one of the most common cancers in the world. The main risk factor for HCC development is cirrhosis of the liver, whereas carcinogenesis also depends on the underlying disease. Due to geographical differences, the incidence may vary from 4 – 15 per 100 000 in Western countries to 120 per 100 000 in Asia and Africa.(1) HCC is the most frequently seen primary cancer of the liver and has a poor 5 year – survival rate of less than 5%.(2)

1.1.2 Definition

HCC is a malignant tumour of hepatocellular differentiation.(3)

1.1.3 Etiology

Infections with hepatitis B (HBV) and hepatitis C (HCV) are playing important roles in the development of HCC. The risk of developing HCC is 100 times higher in patients infected with HBV than in HBsAg negative population.(3;4) After the viral genome has integrated into the hosts genome and under the influence of additional promoters like inflammation and carcinogene substances carcinogenesis takes place.(3) In almost every HBV induced HCC viral genome was found integrated into cellular DNA.(5) The earlier an HBV infection takes place the higher the risk of developing a HCC gets.(3)

	Europe	North America	Asia & Africa	Japan
HCV	60 - 70	50 - 60	20	70
HBV	10 - 15	20	70	10 - 20
ALC	20	20	10	10

Tab.1.1: Most common risk factors for developing HCC depending on geographical distribution.(6)

Besides HBV infections HCV also represents a risk factor for HCC development.(1;3) In comparison to HBV, the HCV is an RNA virus which means it does not integrate into the host genome because it misses a reverse transcriptase enzyme. Despite this, HCV – RNA can be detected in the tumour tissue and surrounding liver tissue. The precise mechanism for carcinogenesis is still unclear but it can be assumed that cirrhosis caused by the virus leads to the development of HCC.(7) Although cirrhosis of the liver is a major risk factor for HCC, it can also develop in a liver without cirrhosis.(3;8) Whereas in western countries patients with HCC are mostly over 40 years old and the HCC is located in a cirrhotic liver, the patients in Africa and Asia are more often younger than 40 and suffering from HCC in a non cirrhotic liver.

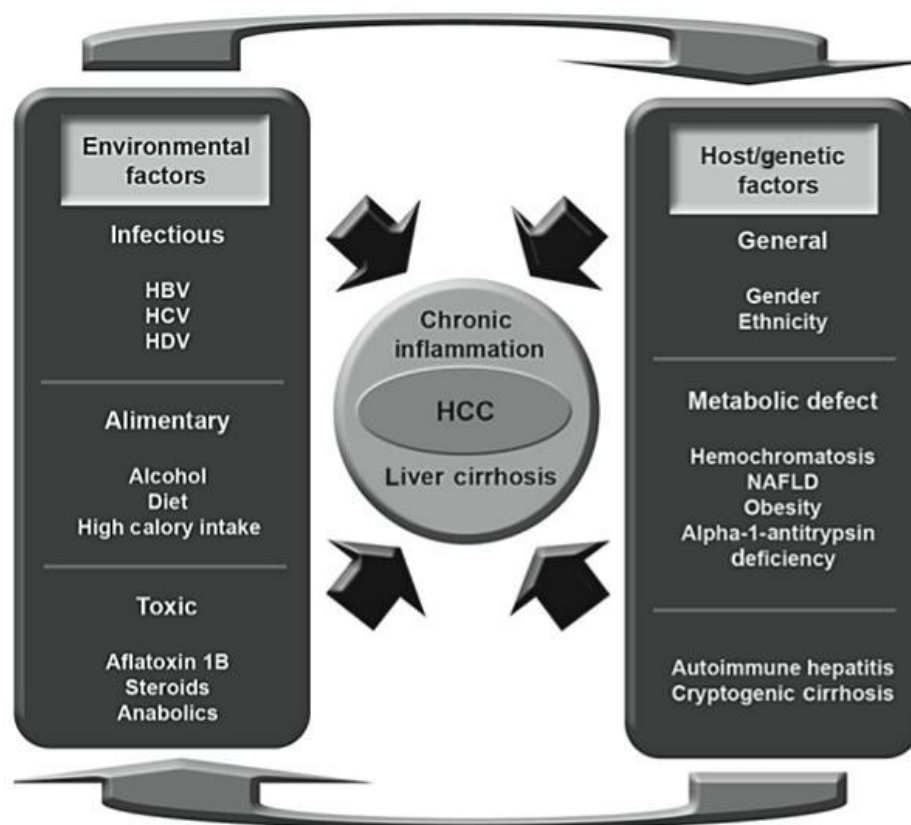


Fig. 1.1: Risk factors for HCC development(9)

Further important risk factors for HCC occurrence are aflatoxin exposure, alcohol abuse, anabolic steroids and metabolic disorders like hereditary hemochromatosis, $\alpha - 1 -$ antitrypsin (AAT) deficiency and glycogen storage disease type 1.(3)

Aflatoxins are hepatotoxins produced by an ubiquitous fungus called *Aspergillus flavus*, which contaminates food, especially peanuts and grain in several tropical regions.(3) It is shown that there is a close association between the mutation of the most frequently mutated gene in human cancer like p53 tumour suppressor gene and aflatoxin exposure. Aflatoxin exposure often results in a mutation of p53 in human HCC cells which also can be found in many other patients with HCC living in regions with high levels of aflatoxin contamination.(7;10)

Hereditary hemochromatosis is an autosomal recessive disorder, which due to increased iron resorption leads to massive accumulation of ferrous pigments, especially in the liver and the pancreas. Due to lipide peroxidation and direct interaction with DNA the iron has a toxic effect on hepatocytes.(3)

AAT deficiency is a quite common autosomal recessive metabolic disorder that affects about 1 of 3500 people. AAT is produced and dispensed by hepatocytes and as its major effect it inhibits elastase of neutrophile granulocytes, which reduces and destroys extracellular matrix. In fact AAT prevents proteolytic destruction of tissue. The causal gene on chromosome 14 forces the liver to produce abnormal AAT which accumulates due to impaired reduction and may play an important role for the development of HCC.(3;7)

1.1.4 Macroscopic Pathology

A macroscopic differentiation between massive tumour nodes, multinodular occurring of tumours and a diffuse infiltration of liver tissue can be made whereby multinodular development may be a possible sign of multicentric genesis.(3) A study from Italy(11) in 1993 showed that the solitary HCC type was the most common cancer type with 54,8%, followed by the multinodular type with 31%, the diffuse type with 7,7% and the least common massive HCC type with 6,5%. Diameters of HCC nodules range from less than 1 cm to more than 30 cm.(11) Depending on

whether the differentiation is high or low macroscopically the colours of HCC nodes vary from brown-red, gray-green-yellow to gray-white and often show spots of hemorrhage, necrosis and common vascular invasion.(3;11;12)

On the one hand cirrhosis of the liver is typical of multinodular HCC, but on the other hand massive HCC nodes are associated with noncirrhotic livers.(11) The diffuse pattern is associated with many small nodules which are often similar to cirrhotic nodules.(13) HCC types can be further distinguished by its behaviour towards adjacent liver tissue. HCCs that show expansive growth characteristics have sharp borders that edge out adjoining liver. In contrast, infiltrative growing HCCs show poorly defined borders which microscopically invade the adjacent liver.(12;14)

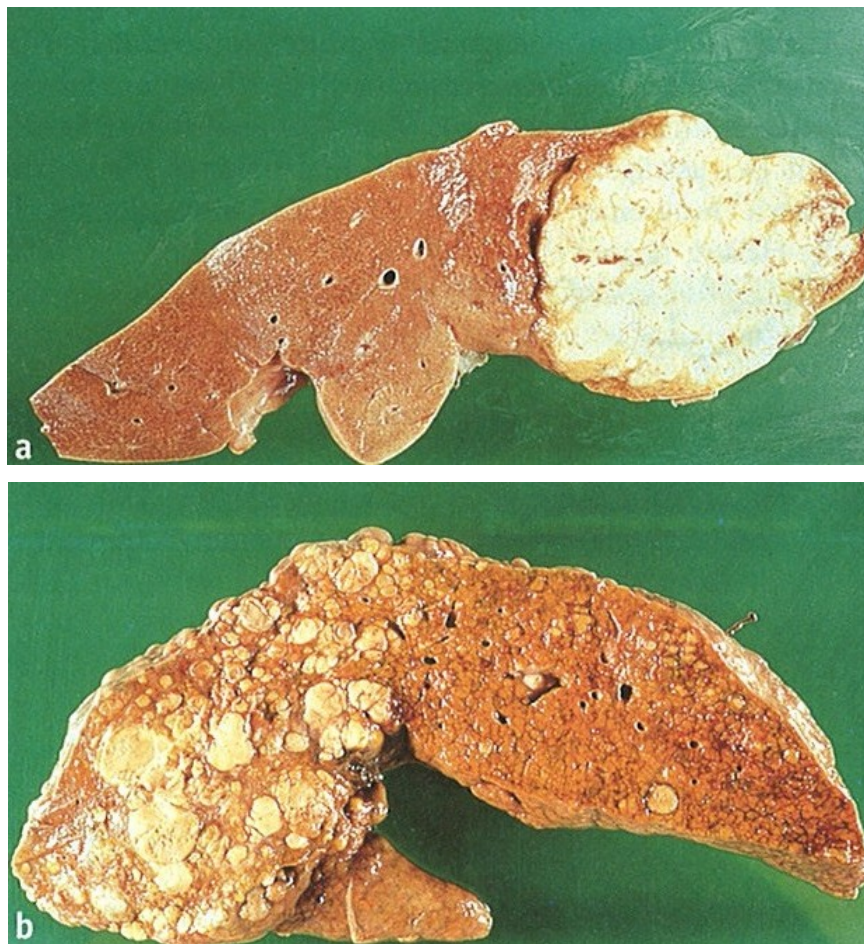


Fig. 1.2a: Massive HCC(3)

Fig. 1.2b: Multinodular HCC(3)



Fig. 1.2c: Infiltrative HCC(15)

1.1.5 Histology and Microscopic Pathology

In most cases HCCs mimic the normal trabecular architecture of hepatocytes. In contrast to normal trabeculae which are one or two cell layers thick, neoplastic HCC trabeculae reach from two to twenty layers of thickness. Besides the most common trabecular arrangement, tubular and papillary patterns can occur. Between these trabeculae of tumourcells, sinusoid like blood caverns appear, which are lined by endothelial cells. Depending on the stage of differentiation, HCC tumour cells resemble more or less hepatocytes and they often show a great variation in cellular and nuclear size and even create multinucleated giant cells.(3;12;16) HCC cells show a more uneven distribution of heterochromatin, have more irregular nuclear membranes, even show different degrees of differentiation inside a single tumour and present an increased apoptotic and mitotic activity.(12) Highly differentiated HCC cells produce bile which can result in the emergence of bile thrombosis. Moreover, miscellaneous intracellular inclusions can be detected, like typical Mallory bodies in 20% of all HCCs independent of the underlying disease.(3;17) Little differentiated HCC often show a decreased number of Kupffer cells whereas higher differentiated tumours may contain regular numbers of Kupffer cells.(18)

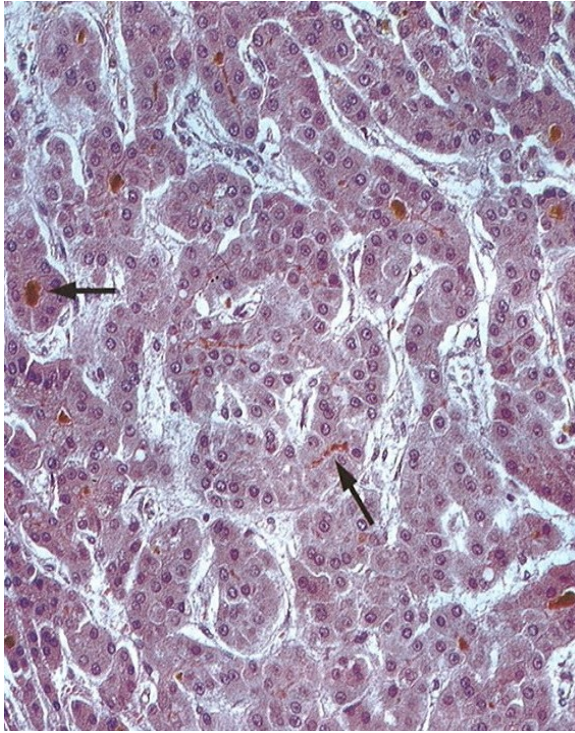


Fig 1.3a: Trabecular – sinusoidal type: This is the most common histological growth pattern. It can be assumed, that other HCC types are derived from this type. The well differentiated tumour cells are polygonal, mostly resemble hepatocytes and grow in multiple layers. They surround endothel lined sinusoid like blood caverns. Arrows indicate intercellular bile secretion.(15)

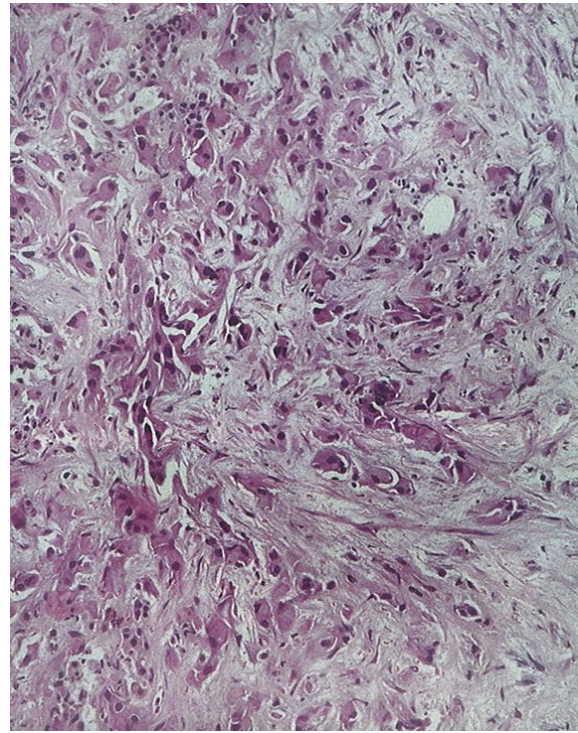


Fig 1.3b: Cirrhotic type (Spindle cell type): This type is characterized by massive deposits of cell lacking sclerotic connective tissue between moderate differentiated tumour cell cords. It is commonly seen after chemotherapy, necrosis and irradiation.(15)

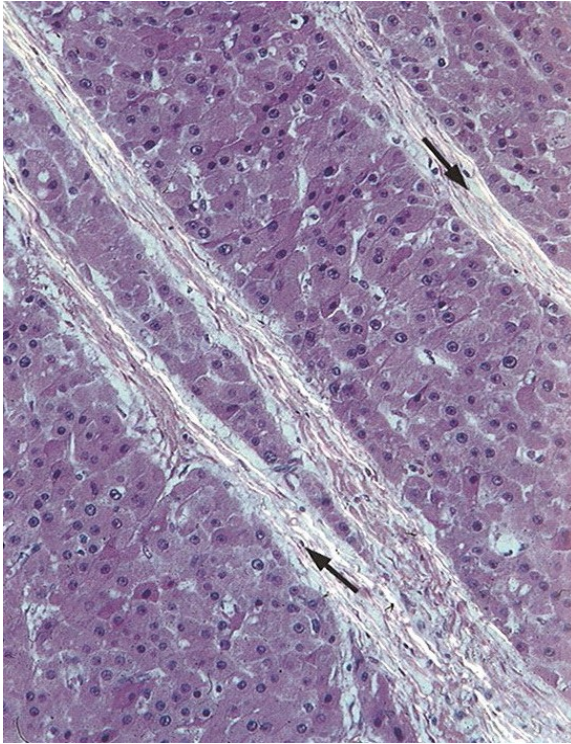


Fig 1.3c: Fibrolamellar type: This rare type occurs in 5% of all cases and hardly develops on the background of underlying cirrhosis or HBV infection. It mostly concerns young patients (90% < 35 years), grows expansively and encapsulates. Thus, it is relatively easy to resect.(15)

