Potential Biomarkers for “Fatty Liver” (Hepatic Steatosis) and Hepatocellular Carcinoma (HCC) and an explanation of their pathogenesis

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Abstract
Hepatic steatosis or “fatty liver” is a characteristic for disorders ranging from Nonalcoholic Fatty Liver (NAFL) to Nonalcoholic Steatohepatitis (NASH) as part of the metabolic syndrome. We hypothesize these liver disorders can due to the hypoxic conditions and its followed pathogenesis been associated with liver cancer or Hepatocellular Carcinoma (HCC). Over the past few decades, the screening for and early diagnosis of HCC has attracted attention worldwide, and especially since HCC is not solely a pathogenesis in Asia evolving from hepatitis infection but also now it is hypothesized it can be correlated with the obesity/type 2 diabetes pandemic related to hepatic steatosis especially in the US and Europe. Because HCC is the fifth most common cancer and the second most common cause of death from cancer worldwide it is an urgent need to find biomarkers for HCC. In the present study using a LCMS Systems Biology lipidomics based approach with a High-Fat diet induced Insulin Resistant casu quo Type 2 diabetes (IR/T2DM) obese C57bl6 mouse model we found several biomarker candidates in the phosphatidylcholine fraction for which a 36:1 PCh could accounted as a clear biomarker for HCC. Other PCs are also potential candidates for novel biomarkers for “fatty liver” in this C57bl6 mouse model such as C32:1 PC, C34:1 PC and C38:2 PC. Our approach highlights new biochemical insights on molecular mechanisms underlying liver cancer disease. This new understanding will promote clinical applications in drug discovery and personalized therapy.

Keywords: Hepatic steatosis; Nonalcoholic Fatty Liver (NAFL); Nonalcoholic Steatohepatitis (NASH); metabolic syndrome; obesity; hypoxia; liver cancer; hepatocellular carcinoma (HCC); C57BL6 mouse model; biomarker; phosphatidylcholine.

Introduction
Hepatocellular Carcinoma (HCC), also called malignant hepatoma, is the most common type of liver cancer. It is an international problem, as the fifth most common cancer and the second most common cause of death from cancers worldwide [1]. In Asia it is an urgent compelling social problem with a country like China where 55% of HCC cases worldwide live. Globally, between 600,000 and 1 million new cases of HCC are diagnosed each year [2]. Most cases of HCC are as a result of either a viral hepatitis infection (hepatitis B or C), metabolic toxins such as alcohol or aflatoxin, conditions like hemochromatosis and alpha 1-antitrypsin deficiency or Nonalcoholic Steatohepatitis (NASH) [3]. HCC was until presently relatively uncommon in the United States and many other developed countries. It occurs most commonly in countries where hepatitis B infections are common [4]. However, HCC is no longer a disease of the Eastern hemisphere (Figure 1). HCC incidence has tripled in the United States in the past two decades. It is the fastest rising cause of cancer mortality in the U.S. and in parts of Western Europe. Presently – as the obesity pandemic sweeps our globe- it is hypothesized Non-alcoholic fatty liver disease (NAFLD) is the most frequent cause of liver cancer or HCC [5]. Due to the obesity and type 2 diabetes pandemic it currently is the fastest-growing cancer in incidence in the
Advances in genomics and proteomics platforms and biomarker assay techniques over the last decade have resulted in the identification of numerous novel biomarkers and have improved the diagnosis of HCC. In this manuscript we want to emphasize that the emergence of HCC has different causes which may vary from excessive alcohol consumption, viral (hepatitis a, b, c) and exposure to toxic components. Non-alcoholic steatohepatitis is part of a disease spectrum, non-alcoholic fatty liver disease, ranging from simple steatosis to cirrhosis, which is the most frequent cause of abnormal liver tests. There is clinical and epidemiological evidence that non-alcoholic fatty liver disease is the precursor hepatic manifestation of Hepatocellular Carcinoma (HCC). Therefore, we hypothesize that HCC can also be the result of a sedentary lifestyle, fatty foods and alcohol abuse leading to metabolic syndrome and obesity. Obesity has emerged as an important risk factor as it can lead to steatohepatitis and Type 2 Diabetes (T2DM) increases the risk of HCC. The overall cancer incidence of liver in nonalcoholic FLD has not yet been quantified, but the association is well-established. Therefore, we can conclude obesity is associated with the incidence and mortality of HCC. As a result, there occurs an accumulation of TGs in the liver (see Figure 3). We hypothesize the major culprit in this model diseases leading to HCC are hypoxic conditions in the “fatty liver” due to the low vascularization degree in excessive White Adipose Tissue (WAT) see Figure 4. Recently biomarkers from metabolomics based on a lipidomics based approach were valuable in case of T2DM in an obese C57bl6 mouse model [25]. These biomarkers are following our hypothesis about