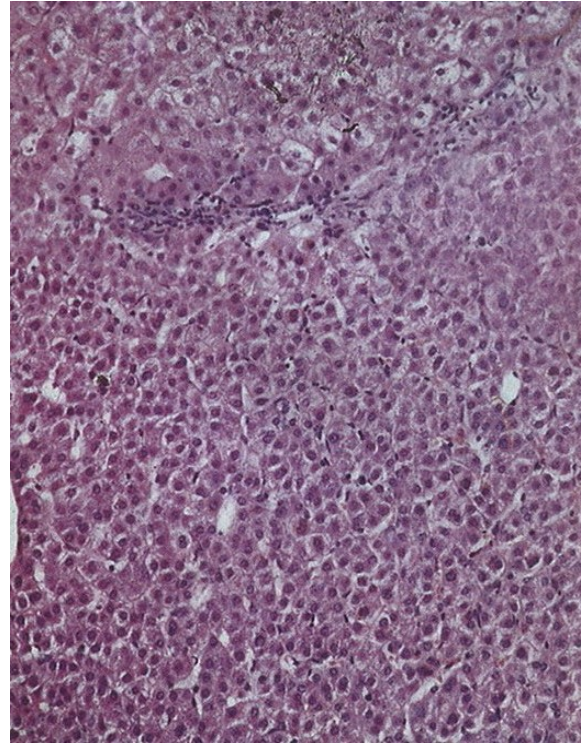


Fig 1.3d: Compact type: This histological variant of HCC consists of multiple layer cell sheets, which are separated from each other through thin septa of connective tissue. Typical hepatic sinusoids are missing.(15)

1.1.6 Metastasis

Due to an aggressive clinical course of HCC, with only 10% of American Joint Committee on Cancer stage I patients surviving 5 years, HCC associated metastasis are generally considered to be rare. Due to improved therapeutic and palliative treatments the survival may be prolonged and metastasis are becoming a relevant factor, especially in developed countries where extensive recourses allow more aggressive and adjuvant treatment options.(19) In most cases of HCC metastasis, it metastasizes to regional lymph nodes, bone and lungs via blood vessels and the lymphatic system. Less frequently metastasis to adrenal glands, stomach, peritoneum, kidney, spleen, heart and brain have been reported. A rare type of spread are metastasis in the small bowel where it is mostly located at the proximal duodenum resulting from direct expansion and invasion from the primary tumour. A lymphatic or hematogenous metastasis to the small bowel is very rare.(20) Lately another rare form of HCC metastasis has been reported, where 3 white native U.S. men developed intracranial metastases and 2 female patients suffered from epidural space metastasis.(19)

1.1.7 Clinical Features

HCC is detected at different stages of the disease and patients show a great variation of symptoms and possible scenarios for the presentation of the tumour.

1.1.7.1 Asymptomatic Presentation

Due to an increasing number of screening programs for cirrhotic patients and improved imaging methods, HCC is often detected at an earlier, asymptomatic, and smaller stage. A study from Italy from 1996 showed, that 23% of 461 cases of HCC were detected at an asymptomatic stage.(21;22)

1.1.7.2 Hepatic Decompensation

Patients with known cirrhosis often become first conspicuous by sudden hepatic decompensation including new onset ascites, recurrent variceal hemorrhage

and/or progressive encephalopathy which should be always an alarm sign for HCC in any differential diagnosis.(22)

1.1.7.3 Gastrointestinal Haemorrhage

In roughly 10% of all HCC cases patients suffer from gastrointestinal bleeding at time of presentation. In relation to malign liver tumours, peptic ulcer disease is with 60% the most common cause of gastrointestinal bleeding, followed by esophageal varices with 40%, which are induced by portal hypertension and portal vein tumour invasion. Occasionally the bleeding is caused by direct tumour invasion into the gastrointestinal system.(22)

1.1.7.4 Classic Triad

This triad which is little regarded in clinical practice, includes right upper quadrant abdominal pain, hepatomegaly and weight loss. In many cases the pain is characterized as a blunt permanent ache that also spreads out towards the shoulder and enhances in progressed stages of the disease when the capsula fibrosa hepatis (Glisson's capsule) is affected. Another common feature of symptomatic HCC is a frequently seen massive hepatomegaly.(22)

1.1.7.5 Tumour Rupture

Hemoperitoneum, caused by a ruptured periphery tumour is a rare (5%) and life-threatening complication of HCC. It may occur spontaneously or with minor abdominal trauma and shows heavy abdominal pain, vascular collapse and signs of peritoneal irritation. To manage the life-threatening situation angiography and embolisation of the bleeding vessel can be an effective method.(22;23)

1.1.7.6 Other Manifestations

Paraneoplastic syndromes and extrahepatic endocrine secretion of biological active substances, synthesized by the tumour, are rare presentations of HCC. Further infrequent manifestations may be massive ascites, which results from hepatic vein spread (Budd-Chiari syndrome)(24) and obstructive jaundice caused by bile

duct compression. Other complications and symptoms of advanced HCC can be symptoms that may be related to and caused by distant metastasis, such as cough and dyspnea, in patients with multiple pulmonary metastases. HCC induced metastasis in bone can result in bone pain and sudden paraplegia when destruction of vertebral column occurs.(22)

HCC associated paraneoplastic syndromes

- Hypoglycemia
- Polycythemia (erythrocytosis)
- Hypercalcemia
- Gynecomastia, Feminization
- Systemic arterial hypertension
- Watery diarrhea syndrome
- Carcinoid syndrome
- Osteoporosis
- Hypertrophic osteoarthropathy
- Thyrotoxicosis
- Hypercholesterolemia
- Thrombophlebitis migrans
- Polymyositis
- Neuropathy
- Cutaneous manifestations

Tab. 1.2 (25)

1.1.8 HCC diagnostics

1.1.8.1 Hepatic Imaging

1.1.8.1.1 Ultrasound

Ultrasound (US) still plays an important role in imaging the liver and detecting HCC, although a controversy, whether US, computed tomography (CT) or magnetic resonance imaging (MRI) is the best modality in tracing and characterizing focal hepatic lesions in patients with chronic hepatic disease, exists. Although some studies have indicated that US is not as accurate as CT, several studies have proven that especially intraoperative US has a higher accuracy than CT in detecting liver lesions.(26) Many factors are responsible for the diagnostic success of HCC surveillance. Among others, diagnostic success depends on the character, the size of the focal liver changes and furthermore on the skills and experience of the examiner. When HCC lesions are still small they appear homogenic and hypoechoic as they do not have a specific morphology on US. As HCC nodes increase and develop central necrosis and microbleeding, they become more and more heterogenic and hyperechogenic. Thus, they become much easier to detect and to characterize. In fact, US sensitivity increases from 70% for lesions of about 1 cm in diameter, towards 90%, for lesions that exceed 5 cm in diameter. CT and/or MRI should always be used to verify any suspect focal hepatic lesion because US is a subjective and a non repetitive method.(6)

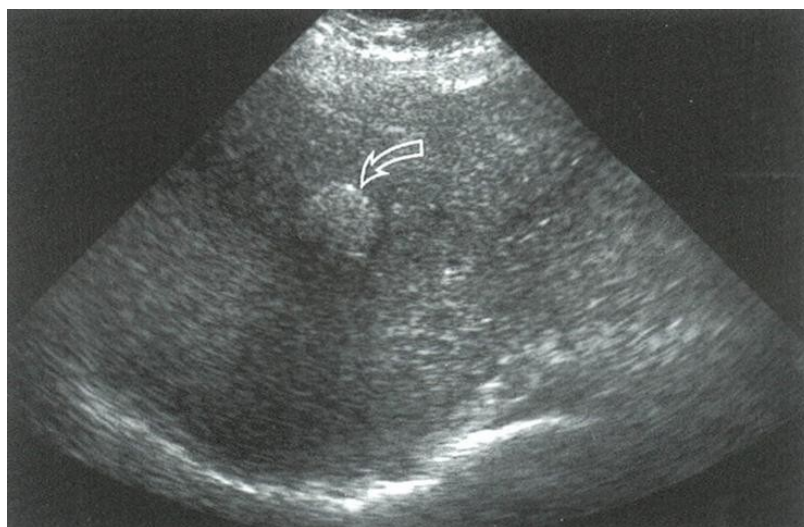


Fig. 1.4: US image of a fat - containing HCC. The arrow indicates an echogenic mass with “acoustic shadowing”.(25)

1.1.8.1.2 Computed Tomography

Computed tomography (CT) is the most frequently used imaging device in diagnosing HCC. Its advantage is widespread availability and short examination time. (27) As different X – ray attenuations between tumour tissue and surrounding liver parenchyma are often not significant, contrast material is used to increase the attenuation of liver tissue. Unenhanced CT scans reveal a large hypodense mass or masses that can demonstrate central spots of lower attenuation, which are results of the tumour necrosis commonly seen in HCC.(28)

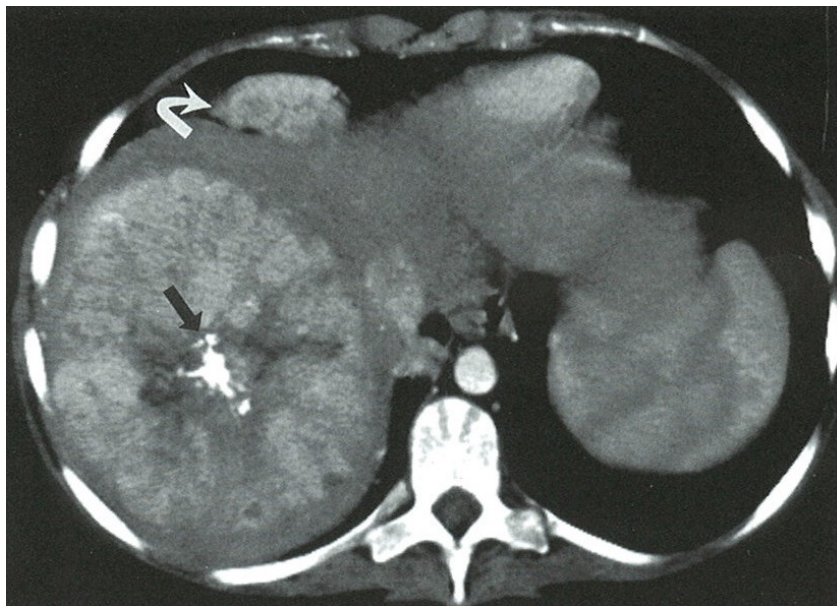


Fig. 1.5a: Contrast enhanced CT scan of a 19 year old patient with fibrolamellar HCC shows a heterogeneous and hypervascular mass in the right lobe of the liver. The black arrow indicates a calcified scar in the centre of the mass. The curved arrow points at a huge cardio-phrenic lymph node, which represents metastasis of the tumour.(25)

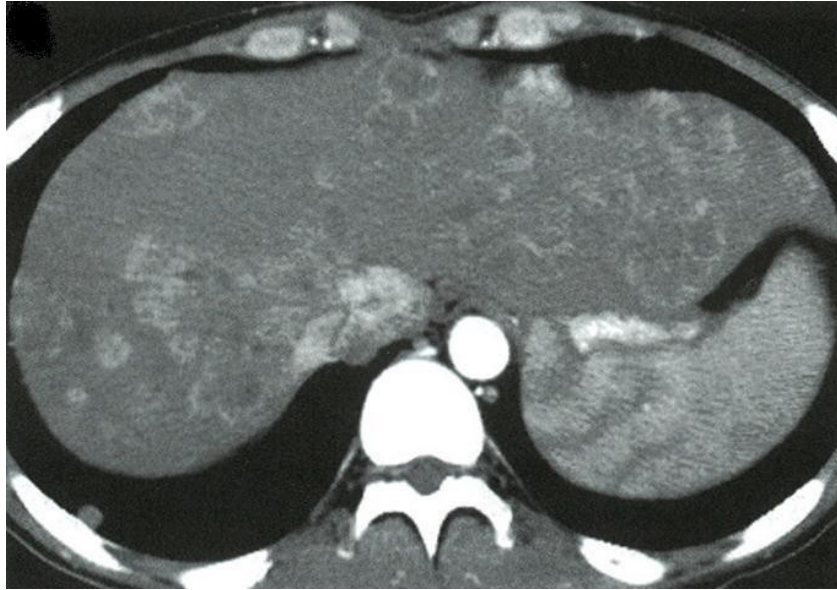


Fig. 1.5b: A multifocal tumour growth throughout the whole liver with several heterogeneous or ring enhanced tumour nodules is shown on this arterial phase CT scan. Hepatic veins and inferior vena cava shows heterogeneous enhancement which suggests tumour invasion.(25)

For an accurate imaging of any hepatic pathology it is important to understand its dual blood supply. The liver receives blood from the hepatic artery and the portal vein. Furthermore the most important liver tumours, like HCC, focal nodular hyperplasia, hepatic adenoma and hypervascular metastasis are supplied by the hepatic artery.(26) HCC enhances early during infusion of contrast agent, in contrast to the liver parenchyma that enhances during the portal venous phase, which happens approximately 50 – 90 seconds after infusing contrast media. A study by Oliver et al.(29) demonstrated that in 9% of patients with HCC the tumour could only be detected on arterial phase imaging and could not be identified on non – enhanced or portal venous phase imaging. To avoid missing small HCCs, it is important that imaging of the cirrhotic liver includes an arterial phase enhanced scan.(28) In fact, a systemic review estimated that the sensitivity of enhanced CT scans in detecting HCC was 68% and specificity was 93% in comparison to pathologic examination.(27)

Despite all technological advances, CT still remains quite insensitive for the detection of HCC lesions smaller than 1 cm in diameter (33 – 45%). It has been shown that numerous benign lesions like hemangiomas or focal confluent fibrosis can

imitate small HCC on CT images. A new invention in CT applications for liver imaging might be volume perfusion imaging. It distinguishes diverse tumour tissue based on its perfusion behaviour. It has been demonstrated that HCCs often show higher perfusion values in comparison to normal liver tissue. Volume perfusion imaging can also reveal a successful treatment, when HCC shows decreased blood volume, blood flow and permeability after chemoembolization or antiangiogenic treatment.(27)

1.1.8.1.3 Magnetic Resonance Imaging

The higher lesion to liver contrast of magnetic resonance imaging (MRI) is a significant advantage over CT. To increase efficiency of detecting and characterizing tumours, new contrast agents and diffusion weighted imaging are being developed.(27) Generally, HCC enhances after application of gadolinium – diethylenetriaminepentaacetic acid (Gd-DTPA). High intensity patterns on T2 – weighted images and low signal intensity in T1 – weighted sequences are distinctive for most tumours.(28) Numerous studies have investigated the accuracy of CT compared to MRI, using the explanted liver as the gold standard and have reported that MRI is slightly better in diagnosing HCC due to improved detection of small lesions of 1 – 2 cm.(27) Despite higher costs and longer examination time, MRI may be useful in patients with renal insufficiency and patients with an allergy against the contrast agent. A further benefit of MRI might be, that in an extremely nodular liver it can distinguish dysplastic nodules from HCC.(28)

1.1.8.2 Serum Markers

1.1.8.2.1 α – Fetoprotein

α – Fetoprotein (AFP) was first mentioned as a useful marker for HCC in 1964. It is a glycoprotein, which has a molecular weight of approximately 70 kDa, is first produced in the endodermal cells of the yolk sac during early fetal development and after that in embryonic hepatocytes. Between the 12 – 16th week of fetal life it reaches a maximum serum concentration of 3 g/L and during the following 18 months the AFP parameter decreases and normalizes. Pathological reappearance is observed when hepatocyte regeneration and/or hepatocarcinogenesis occur.(6)

Elevated AFP values can be seen in about 60% of patients suffering from HCC.(30) Various other liver diseases, like viral hepatitis, liver cirrhosis, hepatoblastoma and metastasis of the liver, are also accompanied by abnormal AFP serum levels. Furthermore it has been shown that AFP serum values correlate with HCC tumour size.(6)

Although it has to be considered that AFP is the “golden standard” for HCC serum markers, the usefulness of testing risk groups should be seriously questioned.(6) It has been reported that the sensitivity of AFP varies widely in HBV – positive and HBV – negative populations. Due to the fact that AFP values also rise in the course of active hepatitis, AFP is not specific for HCC. A direct test of AFP for detecting HCC has demonstrated that the specificity was only 50% in HBV – positive, in comparison to 78% in HBV – negative population. Three studies(31-33) where AFP was used for surveillance, investigated the performance of AFP as a screening test and reported a sensitivity of 39 to 64% and a specificity of 76 to 91%.(34) In fact, the measurement of AFP serum levels is clinically useful in determining the wholeness of tumour resection and in monitoring recidivists. Due to many false – positive and false – negative results AFP does not seem to be a perfect tumour marker. Therefore, numerous alternative tumour markers have been investigated but none of them proved to be more useful than AFP.(35)

1.1.8.2.2 Des-γ-Carboxyprothrombin

An acquired post – translation defect of the prothrombin precursor in HCC cells results in lack of carboxylation of the 10 glutamic - acid ends in the N – terminus, which leads to the emergence of Des-γ-carboxyprothrombin (DCP) (also known as protein induced by vitamin K absence or antagonist II, PIVKA – II), an abnormal and inactive prothrombin.(6) In order to present the usefulness of DCP as an HCC biomarker, Liebman et al. first described an increased DCP level in patients with HCC and in cases of HCC recurrence after surgical treatment. He detected high levels of DCP in 91% of patients (n=76) with biopsy confirmed HCC, in contrast to low levels, detected in patients with chronic active hepatitis or metastatic carcinoma. It was undetectable in healthy subjects.(36) Another study of Nakagawa et al. (37) claimed that the sensitivity of DCP was 48 – 62% and the specificity was

81 – 98%. Due to the fact that AFP and DCP do not correlate, the combination of those serum markers may significantly improve detection of HCC, highlighted by a sensitivity of 74,2% and a specificity of 87,2%.(6)

1.1.8.2.3 α -L-Fucosidase

α -L-Fucosidase (AFU) hydrolyzes sugar containing L – fucose. It is a normal lysosomal enzyme but is over expressed in patients suffering from HCC. It has been proven that AFU serum levels do not show any correlation to tumour size and were commonly seen in early stage HCC. It can be assumed that the sensitivity of AFU in combination with AFP is 82%, which appears to be a valuable serum marker especially in detection of early HCC stages.(6)

1.1.8.2.4 γ -Glutamyl Transferase (GGT)

GGT plays an important role in numerous physiological and pathological processes. During the fetal period it is over expressed by fetal hepatoblasts and later on also when HCC occurs. GGT is a well established serum marker for cholestatic diseases but has little specificity in patients suffering from HCC. Thus, it may be only a supplementary HCC detection marker in combination with other biomarkers, like AFP.(6)

1.1.8.2.5 Transaminases (ALT – AST)

Alanine – aminotransferase (ALT) and aspartate – aminotransferase (AST) are commonly increased in association with HCC and may also have a prognostic factor. A study(38) reported that in 77,4 % of documented HCC cases ALT and/or AST were elevated. Increased transaminases may be a result from direct release of transaminases from hepatocytes, which are destructed by the tumour and may indicate progressing tumour growth.(38) Measuring the activity of ALT is a well established method in determining the extent of necrosis and inflammatory processes in liver diseases. HCC occurs in HCV carriers with normal ALT levels as well as in patients with increased ALT levels. A study by Tarao et al. in 1999(39;40) revealed that 81,8% of the patients who developed HCV induced HCC had persistently increased ALT values. To prevent hepatic carcinogenesis,

maintenance of low inflammatory processes may play an important role in patients with chronic inflammatory liver diseases.(41)

1.1.8.2.6 Bilirubin

High serum bilirubin levels in cases of HCC are not uncommon. Elevated concentrations of bilirubin were reported in up to 40% of patients suffering from HCC.(42) Jaundice, which is associated with increased serum bilirubin levels, has been reported in 19% to 40% of patients suffering from HCC. Massive destruction of liver parenchyma and underlying liver cirrhosis are frequent reasons for HCC related jaundice. Occurrence of tumour thrombi can result in rare cases of obstructive jaundice.(43)

1.1.9 Scoring Systems

1.1.9.1 Child – Pugh Score

Child – Pugh score (CP score) is a well established simple prognostic marker and staging score for patients suffering from cirrhosis of the liver. The CP score ranges from 5 to 15 points and considers 5 criteria, including prolongation of prothrombin time, serum bilirubin level, serum albumin level and the presence and severity of ascites and hepatic encephalopathy. Patients are classified into 3 classes. (Class A 5-6, Class B 7-9, Class C 10-15)(44)

1.1.9.2 MELD – Score

The model of end stage liver disease (MELD) score primordially was invented as a more accurate prognosis tool for patients suffering from decompensated cirrhosis and therefore to simplify the distribution of donor organs depending on the priority. The calculation is based on serum bilirubin, serum creatinine and prothrombin time whereas the prothrombin time is measured based on international normalized ratio (INR). The MELD score ranges from 6 – 40.(44)

1.2 Therapy options for patients suffering from HCC

Treatment options of HCC are selected according to empirical criteria, like number and sizes of tumours, the status of hepatic destruction and whether the disease occurs in a cirrhotic or non cirrhotic liver. It has been shown that the median survival rate from time of diagnosis until death in untreated HCC patients approximately amounts six months.(45) Surgical resection of the liver still is the primary therapy option for patients suffering from HCC, where the tumour is surrounded by regular liver tissue and liver function is still in good condition. Patients who are not qualified for simple resection often undergo orthotopic liver transplantation (OLT). According to the Milan Criteria(46), OLT is the best treatment option for cirrhotic patients with a tumour of less than 5cm in diameter, patients with three tumours each less than 3cm in diameter and less than 60 – 65 years of age. Patients who are matching the Milan criteria but still are inappropriate for OLT or resection can be guided to other treatment options, including tumour thermoablation with radiofrequency, microwaves or laser and tumour injection with absolute ethanol, 50% acetic acid or hot saline. Transcatheter arterial chemoembolisation is another treatment modality for HCC patients incapable of radical therapies but who still have a preserved liver function and no vascular invasion. Application of systemic chemotherapy is an ineffective method for HCC, probably resulting from an over-expression of multidrug resistance genes.(46)

1.2.1 Hepatic Resection

Hepatic resection (HR) is the main part of surgical treatment of HCC. Although it has been proven that patients who undergo OLT have superior outcomes, it is not realizable because the availability of hepatic allografts is limited by the lack of sufficient number of human donor organs. Preoperative evaluation always should include exact appreciation of hepatic reserves, tumour size and tumour distribution. Furthermore it is important to perform hepatic imaging like CT, US, MRI and angiography to get accurate anatomical information about inflow vessels like the hepatic artery, portal vein and intrahepatic vasculature as it relates to the tumour. Further important investigations concern evaluation of viral serological markers HBV, HCV and tumour markers, such as AFP, AFP subtype lectin 3 and protein

induced by vitamin K absence or antagonist (PIVKA). Preoperative patient selection consists of CP score, indocyanine clearance test (ICG R15) and retention rate at 15 minutes.(47) ICG is a sensitive indicator to evaluate liver function.(48) Patients who undergo hepatic resection should have an ICG R15 of less than 35% and should be in CP class A or B. (47)

The strategy for hepatic resection should consider the preservation of hepatic parenchyma to prevent postoperative hepatic failure. The use of anatomical segmental or subsegmental resection and the use of limited resection for small HCCs should be regarded as well. To avoid tumour cell dislodgement and spread, an occlusion of inflow vessels before hepatic mobilisation is recommended. It has been reported that tumour cells have been found in the portal vein in 23% of patients with a tumour larger than 5 cm in diameter.(47;49) To determine and confirm the size and location of the tumour(s), for identifying their relation to intrahepatic vasculature,US should be performed during surgery. Due to the fact that residual liver size is an important parameter of early outcome, patients who undergo extensive hepatic resection can benefit from preoperative portal vein embolisation to induce a compensatory hypertrophy of the unaffected liver. To minimize gastrointestinal bleeding in cirrhotic patients, a gastrointestinal endoscopy should be performed to evaluate esophagogastric shunts before hepatic resection. Preoperative transarterial chemotherapy does not seem to improve the outcome of patients after HR.(47)

Perioperative management includes broad – spectrum antibiotics to avoid infectious complications, which are not uncommon after liver resection. Uncontrolled infections in patients with cirrhosis often lead to hepatic failure. When any signs of infections have been developed in the postoperative course, contrast enhanced CT should be performed in order to exclude abscesses.(47)

Complications after hepatic resection include among other gastrointestinal bleeding, intraperitoneal hemorrhage, atelectasis, ascites, pleural effusion, wound infection, septic complications like subphrenic abscesses and liver failure. Due to excessive resection and infectious or hemorrhagic complications, liver failure is the most common cause of death after hepatic resection.(47)

1.2.2 Liver Transplantation

Orthotopic liver transplantation (OLT) provides an opportunity to cure the underlying disease and to remove all tumours within the liver. It has been shown that patients who suffer from severe cirrhosis and/or portal hypertension frequently do not tolerate specific therapies against the tumour itself and that a diseased liver parenchyma creates a major risk of developing new malignant lesions. Therefore OLT appears to be an optimal treatment because it removes all HCC tumours at once and treats the underlying hepatic disease as well.(50)

To evaluate patients suitable for OLT, candidates have to undergo arterial and venous phase CT scans to rule out any metastatic disease and they have to fit into the pattern of the Milan criteria.(50;51) If a spread of the tumour is found, patients are inappropriate for OLT. If there is any doubt about the diagnosis, it can be confirmed by open, percutaneous or laparoscopic liver biopsy. An eventual bland thrombus of the portal or hepatic vein has to be distinguished from a tumour thrombus. OLT should not be performed on those patients due to rapid recurrence and fatality resulting from HCC, which is often seen during the first year after transplantation. To differentiate between bland or venous thrombosis, CT can be performed because in contrast to bland thrombosis, malignant thrombosis frequently enhance during arterial phase imaging. The main disadvantage of OLT is the shortage of proper organs that causes long waiting times and therefore many patients become unsuitable because of an advanced disease.(50)

1.2.3 Radiofrequency Ablation

Radiofrequency Ablation (RFA) is a localized thermal application that uses radiofrequency energy to produce thermal tissue destruction by heating tumour tissue to temperatures that exceed 100°C. During RFA, a high – frequency alternating current is moving from the tip of a radiofrequency needle electrode into the surrounding tissue. The movement of the ions, which try to follow the direction of the current, results in frictional heating of the tissue. When tumour cells are heated beyond 45° to 50°C, lipid bilayers start to dissolve and melt. Intracellular proteins are denatured and cell membranes are destroyed. Local tissue temperatures that ex-

ceed 100°C produce an area of coagulative necrosis of tumour tissue and adjacent liver parenchyma. The definite size of the area that is heat ablated is proportional to the square of the radiofrequency current. With increasing distance from the electrode the tissue temperature decreases rapidly because the power that is delivered by a monopolar electrode falls rapidly in proportion to the square of the distance to the electrode.(52)

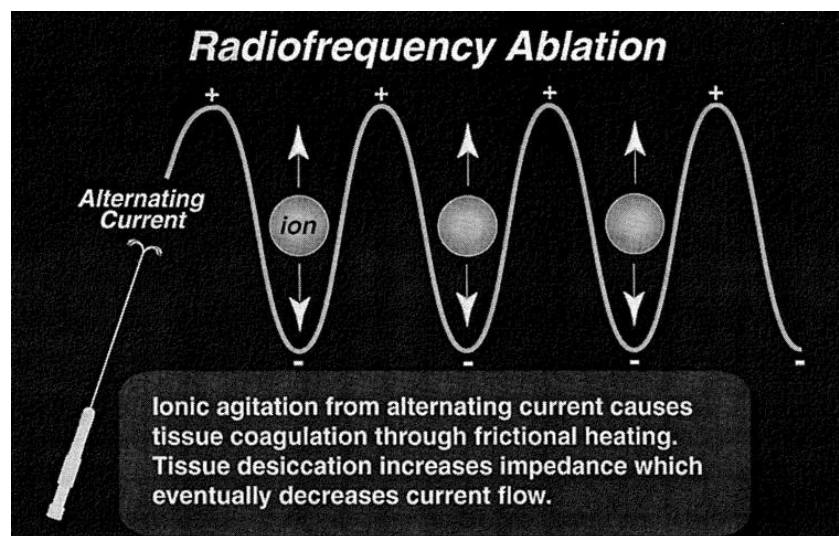


Fig. 1.6: An electrical current moves from the tip of the electrode into surrounding tissue and causes ionic agitation and heating.(52)

RFA is reserved for patients that do not tolerate a required major hepatic resection, patients who are not suited for OLT and patients with unresectable tumours due to tumour size, number, location and inadequate hepatic reserve. It can be further used in patients waiting for OLT in whom a small HCC develops.(53) Due to the fact that patients subsequently develop clinically traceable hepatic or extrahepatic tumour recurrence from their coexistent micrometastatic disease, RFA should only be performed in patients with no evidence of any extrahepatic malignant disease. Furthermore, RFA is not suitable for patients with more than three or four tumours and patients with discrete tumours that exceed these numbers. In fact, absolute contraindications to RFA are any evidence of extrahepatic disease, altered mental status, life expectancy of less than 6 months, active infection and if a tumour is adjoining a major hepatic duct.(52;53)

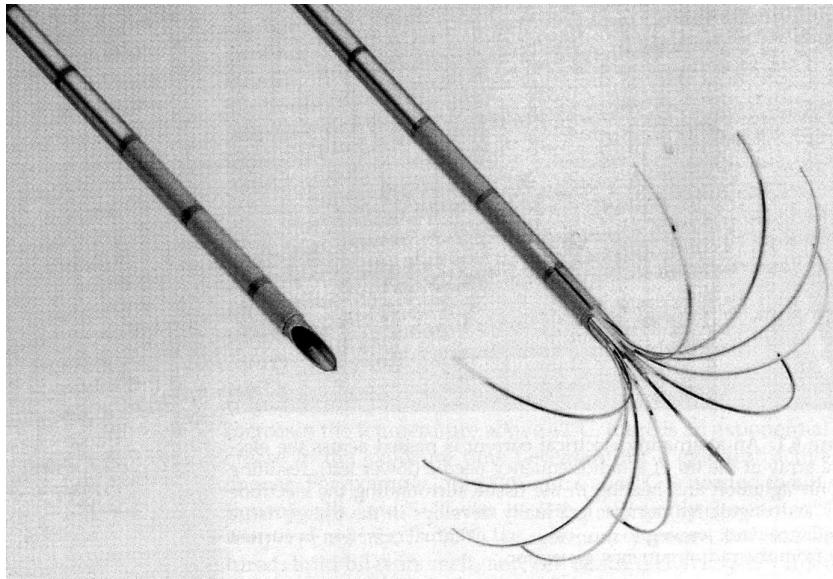


Fig. 1.7: RFA needle electrode (left) pictured with the multiple array retracted into the needle sheath. RFA needle electrode (right) with fully extended multiple array to ablate larger areas.(52)

In general, RFA of hepatic tumours can be performed percutaneously using US or laparoscopic guidance or as a part of an open surgical procedure. The heating of the tissue near the liver capsule causes pain and therefore monitored sedation or general anaesthesia often becomes necessary. When a laparoscopic approach is chosen, a laparoscopic US can be performed, which provides a better visual resolution to determine the location and numbers of lesions. Another advantage is the possibility to explore the peritoneal cavity and to exclude any extrahepatic tumour spread. Furthermore, laparoscopic US provides more exact positioning of the RFA electrode near major intrahepatic blood vessels.(52)