### Table 1a: Some Clinical methodology and biomarkers for NAFDL, Chronic Liver Diseases (CLD) and Hepatocellular Carcinoma (HCC).

<table>
<thead>
<tr>
<th>NAFL/NAFDL/Chronic Liver Disease (CLD)</th>
<th>References</th>
<th>Hepatocellular Carcinoma (HCC)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-scan</td>
<td>[10]</td>
<td>CT-scan</td>
<td>[16]</td>
</tr>
<tr>
<td>CD36 membrane protein</td>
<td>[12]</td>
<td>Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3)</td>
<td>[18]</td>
</tr>
<tr>
<td>breath ethanol, ethane and acetone</td>
<td>[14]</td>
<td>tumor biopsy for confirmation</td>
<td>[17]</td>
</tr>
</tbody>
</table>

United States, and it is expected to continue climbing over the next 20 years [6]. For decades, it was already known that unhealthy food, a sedentary lifestyle and smoking were possible causes for cancer [7]. A very recent study by the International Agency For Research On Cancer Handbook Working Group (IARC) workgroup in the NEJM study proved-based on more than 1000 epidemiologic studies -which were observational studies on cancer risk and excess body fatness- that obesity is directly correlated to several types of cancer including liver cancer [8].

One promise of metabolomics in the identification of biomarkers that could favorably impact disease diagnosis, as well as our ability to assess response to treatment, and thereby, prognosis. In Table 1a several biomarkers for Non-Alcoholic Fatty Diseased Liver (NAFDL), Chronic Liver Diseases (CLD) and Hepatocellular Carcinoma (HCC) are given.

In addition, because human MRI imaging at HCC is common we developed a special MRI program for in vivo MRS imaging of localized hepatic MRI spectra of a high-fat diet obese C57BL6 mouse model [Figure 2].

Although the prognosis of patients with HCC is generally poor, the 5-year survival rate is > 70% if patients are diagnosed at an early stage. However, early diagnosis of HCC is complicated by the coexistence of inflammation and cirrhosis. Thus, novel biomarkers for the early diagnosis of HCC are required. Currently, the diagnosis of HCC without pathological correlation is achieved by analyzing serum alpha-fetoprotein levels combined with imaging techniques.
excessive WAT leading to hypoxia possibly not only useful for early diagnosis of HCC, but also provide insight into the mechanisms driving oncogenesis [26]. In the discussion, we will give a biochemical explanation how this can possibly can lead to HCC. In addition, we will also give a novel biomarker from the Phosphatidylcholine (PC) fraction in an obese C57BL6 mouse model with hepatic steatosis.

Material & Methods

Experimental Animals

In order to find biomarkers in the lipid fraction based on a lipidomics approach of different non-adipose tissues and organs (liver, heart, carcass, hind limb-muscle, brain including blood-plasma). In this manuscript we describe our findings for...
liver tissue. We administered two different diets (one regular and one High-Fat [HF] diet) on a specific mouse model, the mouse strain C57bl6 and compared the lipid profiles using LCMS techniques. Purebred male wild-type C57bl6 mice (age 8-12 weeks), obtained from Charles River (Maastricht, The Netherlands) were used. Animal experiments were approved by the animal experimentation committee of the Leiden University Medical Centre (The Netherlands). Two feeding regimes were compared: 1. a regular diet (Control) and 2. a high-dietary situation in which mice received a High-Fat diet (HF) during 40 days and then were sacrificed (Treatment). In total, 13 rodents were used: 6 mice in the Control group and 7 in the Treatment group. Mice were housed in a temperature-controlled room (21°C) on a 10-hour dark/14-hour light cycle.

**Diet**

Mice in the Control group were fed a standard lab chow (SDS.3, Special Diet Services, Witham, UK) containing about 4.3 energy percent fat (Table 1b). The fatty diet that was fed to mice in the Treatment group contained 21.4 % protein, 36% carbohydrates, 24% fat, 6% fibers and 5.7% water (weight-percentages). Before the experiment started animals of both the control group and the treatment group received unrestricted amounts of food and water. Mice in the Control group fasted for 4 hours before the start of the experiment in order to standardize their metabolic rate.

**LC-MS of Lipids and Fatty Acids**

Thirteen mouse of a C57bl6 mouse strain were used and assigned to the following two treatments. Treatment A was the control group (n=6) and received ad-lib standard lab chow and water during 40 days. Treatment B were animals that received a High-Fat Diet during 40 days (n=7).

**Tissues**

A tissue- or feed- homogenate (~10% wet weight/ vol) in PBS (phosphate-buffered saline) was made by stirring the tissue in a closed tube with small glass beads.

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**Table 1b:** Food composition of the mice chow: “normal” for Control group (Special Diet Services, SDS No.3, Witham, UK) and the High-Fat “fatty diet” (Arie Blok, food code 4032.05, Woerden, The Netherlands) based on bovine lard and 0.25% cholesterol.

<table>
<thead>
<tr>
<th>Proximate Analysis</th>
<th>Standard (SDS.3)</th>
<th>Proximate Analysis</th>
<th>Fatty diet (4032.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>10</td>
<td>Moisture (%)</td>
<td>5.74</td>
</tr>
<tr>
<td>Crude Oil (%)</td>
<td>4.25</td>
<td>Crude Fat</td>
<td>24</td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>22.39</td>
<td>Crude Protein (%)</td>
<td>21.44</td>
</tr>
<tr>
<td>Crude Fiber (%)</td>
<td>4.21</td>
<td>Crude Fiber (%)</td>
<td>6.16</td>
</tr>
<tr>
<td>Ash</td>
<td>7.56</td>
<td>Minerals</td>
<td>2.25</td>
</tr>
<tr>
<td>Nitrogen Free Extract</td>
<td>51.2</td>
<td>Nitrogen Free Extract</td>
<td>36.19</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>99.61</strong></td>
<td><strong>TOTAL</strong></td>
<td><strong>96.03</strong></td>
</tr>
<tr>
<td>Energy (measured bomb-calorimetry) [kJ/g dm]</td>
<td>21.46</td>
<td>Energy (measured bomb-calorimetry) [kJ/g dm]</td>
<td>21.46</td>
</tr>
</tbody>
</table>

**Figure 4:** White Adipose Tissue (WAT), an accumulation of Triacylglycerols (TGs) in the liver (hepatic steatosis) has a low vascularization so that low oxygen conditions (hypoxia) can occur.
LC-MS of lipids and fatty acids in blood plasma: As described earlier [19, 20, 25, 27, 28] fifty \( \mu l \) of the well mixed tissue homogenate was mixed with 1000 \( \mu l \) of a 1% ITPA containing 4 internal standards. In addition blood plasma samples of 10 \( \mu l \) plasma were extracted with 300 \( \mu l \) of Isopropanol (IPA) containing several internal standards (IS: C17:0 lysophosphatidylcholine, di-C12:0 phosphatidylcholine, tri-C17:0 glycerol ester, C17:0 cholesteryl ester and heptadecanoic acid (C17:0)). Samples were placed in an ultrasonic bath for 5 minutes. After mixing and centrifugation (10000 rpm for 3 minutes) the supernatant was transferred to an autosampler vial. Thereafter 10 \( \mu l \) of the sample was injected on the LC-MS instrument (Thermo Electron, San Jose, USA). A Thermo LTQ is a linear ion-trap LC-MS instrument (Thermo Electron, San Jose, USA).