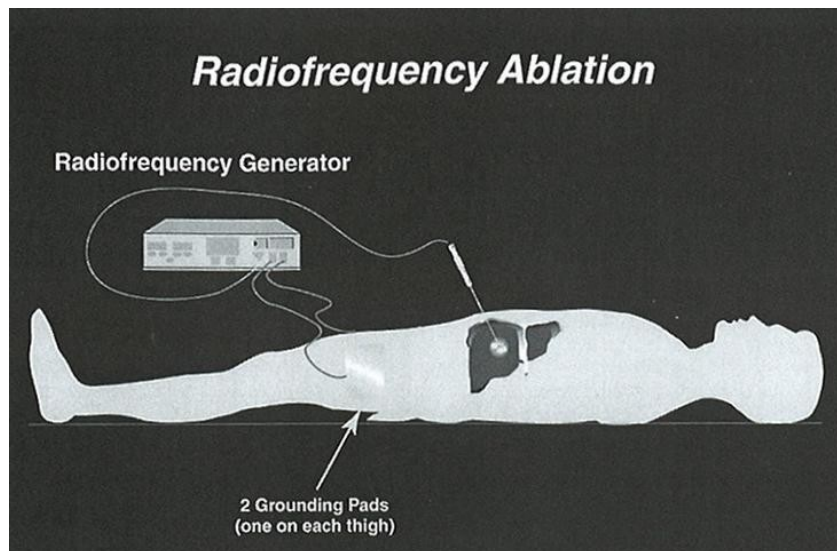


Fig. 1.8: A RFA needle electrode with the multiple array is deployed in a liver lesion. The alternating RFA current moves between the electrode and the grounding pads, which are attached onto the patient. Frictional heating, which is caused by the ionic stimulation, results in coagulative necrosis of the ablated area.(52)

Various RFA associated complications are haemorrhage, biliary stricture or leak, subcapsular haematoma, liver dysfunction and abscess formation. Further complications include pleural effusion, fever, pain, subcutaneous haematoma and ventricular fibrillation.(52;53)

1.2.4 Cryoablation

Cryoablation is another locoregional modality for treating patients with HCC who are inappropriate for HR or OLT due to number and size of lesions, unresectable locations or liver failure. This thermal ablation technique, based on insitu freezing and devitalisation of tissues, destroys target lesions and a proper margin of adjacent normal liver parenchyma. Cryoablation can be applied and controlled exactly to create a predictable area of necrosis.(54) Common cryogen agents for hepatic tumour ablation are liquid nitrogen and argon. Segmentally insulated probes are placed into the tumour, deliver argon gas that rapidly expands and results in fast cooling within seconds (-100°C). Cryoablation is a useful treatment modality when the tumour is adjoining critical anatomical structures or occurrence of the heat

sinking effect, like vicinity to the inferior vena cava is likely. A solitary cryoprobe can create an ice ball of about 3 cm in diameter. Lesions of bigger extent can be treated by application of multiple probes, which can create an ice ball exceeding 8 cm in diameter. CT and MRI are adequate modalities to picture the area of ablation.(55)

1.2.5 Transcatheter Arterial Chemoembolisation

Transcatheter arterial chemoembolisation (TACE) is a popular but controversial treatment option that uses high dose chemotherapy and embolization agents, which are directly injected into the tumour's blood supply. Patients who do not meet the criteria for HR or OLT, who are no candidates for RFA, those with bilobular disease, who suffer from recurrence after OLT and those who did not respond to systemic chemotherapy are often offered this treatment.(56) Furthermore, TACE is appropriate in cases with no evidence of portal tumour thrombus or arterioportal/arteriovenous shunts at the target. Unfortunately, a total response is unusual because sometimes viable malignant cells remain after TACE due to intra – or extracapsular invasion.(57)

To minimize the risk of nontarget organ embolization a precise understanding of visceral arterial anatomy is necessary. It has been reported that 42 % of patients show variation in their hepatic arterial anatomy. Several complications concerning TACE can occur. These include among others, arterial dissection or occlusion, contrast reaction, haematoma, accidental chemoembolized of adjoining organs and hepatic infarction.(56)

1.3 HCC Animal Models

Over the years of studying HCC, various animal models have been developed in order to gain more information on the pathogenesis of this widespread disease as well as in order to be able to test new therapy strategies. Several species have been used to create HCC models, including mice, rats, rabbits, pigs, dogs, cats, tree shrews and woodchucks. For a particular question it is important to choose the most appropriate model.(58) Animal models that are closely similar to human conditions, in relation to physiology, clinical setting and etiology help to improve the treatment of HCC. They can be used as a replacement for a human biological system to study normal and pathological functions. Furthermore, animal models are used to perform preventive or therapeutic interventions in human diseases.(59)

Animals develop HCC in natural circumstances and after artificial treatment. Spontaneous HCC models most closely resemble human liver cancer in terms of antigenicity, cell kinetics, growth patterns and differentiation. The facts that carcinogenesis reflects the accumulation of environmental carcinogens and that the extent of cancer susceptibility in natural conditions resembles clinical human liver cancer, are major advantages of spontaneous HCC models. Artificially induced hepatocyte carcinogenesis is frequently triggered by chemical agents like carbon tetrachloride, diethylnitrosamine (DEN), 2-acetylaminofluorene, N-nitrosodimethylamine, arsenic, o-aminoaztoluene, N-nitrosomorpholine and 1,2-dichloroethane. The most commonly used application modalities are food, drinking water, intraperitoneal or subcutaneous injections and inhaled gases.(58)

1.3.1 HCC in Pigs

Pigs provide a rather close model to humans because a pig model mimics physiology, molecular biology and liver anatomy. Porcine animal models act as a well established human scale device because they are quite similar to human size, which provides better opportunities of surgical and interventional manipulations than small animal models.(59;60) Recently two methods have been reported to induce HCC in pigs.(59)

Li et al. in 2006 (60) created a drug induced HCC model, where liver multicentric HCC with cirrhosis was induced in China Taihu pigs. Once a week, intraperitoneal injection of 10 mg/kg of N – nitrosodiethylamin was performed for 3 months. After that, N – nitrosodiethylamin treatment was interrupted for the next 10 – 12 months. CT scans revealed that the animals developed multiple encapsulated tumour nodules of various sizes. CT angiography and digital subtraction angiography proved that the tumours primarily received their blood supply via the hepatic artery, which is usual for HCC. Confirmation that the tumours were of hepatocytic origin was delivered by immunohistochemistry.(60)

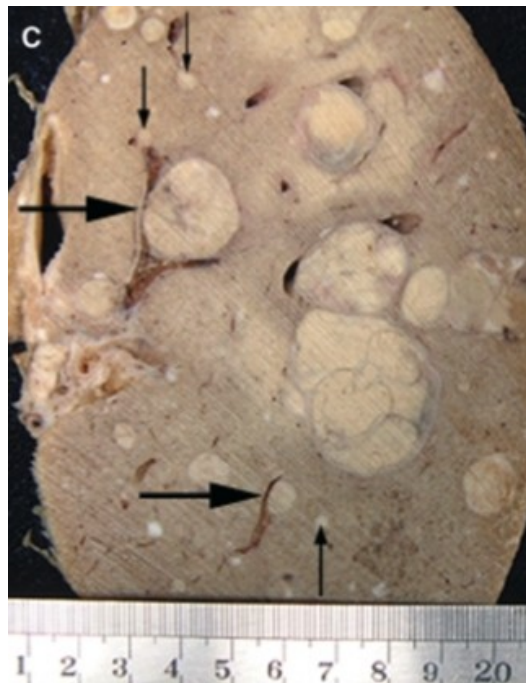


Fig. 1.9: This picture of Li et al. DENA induced tumour model shows multiple encapsulated nodules of various sizes. Thick arrows indicate invaded and distorted intrahepatic vessels.(60)

Another tumour pig model creates a defined tumour lesion in the isogenic host animal. Normal fibroblasts from an ear notch cartilage are changed with diverse mutations. The altered cells are returned to the original source animal which is treated with mild immune suppression to force tumour growth. This modality can rapidly and genetically induce tumours of sizes resembling to those which are treated in humans. This method has the advantage that it creates a genetically defined tumour.(59;61)

1.3.2 HCC in Mice

Due to several reasons mice have been the most popular animals for animal model cancer studies. Experiments with mice are cheap and due to the small size the handling is well manageable. Although certain advantages exist, it has been shown that mice are unsuitable for understanding molecular mechanisms and signal transduction pathways. Furthermore, it has been proved that the application of visualization techniques, like US for percutaneous advices is quite unrealistic. The most frequently used modalities for inducing HCC in mice are models with immune – suppressed strains with xenograft and drug induced models.(59)

1.3.3 HCC in Rats

HCC animal models in rats have been created by continuously feeding rats with a diet that lacks choline and methionine. It has been reported that in this case rats develop HCC without any exogenous carcinogen. Chemically induced carcinogenesis has been shown to be a popular procedure to create HCC animal models in rats. Attempts to create valuable HCC models by implantation of carcinogens have shown very little success.(59) A study by Al-Rejaie et al. in 2009 reports HCC carcinogenesis in adult male Wistar albino rats induced by a single dose of intraperitoneally injected diethylnitrosamin (DENA).(62)

1.3.4 HCC in Woodchucks

In 1950 it was first mentioned, that woodchucks who suffer from woodchuck hepatitis virus (WHV) frequently develop hepatomas. Due to the fact that WHV resembles HBV in humans, it might be useful to carry out mechanistic and preventive studies on a woodchuck HCC model. Unfortunately it takes about 2 – 4 years before tumours develop and woodchucks typically do not develop cirrhosis. To consider these animals as a valuable HCC animal model, several improvements have to be made, including establishing a background of cirrhosis and abridging time of development.(59)

1.4 Diethylnitrosamin

Diethylnitrosamin (DENA), also called N – nitrosodiethylamin (NDEA), is a strong carcinogen, which can induce various liver lesions in animal cancer models.(60;63) It has also been detected in tobacco smoke, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics, pharmaceutical products and numerous alcoholic beverages.(62;63) It has been proved, that DENA is a hazardous material for humans and can create various tumours in all animal species.(63)

1.4.1 Metabolism of DENA

After application of DENA it is primarily metabolized in the liver through cytochrome P – 450 to ethyl – acetoxyethyl – nitrosamine, which is an α – hydroxy derivative. Either this intermediate is now conjugated to a non – poisonous combination or it might spontaneously produce an ethyl – diazonium ion, which is primarily responsible for the major biological effects of DENA. The ethyl – diazonium ion ethylates cellular macromolecules and reacts with nucleophilic sites, which produces adducts in the DNA. Cell repair mechanisms are not able to repair the DENA induced DNA damage. The magnitude of carcinogenesis is related to the extent of DNA adduct formation.(60) Furthermore, it has been reported that DENA seriously involves cell cycle regulatory proteins in terms of initiation and promotion of carcinogenesis.(64)

1.4.2 DENA induced Oxidative Stress

The influence of DENA on an organism induces oxidative stress through the formation of reactive oxygen species (ROS). This oxidative stress causes damage of DNA, proteins and lipids. It mutates important sequences of intracellular signaling pathways and gene expression. Important carcinogenic effects caused by the application of DENA also provide the formation of 8 – hydroxydeoxyguanosine, structural and functional membrane modifications, protein oxidation and other carcinogenic products of oxidation, including acrolein, crotonaldehyde and malondialdehyde. Added up, all modifications which are caused by oxidative stress after the

administration of DENA act as promoters of abnormal cell growth and carcinogenesis.(63)

2 AIM OF THE STUDY

Animal models are useful to investigate new therapy options and to improve already existing therapy modalities of various diseases. Many research groups created numerous more or less reasonable HCC animal models. Pigs represent adequate human scale models, as they have biological conditions similar to those of humans and a proper size to test various interventional treatments for HCC. Thus, we hypothesize that pigs are the most appropriate animals in terms of HCC research.

Based on a study by Li et al(60) from 2006, where they used 6 China Taihu piglets, we tried to create and establish a resembling model in conventional domestic pigs.

The goal of our study was to induce a HCC in pigs by intraperitoneal injections of DENA once a week for an undefined period. In contrast to Li et al where DENA was administered for a determined time of 3 months once a week, followed by 10 – 12 months without DENA treatment, we applied DENA weekly as long as CT scans revealed serious changes in terms of HCC development. Blood samples which were collected every second week should monitor changes in terms of liver functions and general health status of the animals.

Furthermore, we increased the dose of DENA from 10 mg/kg to 15 mg/kg. By courtesy of continuous administration of the toxic agent and augmentation of the dose, we intended to shorten the time of HCC development, which has been a major disadvantage in the study by Li et al.

3 MATERIALS AND METHODS

3.1 Overview

The study took place from January to December 2009. All experiments and additional investigations were carried out at the Medical University of Graz. Pigs were kept in the stables at the Institute for Biomedical Research and were transferred to the operation room of the department for surgical research for surgical procedures or to the pediatric clinic for CT scans. The experiment was part of the IMPPACT project and received funding from the European Community's Seventh Framework Programme under grant agreement n° 223877. The study was carried out according to the guidelines of the Austrian animal protection law.

3.2 Animals

This study was performed on 6 domestic pigs (3 female, 3 male) which were purchased from pig breeders from the surroundings. They were kept according to their size either in single or double boxes, were fed twice a day with the usual diet and had continuous access to water. When the experiment started, the animals had an average weight of $33,3 \pm 3,2$ kg and they gained weight as fast as a normal pig without any treatment is expected to.

3.3 Sedation for DENA Treatment and Blood Sampling

Animals were sedated for the administration of DENA and for blood sampling. A short general anaesthesia was maintained by application of 1 mg/kg midazolam (ERWO Pharma GmbH, Brunn am Gebirge, Austria) and 10 mg/kg ketamin (Ketasol®, aniMedica GmbH, Senden – Bösensell, Germany) into the neck musculature. Depending on the weight of the animals, sedative substances were poured into 10 or 20 ml single use syringes (Braun, Melsungen AG, Melsungen, Germany) which were connected to an extending hose to maintain a sufficient range for ad-

ministration. On the other side the hose was connected to a disposable needle (Sterican®, Braun Melsungen AG, Melsungen, Germany). After completing blood sampling and administration of DENA, pigs were kept in separate boxes until they woke up from narcosis. Due to different effects and durations of the narcosis, this was necessary to avoid any kind of cannibalism among the pigs, especially in cases where a single animal is defenseless.

3.4 Administration of DENA

Adapted to the literature we administered a single dose of DENA (Sigma Aldrich®, USA) once a week. Each animal received 15 mg/kg DENA per week. Under the guidance of a professional veterinarian, DENA was injected intraperitoneally into sedated pigs. Before this, DENA had been kept cool and protected from light. Due to the immense toxicity of DENA, any open processing with the agent was performed under a fume hood and with chemical resistant gloves (HSO - NITRILE®, HSO Health Care GmbH, Wr. Neudorf, Austria). DENA was poured into 1 ml single use syringes (Primo®, Codan Medical ApS, Rodby, Denmark) or 2ml single use syringes (Braun, Melsungen AG, Melsungen, Germany), which were connected to disposable hypodermic needles (Sterican®, Braun Melsungen AG, Melsungen, Germany). To avoid puncturing of any lower abdominal organs, needle sizes were adapted to the thickness of the rind. After the sedation of the animals, they were placed on their left side to achieve a proper position of the mesenterium and the intestines.

To avoid contamination with DENA and putting human life at risk, security measures were observed. Before injecting DENA into a pig, the examiner had to put on a face mask, protective goggles, chemical-resistant gloves and a safety apron.

Before injecting DENA, the spot on the skin most appropriate for intraperitoneal injection was intensively disinfected several times with alcohol. The most appropriate area for needle insertion is located in the right lower abdominal region lateral of the second and third teat. To achieve a proper angle, the needle tip was pointed at the opposite shoulder. (left shoulder) After aspiration in order to ensure

that the tip of the needle is not located in the intestines or the bladder, DENA was injected.

3.5 Blood Samples

Every second week blood samples were taken to monitor changes in liver function and general health conditions of the animals. Whether the animals were equipped with a Port – a – Cath (PAC) system or not, blood sampling was performed through puncture of the PAC or direct approach via the vena jugularis. Both methods required a short general anaesthesia to guarantee a safe handling.

For surveillance of liver functions and general health conditions, 2 tubes per pig were sampled every second week. One EDTA tube (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria) was collected to determine a general blood count and one lithium heparin plasma gel tube (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria) was collected to detect any changes in liver functions.

EDTA tube	Lithium heparin tube
Leucocytes	Sodium
Erythrocytes	Potassium
Haemoglobin	Calcium
	Phosphate
	Creatinine
	Urea
	Bilirubin
	AP
	GGT
	CHE
	AST
	ALT
	LDH
	P - Amylase
	Lipase

Tab. 3.1: Blood parameters associated to the particular blood tube.

3.5.1 Blood Sampling via Port – a – Cath System

One pig in this study was equipped with a PAC for collecting blood and for the administration of sedative substances during CT scans. The sedated animals were placed PAC side up to ensure a good approach. Furthermore the PAC area was inspected for any sign of infection and was cleaned and thoroughly disinfected with alcohol. After disinfection, the PAC was punctured with a Gripper PAC needle (Baxter, Halle, Germany). A single use syringe (Braun, Melsungen AG, Melsungen, Germany) was connected to the Gripper needle and at least 5 ml of blood and heparin solution were aspirated and then disposed of. Then, the Gripper needle connection was connected to a Luer adapter (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria), which was screwed into a standard tube holder (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria). Furthermore, tubes were put into the tube holder to be filled with blood. After blood sampling the PAC was blocked with 3ml of 100 IE/ml heparin solution (EBEWE Pharma Ges.m.b.H Nfg. KG, Unterach, Austria) to avoid obstruction of the system.

3.5.2 Blood Sampling via Jugular Vein

Animals without a PAC system were blood sampled through direct puncture of the vena jugularis. Sedated pigs were placed on their backs and an assistance investigator pulled the front legs in caudal direction to achieve a suitable and firm approach for the procedure. The area for puncture was thoroughly disinfected with alcohol. To determine the appropriate spot, an imagined line between the manubrium sterni and a shoulder articulation was halved. A single use syringe (Braun, Melsungen AG, Melsungen, Germany) with 5 ml 9% saline solution to simplify aspiration was attached to a Strauss cannula (Dispomed Witt oHG, Gelnhausen, Germany) and the skin was punctured. While continuous aspiration the cannula was inserted in mediocaudal direction until the vena jugularis was punctured. Furthermore, the syringe was removed and replaced by a Luer adapter (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria) which was screwed into a standard tube holder (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria). Afterwards blood tubes were mounted carefully onto the standard tube holder one

by one and filled with blood. Then the Strauss cannula was removed and the vessel was compressed for a few minutes to break the haemorrhage.

3.6 Implantation of Port – a – Cath System

In order to simplify blood sampling a Port – a – Cath system (PAC) was implanted into one animal. Still in the transporting box, the pig was premedicated with 1 mg/kg midazolam (ERWO Pharma GmbH, Brunn am Gebirge, Austria), 10 mg/kg ketamin (Ketasol®, aniMedica GmbH, Senden – Bösensell, Germany) and 2 mg/kg azaperon (Stresnil®, Janssen – Cilag Pharma, Vienna, Austria) which were injected into neck musculature. To infuse saline solution for managing the fluid balance an i.v. cannula (TriCath®, CODAN Medizinische Geräte GmbH, Lensahn, Germany) was installed into an ear vein. Further, the swine was placed on its left side onto the operating table, whereas the PAC set was implanted into the right retroauricular region. To maintain a sufficient respiration and narcosis, the animal was intubated with a 8,0 spiral tubus (Willy Rüschi GmbH, Kernen, Germany) and ventilated by a narcosis device (Sulla® 808v, Dräger Medical, Best, Netherlands) with oxygen and 1,8 – 2% sevofluran (Sevorane®, Abbott AG, Baar, Switzerland). To ensure an adequate analgesia during the procedure 30 µg/kg fentanyl (Janssen – Cilag Pharma, Vienna, Austria) per hour was applied through the ear vein by a syringe pump (Perfusor® F, Braun Melsungen AG, Melsungen, Germany).

After sterile ablation of the operation area and sterile covering of adjoining areas two incisions were made to implant the PAC system. At first, at the ventral side of the neck, the vena jugularis dextra was explored to create an approach for the PAC tube. Then, at a retroauricular spot, a subcutaneous pocket was created to install the PAC chamber. The distance (approximately 20 cm) from the PAC chamber to the vena jugularis dextra was tunneled and the PAC tube was pulled through that tunnel. On the one side the tube was inserted into the vena jugularis dextra and on the other side the tube was connected to the PAC chamber. After a thorough test of the installed PAC with 9% saline solution, wounds were cleaned and sewed. Finally the PAC was blocked with 3 ml of 100 IE/ml heparin solution. To avoid any infectious complications after the implantation, the animal received

550 mg amoxicillin/clavulanacid (Augmentin®, GlaxoSmithKline Pharma, Vienna, Austria) intravenously before and after surgery. Furthermore, an intramuscular injection of 5 ml benzylpenicillin – procain (Peni – Strepto®, Virbac Laboratoires, Carros, France) was administered on the same day. In terms of a further oral prophylactic antibiotic treatment the swine received 20 mg/kg amoxicillin (Amoxi – Mix®, AniMed Service AG, Dobl, Austria) for the following 4 days.

3.7 Computed Tomography

In order to investigate and monitor the state of the liver a CT scan was performed on each pig every 2 – 3 months. Pigs were transferred to the operation room of the facility for surgical research for premedication and intubation. Animals were pre-medicated with 1 mg/kg midazolam (ERWO Pharma GmbH, Brunn am Gebirge, Austria), 10 mg/kg ketamin (Ketasol®, aniMedica GmbH, Senden – Bösensell, Germany) and 2 mg/kg azaperon (Stresnil®, Janssen – Cilag Pharma, Vienna, Austria). To maintain a continuous general anaesthesia an i.v. cannula (TriCath®, CODAN Medizinische Geräte GmbH, Lensahn, Germany) was installed into an ear vein. Animals were intubated with a 8,0 spiral tubus (Willy Rüscher GmbH, Kernen, Germany) and ventilated with an mobile ventilator (Oxylog® 2000, Dräger Medical, Best, Netherlands). A continuous sedation during the whole procedure was maintained by a sustainable application of 5 – 10 mg/kg/h propofol (Diprivan®, Astra Zeneca, Vienna, Austria) through a syringe pump (Perfusor® F, Braun Melsungen AG, Melsungen, Germany).

CT scans were performed at the pediatric clinic of the Medical University of Graz using a dynamic volume computed tomography scanner (Aquilion ONE®, Toshiba Medical Systems, Japan). By switching of the ventilator, an artificial apnoea was induced before the contrast agent 2,5 ml/kg Iopamidol (Jopamiro® 300mg, Bracco Austria, Vienna, Austria) was injected through the ear vein and the scan was started. Each pig underwent an arterial phase, a portal venous phase and a hepatic venous phase contrast enhanced CT scan. CT images were processed and interpreted at a 3D workstation (Vitrea®, Toshiba Medical Systems, Japan)

3.8 Sacrification

As CT scans revealed changes in liver morphology in terms of HCC development animals were euthanized through an intravenous injection of 100 mg/kg phenobarbital (Nembutal®, Sanofi Ceva, Düsseldorf, Germany) by a professional veterinarian. A median laparotomy was performed to explore and to remove the liver. In addition, a thorough investigation of adjoining organs was performed to investigate possible extrahepatic tumor spread or other changes that could have been related to the preceding long period poisoning treatment. The liver was removed and dissected to detect regions of HCC development which were trimmed into small tissue blocks to fit into tissue cassettes.

3.9 Histological Processing – Paraffin Sections

All tissue blocks were fixed in 4% formalin solution (Sigma Aldrich, Vienna, Austria) for 24 hours. Afterwards, tissue blocks were dehydrated in a Tissue – Tek® VIP tissue processor (Sakura Finetek Germany GmbH, Staufen, Germany). Next, dehydrated tissue blocks were paraffin embedded (60°C melting temperature) with an embedding console system (Tissue - Tek® tec 5, Sakura Finetek Germany GmbH, Staufen, Germany). Then, paraffin tissue blocks were sliced to 1,5 µm sections with a motorized rotary microtome HM 360 (Microm GmbH, Wallendorf, Germany). Before paraffin sections were transferred to microscope slides (26x76x1 Menzel GmbH & Co KG, Braunschweig, Germany) they were stretched in a water bath.

3.9.1 Haemalaun Eosin Stain

To de – paraffinize sections they were stored in an incubator (70°C) for 30 min before they were inserted twice into xylol and into 100%, 70%, 50% alcohol. Then, sections were stained for 3 min with haemalaun (Mayer's method) (Hospital pharmacy, LKH Graz, Graz, Austria) and after 3 min under hot floating water they were continued to be treated with eosin - phloxin (Hospital pharmacy, LKH Graz, Graz, Austria) for 2 min. Afterwards sections were washed in 90%, 100% alcohol and in

n – butylacetat (Merck KGaA, Darmstadt, Germany) Finally sections were automatically covered with a coverslipper (Tissue - Tek® GLC 500TM Film Coverslipper, Sakura Finetek Germany GmbH, Staufen, Germany).

3.9.2 Gomori Stain

To de – paraffinize sections they were treated as described in 3.9.1 above. Then, they were stored in 1% potassium – permanganate (Hospital pharmacy LKH Graz, Graz, Austria) for 1 minute. Further, sections were rinsed with distilled water before they were treated with 2% potassium – metabisulfite (Hospital pharmacy LKH Graz, Graz, Austria). Once more sections were rinsed with distilled water before they were stained with 2% iron – alum (Hospital pharmacy LKH Graz, Graz, Austria) for 1 minute. Furthermore, sections were rinsed with distilled water again and then stained with silver – solution (10 ml 10 % silver – nitrate (Hospital pharmacy LKH Graz, Graz, Austria), 2 ml 10% potassium – hydroxide – solution (Hospital pharmacy LKH Graz, Graz, Austria)) for 1 – 2 minutes. After that sections were rinsed twice with distilled water and treated with 3 – 5% formalin (Sigma Aldrich, Vienna, Austria). Then, sections were rinsed with distilled water one last time and washed in 90%, 100% alcohol and in n – butylacetat (Merck KGaA, Darmstadt, Germany). Finally sections were automatically covered with a coverslipper (Tissue - Tek® GLC 500TM Film Coverslipper, Sakura Finetek Germany GmbH, Staufen, Germany).

All histological sections from DENA treated animals were examined and recorded by a professional pathologist at the Medical University of Graz.

4 RESULTS

4.1 Overview

6 animals were treated with DENA for an average time of 30 weeks. Two pigs died of an inexplicable reason after 20 weeks of DENA administration. Since the remaining 4 pigs were euthanized as their health conditions got worse in terms of signs of a decreased appetite and epistaxis the experiment was not carried out until the predicted end. One animal showed signs of melaena and lethargic behaviour. Before euthanasia all animals underwent a CT scan to perform a radiological evaluation and documentation. Although the animals showed cirrhosis and tumorous changes in the liver the experiment did not fulfil the original goal of inducing a HCC in conventional domestic pigs.

4.2 Blood Parameters

All blood parameters harvested to monitor the state of liver functions and health conditions of the animals did not significantly change in the first 8-9 months. Only one animal (denominated as DENA 3) was part of the experiment as long as significant changes in liver enzymes and blood count in terms of hepatic failure occurred.

The red blood count (erythrocytes, haemoglobin) significantly decreased during the last month whereas bilirubin, urea and AST reached peak values. Contrary to all expectations ALT paradoxically decreased quite constantly in the whole course of the experiment. Furthermore alkaline phosphatase and GGT levels did not bring the results which were expected. Either they did not change or they decreased. (cf. Table 4.1 next page)

DENA-3	Leuco	Ery	Hb	Phos	Crea	Urea	Bili	AP	GGT	CHE	AST	ALT	LDH	Lipase
15.01.2009	21,15	4,39	7,7		0,91	26	0,13	101	167	862	97	79	1090	16
22.01.2009	15,82	5,65	10	2,28	1,23	14	0,15	107	49		61	92	853	7
29.01.2009	14,88	5,78	10,6	1,82	1,44	31	0,15	98	63	325	76	110	849	8
19.02.2009	20,04	5,57	10,2	2,42	1,21	21	0,15	133	56	316	67	116	766	10
12.03.2009	13,54	5,63	10,8	2,05	1,27	19	0,08	159	60	402	46	97	576	6
26.03.2009	11,11	5,86	11,2	2,46	1,54	13	0,16	183	64	171	73	85	657	8
16.04.2009	25,25	6,37	12,1	2,39	1,38	17	0,21	229	69	584	71	53	592	9
29.04.2009	11,34	5,85	11	2,42	1,58	15	0,17	181	97	433	95	72	619	8
14.05.2009	11,05	5,55	10,5	2,48	1,39	16	0,21	139	62	495	76	72	545	9
28.05.2009	13,41	5,67	10,7	2,53	1,54	12	0,16	130	81	544	51	51	443	7
10.06.2009	13,81	5,56	10,6	2,26	1,7	22	0,18	164	70	435	78	60	443	7
24.06.2009	12,46	5,47	10,3	2,48	1,45	12	0,28	173	71	338	66	43	355	7
09.07.2009	9,55	5,99	11,4	2,36	1,44	22	0,2	126	98	414	83	57	366	7
23.07.2009	12,48	5,36	10,2	2,49	1,56	17	0,09	114	94	400	81	54	396	7
05.08.2009	12,91	5,65	10,8	2,38	1,95	21	0,23	112	100	456	108	47	378	6
20.08.2009	10,75	6,22	11,8	2,39	2	18	0,29	147	91	414	99	53	413	9
03.09.2009	9,84	5,53	10,7	2,28	1,78	16	0,25	149	93	595	80	44	344	7
17.09.2009	8,91	5,61	10,7	2,4	2,04	23	0,36	116	108	543	101	45	316	8
30.09.2009	9,34	5,33	10,1	1,98	1,65	23	0,26	111	110	702	100	40	294	7
15.10.2009	9,34	5,45	10,5	2,21	1,35	29	0,6	111	103	649	109	48	272	8
29.10.2009	9,19	5,18	9,9	1,8	1,32	32	1,4	80	94	876	129	35	316	12
18.11.2009	9,4	1,7	2,6	3,32	2,07	69	1,34	37	50	458	331	20	446	25

Tab. 4.1: Chronological table of blood parameters of the pig with the longest period in this study (denominated as DENA 3). The last 3 highlighted rows indicate the last 3 blood samples where all significant changes happened. Note the increase of AST and bilirubin during the last 3 blood samples whereas in contrast to all expectations ALT decreases dramatically! Abbreviations: Leuco: leucocytes x 1000/ μ l; Ery: erythrocytes in mill/ μ l; Hb: haemoglobin in g/dl; Phos: phosphate in mmol/l; Crea: creatinine in mg/dl; Bili: bilirubin in mg/dl; AP: alkaline phosphatase in U/l; GGT: gamma – glutamyl transferase in U/l; CHE: choline esterase in U/l; AST: aspartate – aminotransferase in U/l; ALT: alanine – aminotransferase in U/l; LDH: lactate dehydrogenase in U/l; Lipase in U/l