Lipids were separated on a 150 x 3.2 mm i.d. C4 Propherase column (Alltech, USA) using a methanol gradient in 5 mM ammonium acetate and 0.1% formic acid (mobile phase A: 5% methanol, mobile phase B: 90% methanol). The flowrate was 0.4 ml/min and the gradient was as follows: 0-2 min – 20%B, 2-3 min – 20% to 80%B, 3-15 min – 80% to 100%B, 15-25 min – hold 100%B, 25-32 min – condition at 20%B. The instrument used was a Thermo LTQ equipped with a Thermo Surveyor HPLC pump. Data were acquired by scanning the instrument from m/z 300 to 1200 at a scan rate of approximately 2 scans/s in positive ion ESI mode.

LC-MS of lipids and fatty acids in blood plasma: As described earlier [19, 20, 25, 27, 28] Lipids and Free Fatty Acids (FFA) in blood plasma were analyzed with electrospray LC-MS using samples with a volume of 10 \( \mu l \) plasma. This was extracted with 300 \( \mu l \) of Isopropanol (IPA) containing several internal standards (IS: C17:0 lysophosphatidylcholine, di-C12:0 phosphatidylcholine, tri-C17:0 glycerol ester, C17:0 cholesteryl ester and heptadecanoic acid (C17:0)). After mixing and centrifugation the supernatant was transferred to an autosampler vial. Lipids were separated on a 150 x 3.2 mm i.d. C4 Propherase column (Alltech, USA) using a methanol gradient in 5 mM ammonium acetate and 0.1% formic acid (mobile phase A: 5% methanol, mobile phase B: 90% methanol). The flowrate was 0.4 ml/min and the gradient was as follows: 0-2 min – 20%B, 2-3 min – 20% to 80%B, 3-15 min – 80% to 100%B, 15-25 min – hold 100%B, 25-32 min – condition at 20%B.

The instrument used was a Thermo LTQ equipped with a Thermo Surveyor HPLC pump. Data were acquired by scanning the instrument from m/z 300 to 1200 at a scan rate of approximately 2 scans/s in positive ion ESI mode.

The FFA LC-MS platform employs the same sample and similar HPLC conditions as the lipid method. The ammonium acetate concentration is 2 mM instead of 5 mM and no formic acid was added. The gradient: 0-2 min – 30%B, 2-3 min – 30% to 70%B, 3-10 min – 70% to 100%B, 10-15 min – hold 100%B, 15-20 min – condition at 30%B. Detection of FFA is performed in negative ion ESI mode. Combined the two methods provide (semi)quantitative data for approximately 200 different identified lipids and FFA.

Each extract was injected three times (10 \( \mu l \)), once for the LC-MS FFA platform and two times for the LC-MS lipid platform. Furthermore, a Quality Control (QC) sample was prepared by pooling the samples. The pool was divided into 10 \( \mu l \) aliquots that were extracted the same as the study samples. The QC samples were placed at regular intervals in the analysis sequence (one QC after every 10 samples). The QC samples served two purposes. The first is a regular quality control sample to monitor the LC-MS response in time. After the response has been characterized, the QC samples were used as standards of unknown composition to calibrate the data.

In plasma samples, the 6 dominant lipid classes observed with these two methods are the lysophosphatidylcholines (IS used: C17:0 lyso-phosphatidylcholine), phosphatidylcholines (IS used: di-C12:0 phosphatidylcholine), sphingomyelins (IS used: di-C12:0 phosphatidylcholine), cholesterylesters (IS used: C17:0 cholesteryl- ester), triacylglycerols (IS used: tri-C17:0 glycerol ester), and free fatty acids (IS used: C17:0 FFA). (Figure 1, Annex 1). In addition to these lipids, the extracts also contain minor lipids, but these were either not detected (concentration too low relative to very abundant lipids like phosphatidylcholines and diacylglycerols, DG) or they were not included in data processing with exception of the DG. The LC-MS lipid and LC-MS FFA data were processed using the LC-Quan software (Thermo).

Definition of a Biomarker for the several compounds measured by LCMS techniques from the lipid fraction: At experimental animal (mouse) level a prerequisite for a biomarker is that it can be distinguished from the Control group fed a Control-Chow e.g. in comparison to our, for 40 days fed a High Fat diet for our C57Bl6 mouse model resulting in obese IR/T2DM animals e.g. by an elevated concentration in an organ or tissue:

A). So in terms of product-precursor ratio the metabolic route is obstructed, or the enzyme cannot convert in its metabolic route the precursor to its end product resulting in an accumulation of the precursor: the detected Biomarker. This means that the metabolic pathways or enzymes are blocked in this way resulting in an accumulation of the end product => the biomarker.

B). A second prerequisite is that it may not be diet induced which means that it may not be found in the Control-Chow or High-Fat diet.

C). A third requisite for a biomarker in the field is that organ/tissue sampling by biopsy is very uncomfortable, not practical and sometimes dangerous for e.g. a liver biopsy or impossible in case of heart muscle. Therefore the third precondition for a suitable biomarker is that it not only accumulates in the specific organ or tissue but also in the blood-plasma. So accumulation takes place because of a blockage in a specific pathway or metabolic route [25, 27, 28]. In case of Lipidomics these are the major metabolic pathways of n-3 and n-6 PUFA (see Figure 4). An approach to get an impression of enzyme activities (endogenous conversion of desaturases and elongases) in the liver (expressed in: change in hepatic expression) can be estimated from product-to-precursor ratios as given in below according to van [29] (see also Figure 5). Activity of desaturases and elongases of
the Cholesteryl esters (ChE) of the blood plasma fraction can be performed using the product-to-precursor ratios of individual LC-MS measured fatty acids as follows:

- C18:3\text{n}6/C18:2\text{n}6 ratio = Δ6-desaturase
- C20:3\text{n}6/C18:3\text{n}6 ratio = elongase
- C20:4\text{n}6/C20:3\text{n}6 ratio = Δ5-desaturase
- C16:1\text{n}7/C16:0 ratio = Δ9-desaturase

**D): Toxic lipid species:** According to our definition for a suitable biomarker toxic compounds are excluded from our bio-medical definition [30, 31]. Thus we exclude Sphingomyelin because it can be converted to the toxic Ceramide which can cause cellular apoptosis [25].

**Calculations and statistics:** For all parameter, the mean value of the control mice group was compared to the mean value of the fatty-diet group. Statistics were performed via SPSS [32] using a two-tailed T-Test for differences between the Control group and the Treatment (fatty-diet) group. P ≤ 0.05 was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov-Smirnov and Fmax tests, respectively.

**Results**

**Control Chow and the High-Fat Diet**

The chromatogram of a sample with run on the LC-MS with a Control-Chow food sample and a High-Fat diet food sample is displayed in Figure 6. Three groups of chemical compounds can be clearly distinguished in this Figure 6: A). After 9-11 minutes’ retention time the Lysophosphatidylcholines (LPC) become visible with at 11.5 minutes the Internal Standard Di-Lauroyl-Phosphatidylcholine (IS); B). After 13-16 minutes the Plasmalogens, Phosphatidyl Cholines (PC), Sphingomyelins (SPM) and Phosphatidylethanolamines (PE) become visible; and C). After 18-19 minutes the TG’s and Cholesteryl-Esters (ChE).