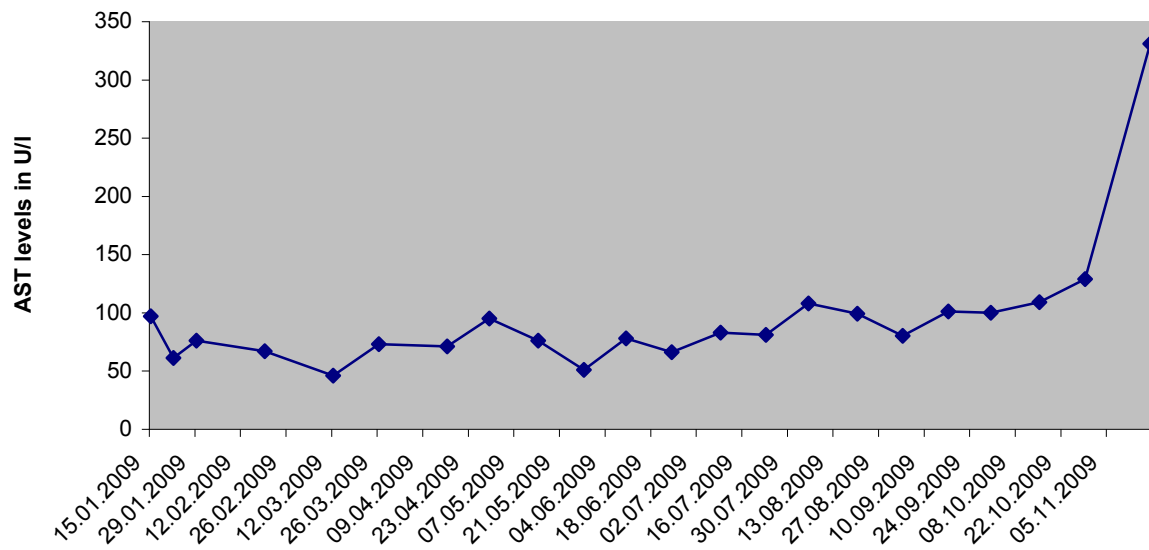


Fig. 4.1: This diagram shows the course of the AST level of the pig with the longest period in this study. (DENA 3)

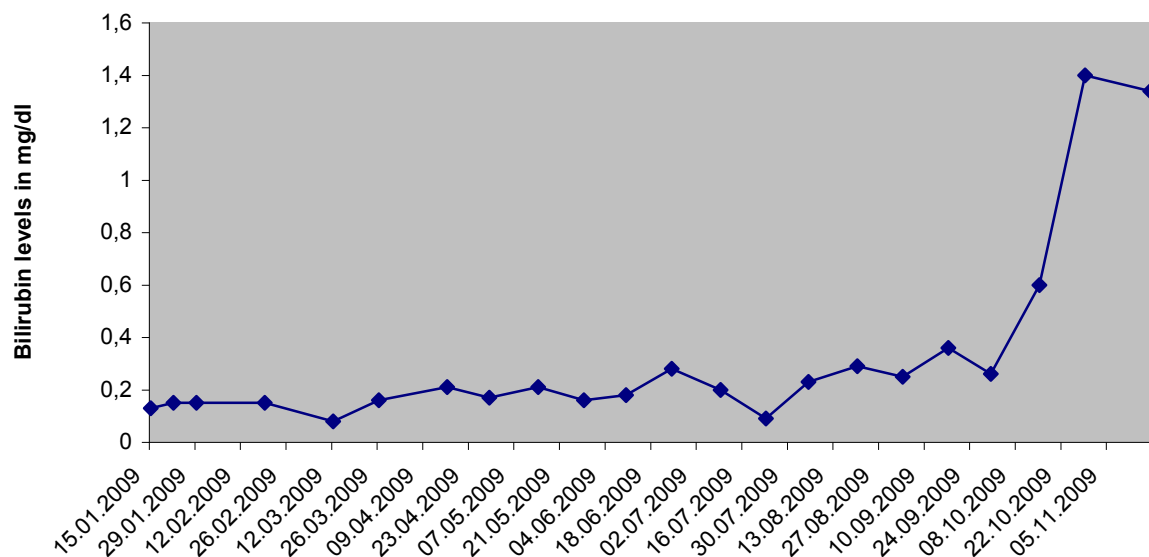


Fig. 4.2: This diagram shows the course of the bilirubin level of the animal with the longest period of DENA treatment. (DENA 3)

4.3 Macroscopic Results

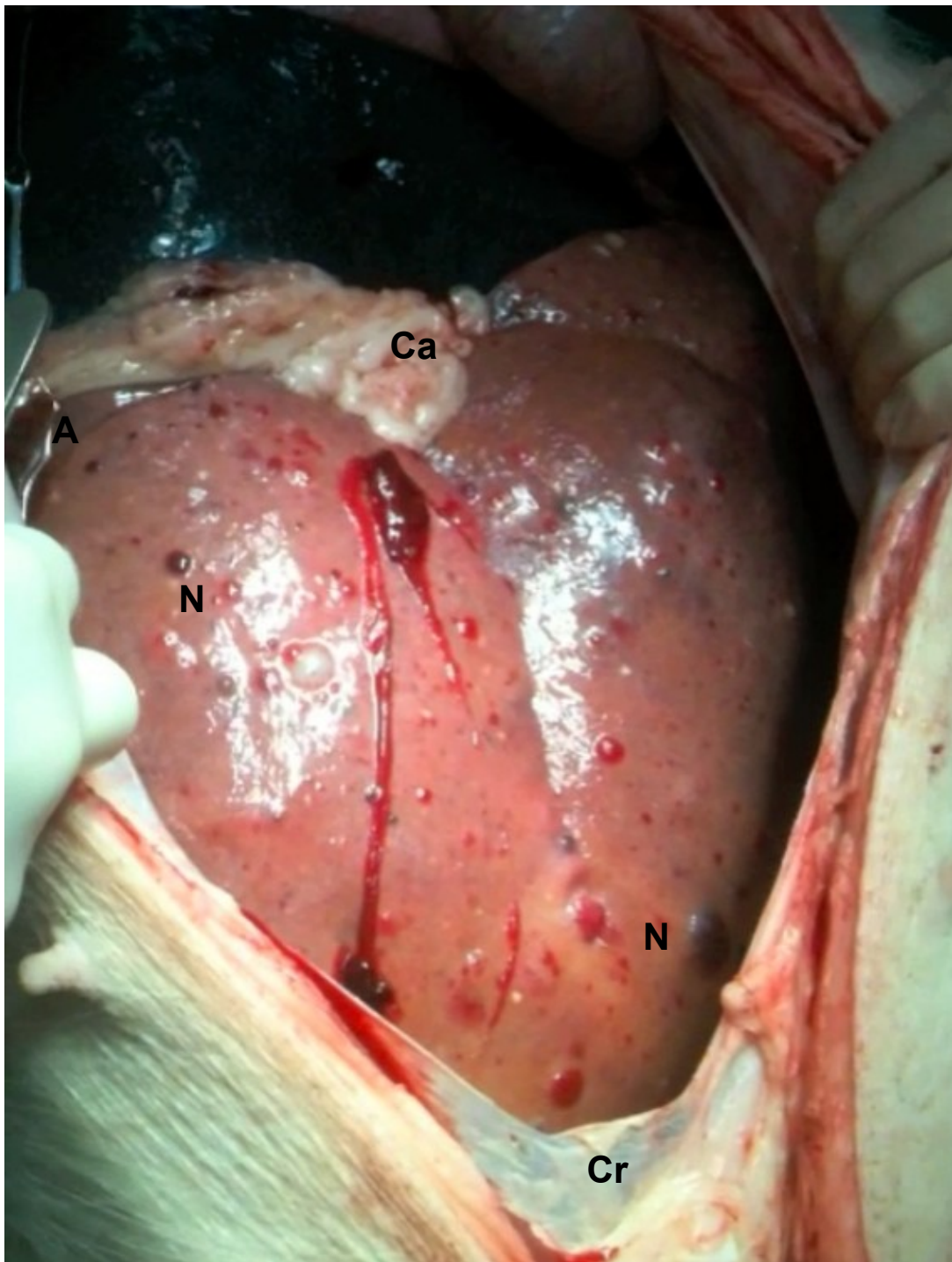


Fig. 4.3a: This pig (denominated as DENA 3) was treated with DENA for 10 months (longest period in this study). A median laparotomy revealed a massively enlarged liver with multiple miscellaneous coloured tumour nodules (N) distributed on its surface. As the animal suffered from melaena, lethargic behaviour, elevated levels of AST and bilirubin, a further indicator for hepatic failure may be ascites (A) seen at the left distal border of the liver. Caudal → Ca; Cranial → Cr



Fig. 4.3b: This image shows a transverse section from the liver of the pig with the longest period in this study.(DENA 3) Various tumour lesions (T) of different colours and sizes are spread out through the whole section. The colour of the parenchyma changes in an undefined pattern.

4.4 Histological Results

As pigs were euthanized at different stages of tumour induction we saw a lot of different histological phases in the course of the experiment. We classified our histological results in early changes in liver morphology without or with little cirrhosis, in stages of moderate and severe cirrhosis and in a stage with different tumours.

4.4.1 Early Changes

The first changes in cellular liver morphology appear as a ballooning degeneration of hepatocytes of presumably toxic origin (DENA). Furthermore images show areas of hepatocellular necrosis and centrilobular pericellular fibrosis.

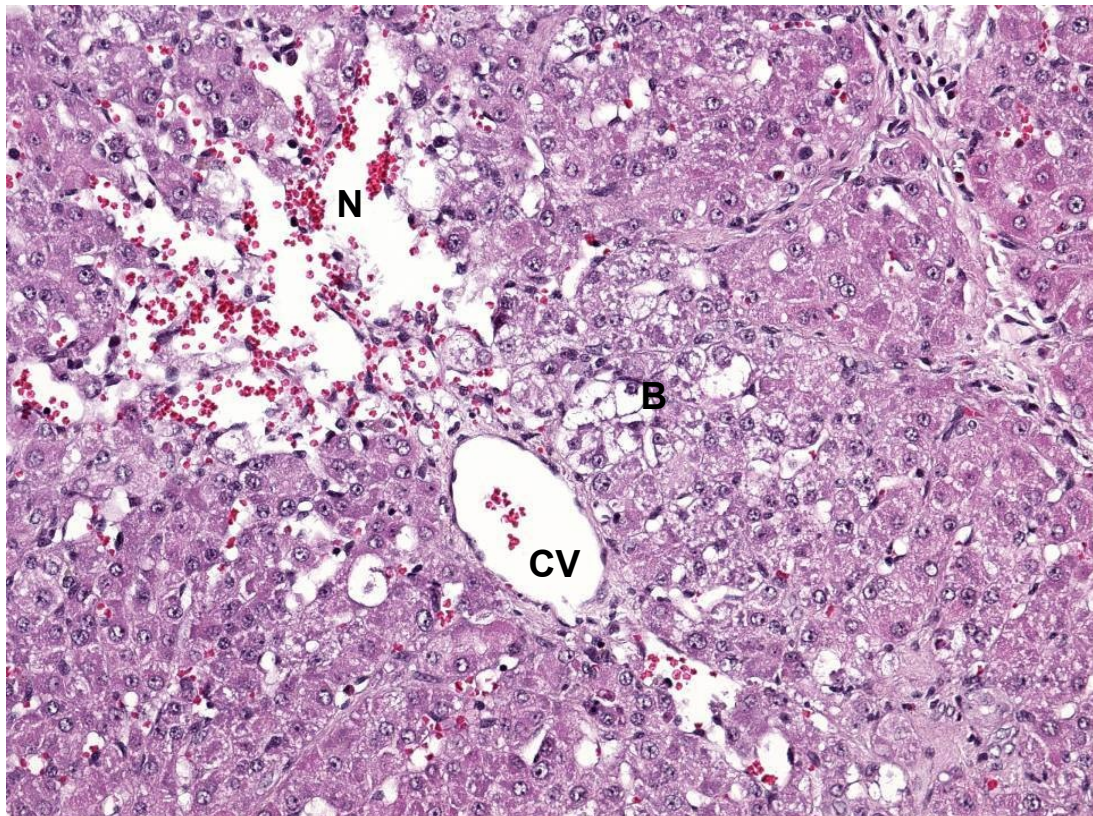


Fig. 4.4a: Histological image (haemalaun eosin stain) of DENA induced early changes in liver parenchyma. B → ballooning degeneration of hepatocytes; N → hepatocellular necrosis; CV → central vein

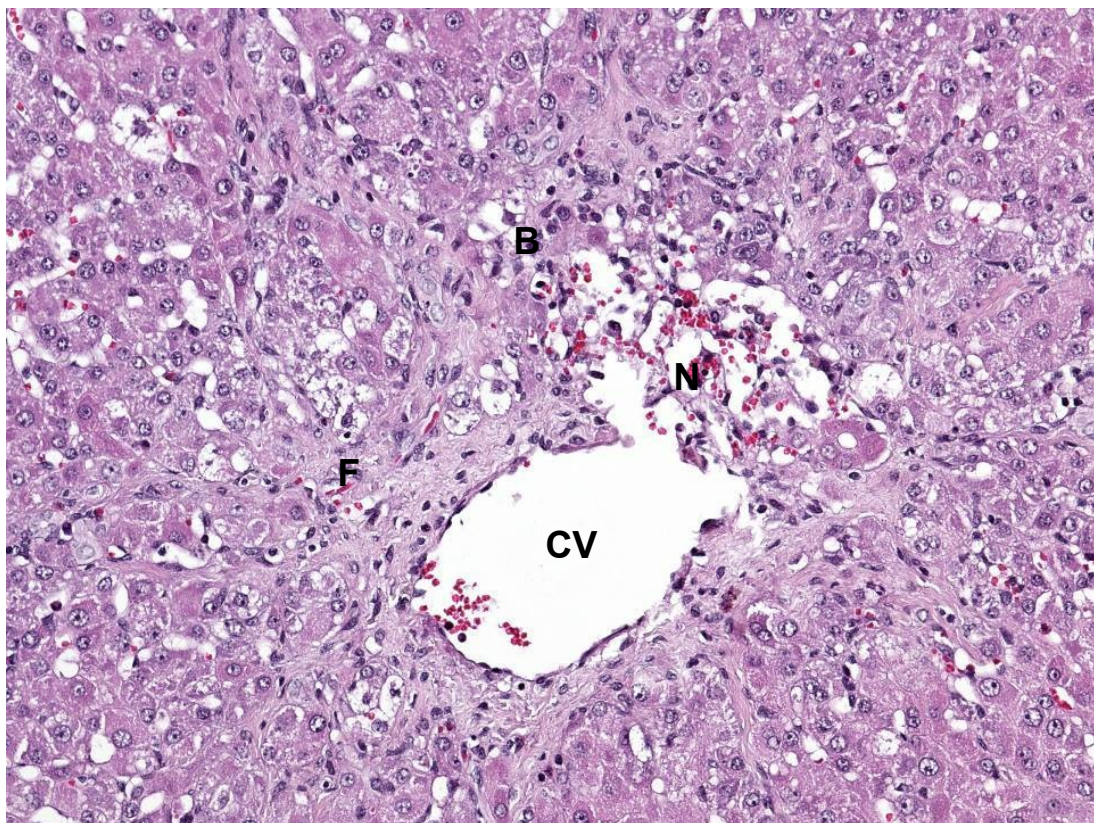


Fig. 4.4b: Histological image (haemalaun eosin stain) of DENA induced early changes in pig liver parenchyma. F indicates an obvious area of centrilobular pericellular fibrosis; B → ballooning degeneration of hepatocytes; N → hepatocellular necrosis; CV → central vein