In Annex 1 are in detail the different lipid compounds for both diets given. The following highly significant differences between the two diets can be observed:

i. **1000%-10,000%**: The following compounds are in the range 1000%-10,000% higher in the High-Fat diet in comparison to the Control-Chow diet: C26-1-DG, C36-0-DG, C22-0-SPM, C44-2-TG, C44-3-TG, C46-3-TG, C46-4-TG, C48-0-TG, C48-3-TG, C48-4-TG, C50-1-TG, C50-2-TG, C50-3-TG, C50-4-TG, C52-1-TG, C54-2-TG, C56-1-TG, C56-9-TG.

ii. **> 10,000%**: The following compounds are in the range > 10,000% higher in the High-Fat diet in comparison to the Control-Chow diet: C44-0-TG, C44-1-TG, C46-0-TG, C46-1-TG, C46-2-TG, C48-1-TG, C48-2-TG, C50-0-TG, C54-1-TG.

So comparing the diet composition of the two used diets for lipid compounds (Annex 1), we can conclude that the major differences in the High-Fat diet, in comparison to the Control-Chow diet, are mainly in the TG’s compounds and mainly the elongated ones (>10,000% increase).

**LC-MS-results**

The chromatogram of mouse liver (Control animal) is displayed in Figure 7. Three groups of chemical compounds can be clearly distinguished in this Figure 7: A). After 9-11 minutes’ retention time the Lysophosphatidylcholines (LPC) become visible with at 11.5 minutes the Internal Standard Di-Lauroyl-Phosphatidylcholine (IS); B). After 13-16 minutes the Plasmalogens, Phosphatidyl Cholines (PC), Sphingomyelins (SPM) and Phosphatidylethanolamines (PE) become visible; and C). After 18-19 minutes the TG’s and Cholesteryl-Esters (ChE).

**Figure 5:** Elongase- and Desaturase- activity of enzymes based on product-to-precursor ratios of individual LC-MS measurements of fatty acids [30, 31].

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In this study, we observed clearly tissue specific differences between liver in comparison to blood-plasma in the High Fat (HF) diet group in comparison to the control group. As described in the result section (Annex 2 & 3), these differences consider mainly Cholesteryl Esters (ChE), Lysophosphatidylcholines (LPC), Phosphatidylcholines (PC) and Sphingomyelin (SPM) but due to interconversions and/or toxic compounds we clearly restricted the definition of a bio-medical biomarker. Comparisons of LPC, PC and SPM show no general pattern. Sometimes these compounds were significantly higher or lower in the several non-adipose tissues and organs. Related to hepatic enzymatic activity as defined under M & M we will concentrate on elongase activity because this will result in increased fat and a “fatty liver” (hepatic steatosis). From table 2 we see that under conditions of High Fat diet elongase activity is significantly (P<0.00023**) increased with 309%. So
High-Fat diet conditions lead to fat formation according to elongase/desaturase enzymatic activity (see Table 2).

Biomarkers

Non-alcoholic steatohepatitis is part of a disease spectrum, non-alcoholic fatty liver disease, ranging from simple steatosis to cirrhosis, which is the most frequent cause of abnormal liver tests. There is clinical and epidemiological evidence that non-alcoholic fatty liver disease is the hepatic manifestation of the metabolic syndrome and finally can result in Hepatocellular Carcinoma (HCC). Therefore, new biomarkers as a result of hepatic steatosis [25,27,28] might be applicable under both conditions (Table 1). This study was designed to find via a High-Fat (HF) diet induced Insulin Resistant (IR) and/or Type-2 diabetes (T2DM) C57Bl/6 mouse model potential novel biomarkers. Major aiming was to find following this lipidomics based approach novel safe biomarkers applicable for humans with Hepatocellular Carcinoma (HCC) that can be used in the assessment of diagnosis, intensive treatment, clinical use and new drug development. In addition the biomarker has to be found in blood-plasma simultaneously while is not a component of the HF-diet.