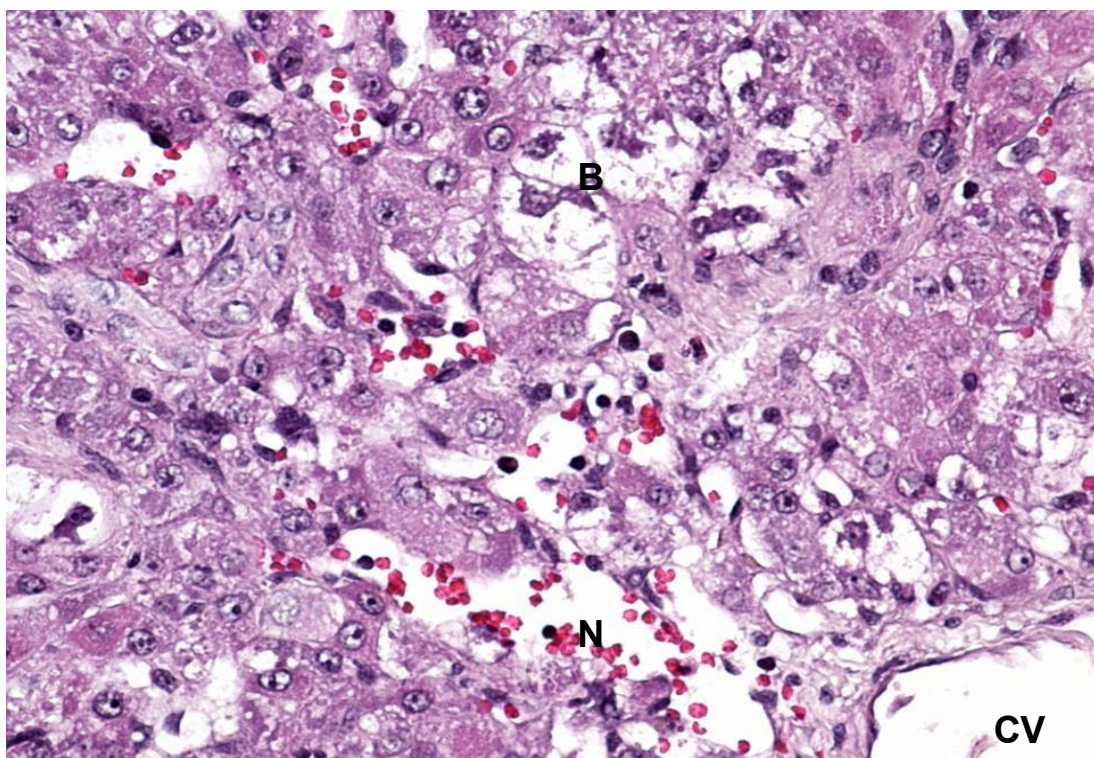


Fig. 4.4c: Greatly enlarged histological image (haemalaun eosin stain) of early changes. B indicates giant ballooned and degenerated hepatocytes; N → necrosis zone; CV → central vein

4.4.2 Early Stage Cirrhosis

Depending on the duration of DENA treatment, animals show different stages of cirrhosis. Early stage cirrhosis is characterized by the appearance of numerous regenerative nodules separated by fibrous septa.

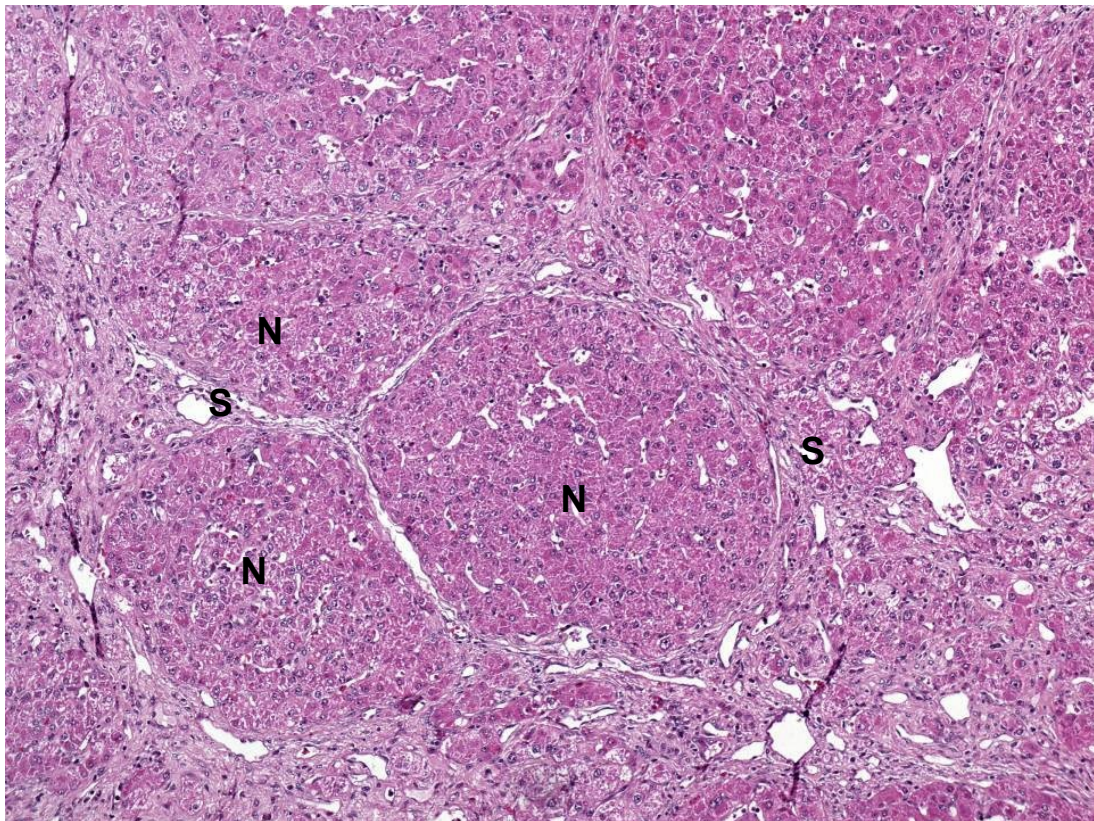


Fig. 4.5: This histological picture (haemalaun eosin stain) shows a section of a porcine liver with an early state of cirrhosis. Regenerative nodules (N) are separated from each other by fibrous septa.(S)

4.4.3 Late Stage Cirrhosis

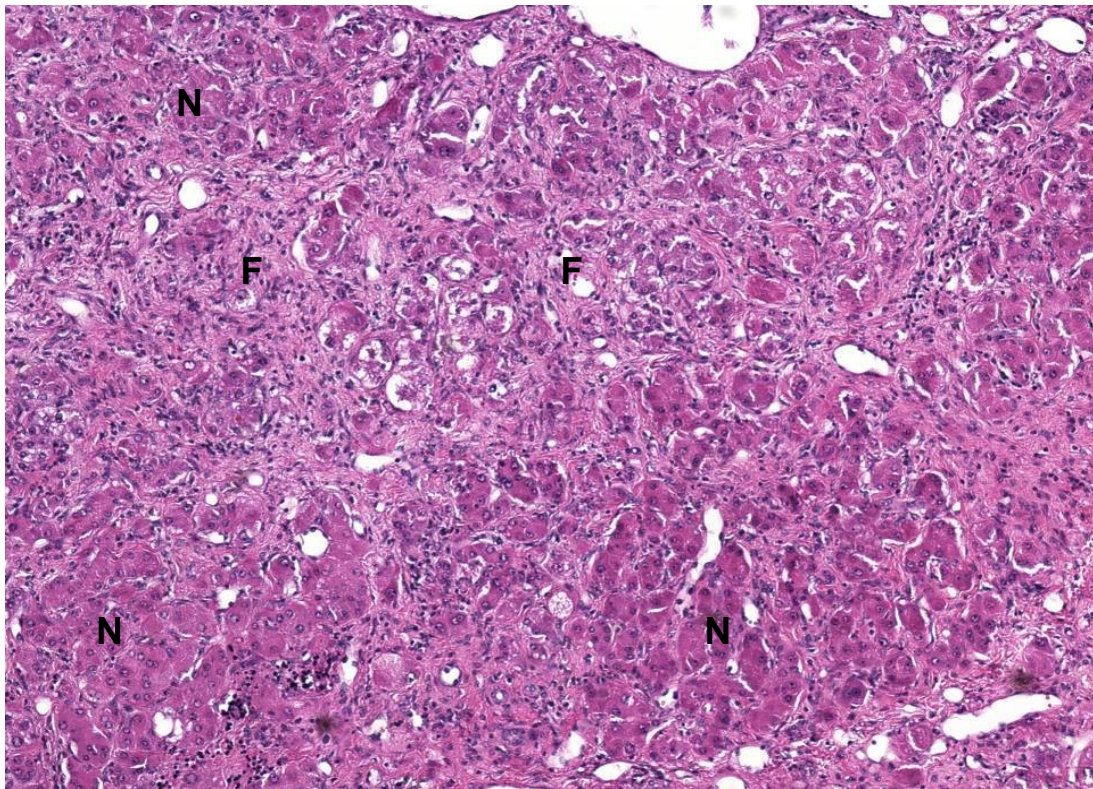


Fig. 4.6a: Histological picture (haemalaun eosin stain) of a porcine late stage cirrhosis. Micronodules (N) are surrounded by massive pericellular fibrosis.(F)

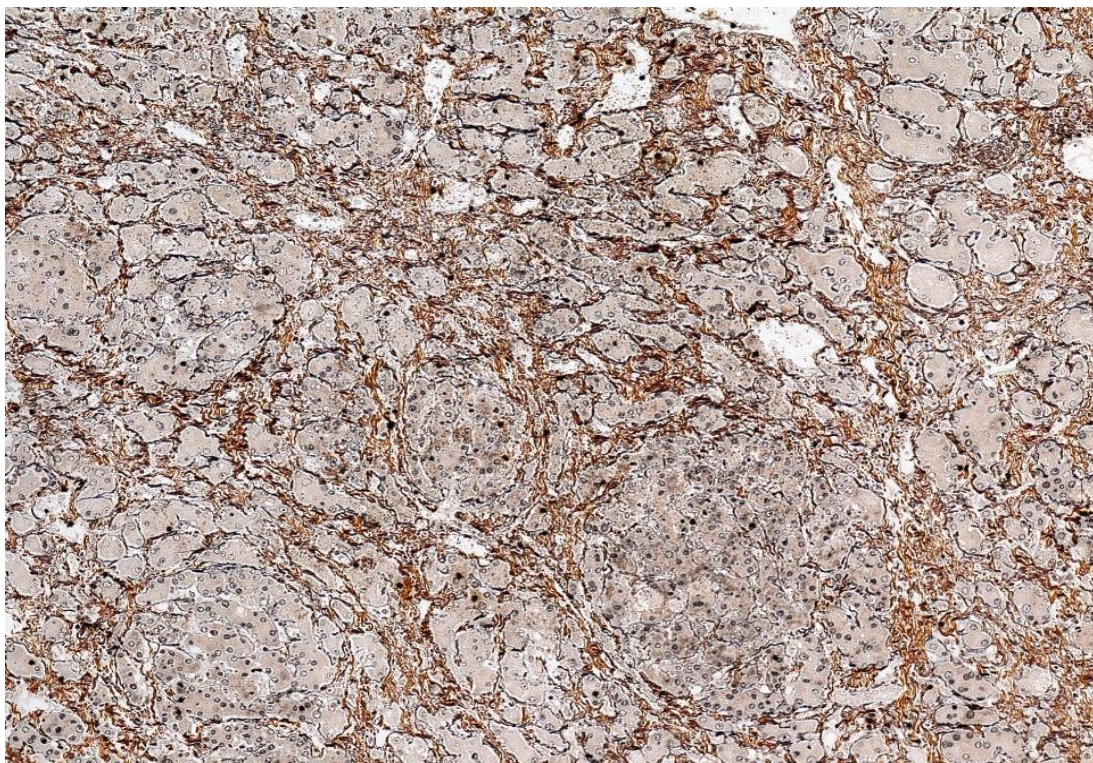


Fig. 4.6b: Reticulin fibre stained (gomori stain) histological section of a porcine late stage cirrhosis. Reticulin fibres → black; Collagen fibres → orange; Hepatocytes parenchyma → light brown (black nuclei within the light brown stained parenchyma)

4.4.4 Tumours

The original goal of the experiment was not fulfilled as the animals did not develop HCCs. Instead numerous other tumours were found including leiomyomas and leiomyosarcomas but more frequently angiosarcomas. In all histological sections there were no signs of any clear cut HCCs.

4.4.4.1 Angiosarcoma

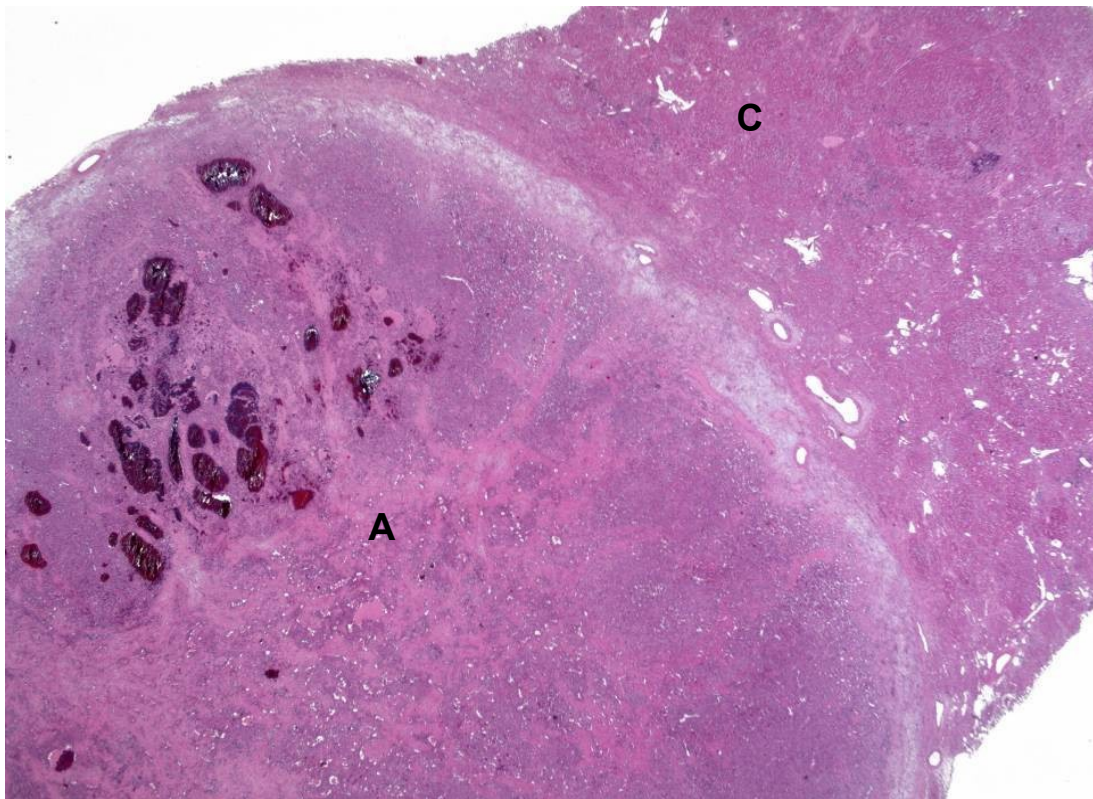


Fig. 4.7a: Histological image (haemalaun eosin stain) of an angiosarcoma arising in a cirrhotic porcine liver after intraperitoneal injections of DENA for 10 months. A → angiosarcoma; C → cirrhotic liver

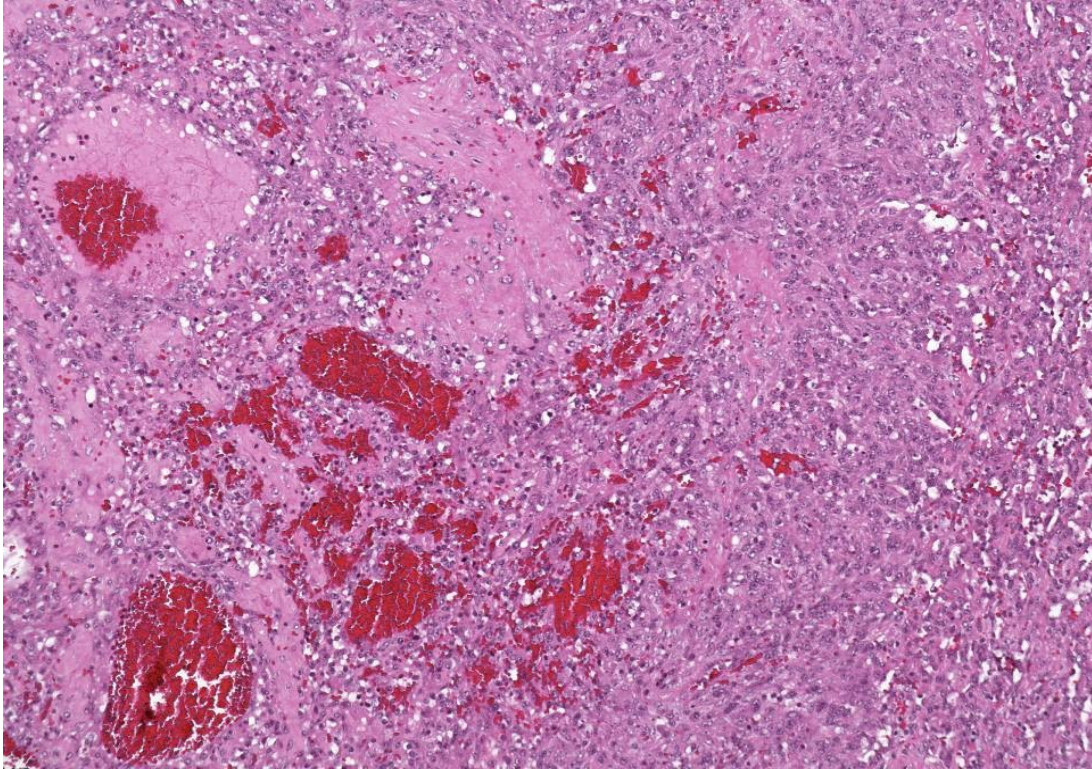


Fig. 4.7b: Histological section (haemalaun eosin stain) shows an angiosarcoma in a pig liver.

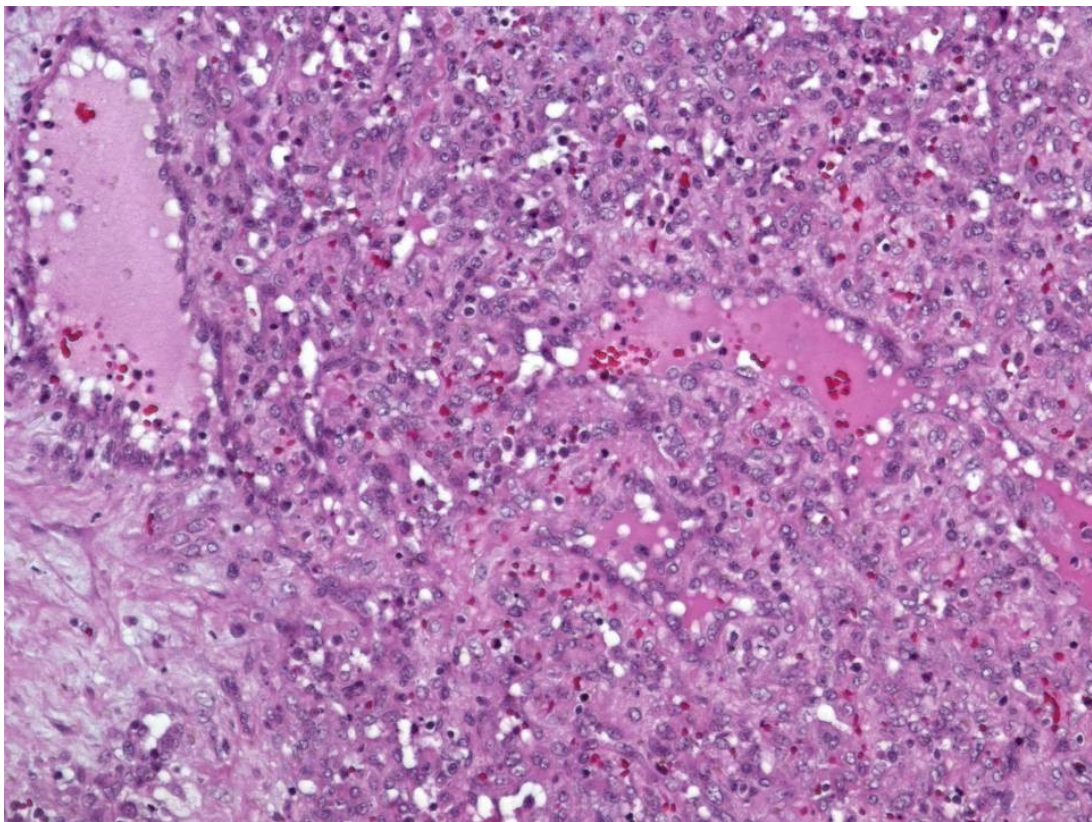


Fig. 4.7c: Greatly enlarged histological section (haemalaun eosin stain) of an angiosarcoma arising in a porcine liver.

4.4.4.2 Leiomyoma

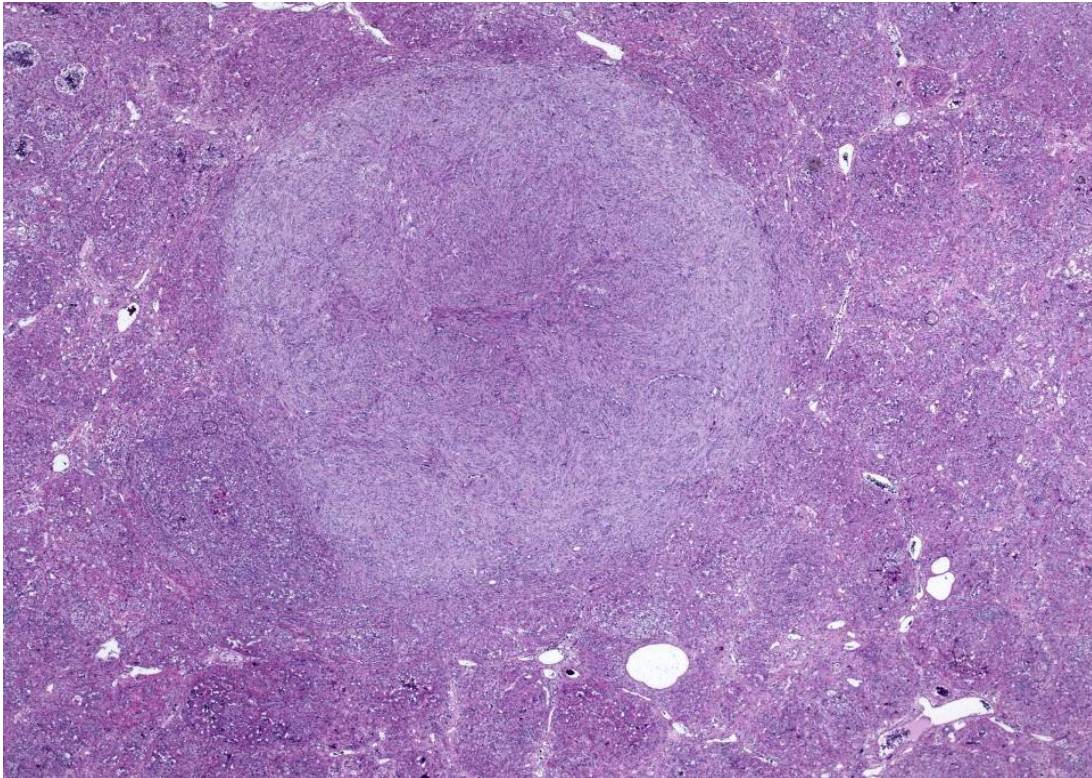


Fig. 4.8a: Microscopic image (haemalaun eosin stain) of a leiomyoma in a cirrhotic porcine liver.

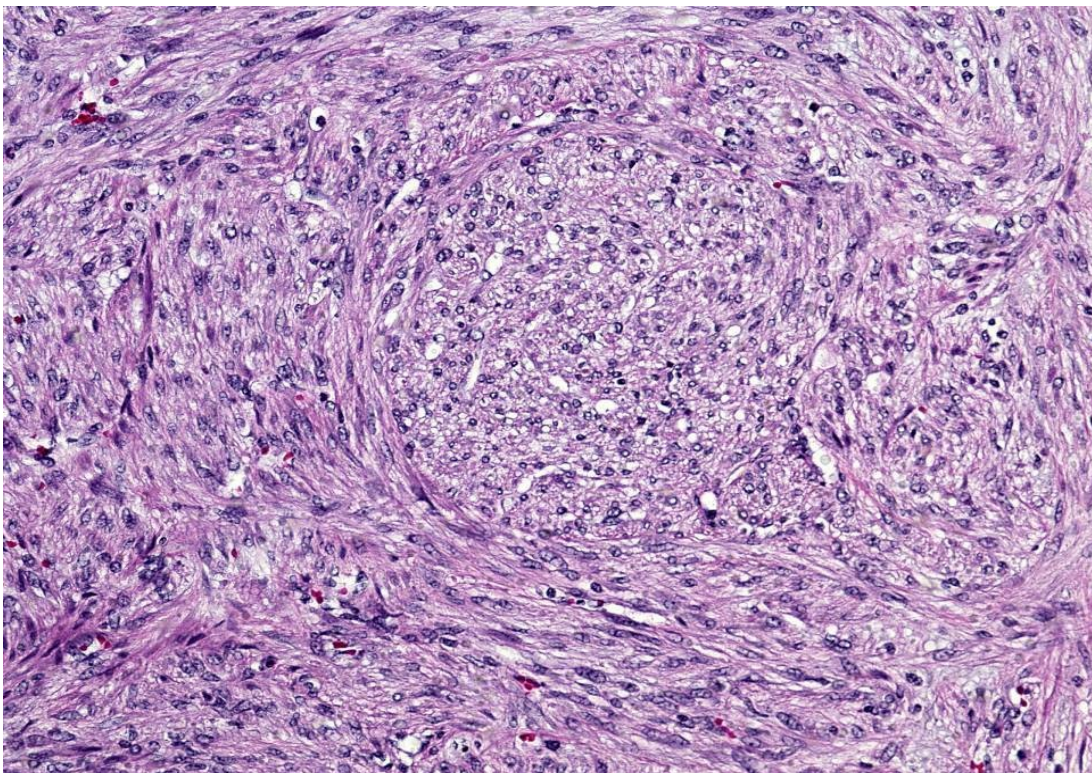


Fig. 4.8b: Detailed microscopic view (haemalaun eosin stain) of a leiomyoma in a pig liver.

5 DISCUSSION

5.1 Carcinogenesis

5.1.1 Duration of DENA Treatment

We intended to create an HCC animal model by continuous weekly application of an intraperitoneal injection of DENA as long as CT scans reveal changes in liver parenchyma in terms of HCC development and cirrhosis of the liver. Every time DENA was applied a short general anaesthesia was required, which provides an enormous organic stress for the laboratory animals that may cause serious collateral damages in other organs apart from the liver. Thus, collateral damages may limit the endurance of the experiment although a HCC is not visual yet or still in molecular development. Shortening the time of DENA treatment and recurrent general anaesthesias to a defined period of 3 or 4 months may improve the chances to keep the animals in acceptable health conditions as long as a HCC develops.

5.1.2 DENA – Dosage

DENA is a well known and well described potent carcinogen that can induce HCC in various animals. In terms of chemically induced HCC by injecting DENA in pigs literature only provides the study by Li et al(60) where they used China Taihu piglets to perform their research. We used conventional domestic pigs and hypothesized that DENA expresses its hepatocarcinogen effect in related species too. A possible explanation of a delayed or inhibited development of HCC may be that domestic pigs are more robust and tougher in handling carcinogen substances. A further increase of the dosage from 15 mg/kg may shorten the time of carcinogenesis but may also cause serious side effects.

5.1.3 Promoters

To enhance carcinogenesis additional chemical agents and HCC promoters are worth mentioning. Phenobarbital is a well known promotor to boost HCC development. Adding Phenobarbital to the diet of the animals may shorten the time of carcinogenesis and provide better results.

5.2 Blood Sampling

Every second week blood samples were taken to monitor hepatic functions and general health conditions of the animals. In the course of the sedation for DENA application, blood was sampled from sedated animals. As mentioned in 5.1.1 weekly anaesthesias may cause serious side effects. In case of future studies where DENA is only applied for 3 or 4 months followed by a period without any treatment, blood sampling without sedation would spare the animals harmful side effects. Pigs that are equipped with a PAC system could be trained to become more trusting and then they would endure the puncturing of the PAC chamber without the use of sedative substances. The swine in our study which was equipped with a PAC became that trusting that it was possible to puncture the PAC chamber without a general anaesthesia.

5.2.1 PAC vs Jugular Vein

In this study it has been shown that blood sampling via a PAC system is an efficient and easy way to collect blood from an animal. Unfortunately PACs can become obstructive and therefore useless. Furthermore, the implantation of the PAC is an invasive procedure which can cause a large amount of post – operative complications. Blood sampling through a direct puncture of the vena jugularis is an acceptable alternative to PAC blood sampling whereby, however, an assistance is needed and the success of the procedure depends on the skill of the investigator. The accidental puncturing of the arteria carotis was not an uncommon complication which required a thorough compression of the injured vessel for a few minutes.

5.2.2 Blood Parameters

Blood parameters did not change significantly in the first 8 – 9 months and were not useful to monitor the development of a hepatic malignancy or to predict a soon death of an animal in the above mentioned period. As mentioned before two pigs died in this study without preceding announcement by changing blood parameters. It can be assumed that the laboratory animals tolerate the continuous poisoning treatment for 9 – 10 months without mentionable alterations of the blood parameters in terms of a compensated but continuously advancing liver damage. As the course of the experiment exceeded a particular point, blood parameters changed rapidly and dramatically. Presumably this phenomenon is associated with acute decompensation. Future similar investigations and studies in this direction may abstain from blood sampling in the first months as they do not lead to valuable results. Evaluating tumour markers like AFP may be an interesting and worthwhile addition.

Blood sampling was performed in the stables of the facility of biomedical research, which is far from the blood laboratory. Thus, samples often were not cooled and remained unprocessed for a certain time, which probably weighted some parameters.

5.3 Alternative Detecting Methods

Periodical hepatic imaging with CT provided excellent high resolution and well interpretable pictures. Unfortunately this method is associated with lots of logistical problems and requires a long and organ stressing general anaesthesia with propofol. US may be an interesting alternative to CT. As US is a mobile and radiation free device, HCC tumour nodules and cirrhosis of the liver could be detected and observed as well, without or with superficial sedation.

The biopsy of the liver could be used to determine the exact histological status of the liver in terms of the state of cirrhosis and/or possibly arising but still too small tumour nodules. Thus, future similar studies may be able to document the exact course of the disease until a HCC develops but without sacrificing the animal.

5.4 Future Perspectives

As HCC is a major cancer problem worldwide valuable research models are very important and promising instruments for inventing and improving new therapy strategies. This study has shown that DENA has extreme toxic and harmful effects on porcine organisms and especially porcine livers. Further similar studies at the Medical University of Graz attempt to improve the study design of this study to receive a repeatable HCC model in pigs that resembles human HCC.

In the next study the duration of DENA treatment will be shortened to a period of 3 months and the dosage of DENA will be reduced to 10 mg/kg per week. Hopefully, this will guarantee an adequate accumulation of DENA in the porcine organism to create a carcinogenic effect but will also reduce dangerous side effects, which harm the animals and therefore limit the endurance of the experiment. Blood samples still will be taken to monitor liver functions, health conditions of the animals and the possible development of a HCC.

If the above mentioned modifications of the study design are still not enough to induce a clear cut HCC, every possible effort will be taken to finally create this valuable research model. This includes further alterations of the dosage, the duration of DENA administration and additions of HCC promoters like phenobarbital.

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