In Figure 8 is the whole factor spectrum depicted of the five major lipid groups of the LCMS measurements following a Systems Biology lipidomics based approach of the lipid fractions in blood plasma in the comparison of a high fat diet C57bl6 mouse model versus a control group.

From figure 9 we can see that the only molecular weight compound that meets the previously set requirements for a biomarker is C36:1 Phosphatidylcholine. The concentration in the blood of this 36:1 PC of the control group is marginal namely 0.508 ± 0.104 while that of the high fat diet group 7584 ± 0481 is a very strong significance of P <0.00001 and a concentration difference in rates of this biomarker in blood plasma 1493.3% in the high fat diet group. Other PCs are also potential candidates for novel biomarkers for “fatty liver” in this C57bl6 mouse model such as C32:1 PC, C34:1 PC and C38:2 PC.

Related to hepatic enzymatic activity as defined under M & M we will concentrate on elongase activity because this will...
result in increased fat $\Rightarrow$ White Adipose Tissue (WAT). From table 2 we see that under High-Fat diet conditions hepatic elongase activity significantly ($P<0.0023^{***}$) increased with 309%. So High-Fat diet conditions lead to fat formation according to elongase/desaturase enzymatic activity (see Figure 5 & Table 2).

**Discussion**

Liver disease is a growing global health problem, as deaths from end-stage liver cirrhosis and cancer are rising across the world. At present, pharmacologic approaches to effectively treat or prevent liver disease are extremely limited. The prevalence of Hepatocellular Carcinoma (HCC) worldwide parallels that of persistent infection with the Hepatitis B Virus (HBV) and/or Hepatitis C Virus (HCV). In the United States, about 26,000 to 27,000 cases of liver cancer are diagnosed each year, and that number is increasing year after year [2]. In fact, HCC is one of the few cancers that is increasing in incidence in the United States possibly as a consequence of obesity and type 2 diabetes. Obesity has emerged as an important risk factor as it can lead to steatohepatitis [22, 23]. In addition, Type-2 Diabetes (T2DM) increases the risk of HCC [23]. The overall cancer incidence of liver in nonalcoholic FLD has not yet been quantified, but the association is well-established [24]. Therefore, we can conclude obesity is associated with the incidence and mortality of HCC although the Cartesian model for the mechanism of obesity induced liver disease needs further to be investigated and proven. For the establishment of such a model, figure 10 can be very useful.

According to recommendations by the World Health Organization guidelines for HBV/HCV, Alpha-Fetoprotein (AFP) testing and abdominal ultrasound should be performed in routine surveillance of HCC every 6 mo for high-risk patients. These examinations have also been recommended worldwide by many other HCC guidelines over the past few decades. In recent years, however, the role of AFP in HCC surveillance and diagnosis has diminished due to advances in imaging modalities [33] AFP was excluded from the surveillance and/or diagnostic criteria in the HCC guidelines published by the American Association for the Study of Liver Diseases in 2010, the European Association for the Study of the Liver in 2012, and the National Comprehensive Cancer Network in 2014 [34]. More frequent non-invasive surveillance for HCC via blood biomarkers may be warranted in obese patients with fatty liver and attempts should be made to interrupt the progression from simple hepatic steatosis to steatohepatitis, cirrhosis and ultimately HCC.
Other biomarkers, including theLens culinarisagglutinin-reactive fraction of AFP (AFP-L3), des-γ-carboxyprothrombin, Dickkopf-1, midkine, and microRNA, are being studied in this regard [33].

In the next section we will outline our “hypoxia model” in the transition from NAFL/NAFDL/Chronic Liver Disease (CLD) towards Hepatocellular Carcinoma (HCC) based on some biochemical modelling [30, 31]. In order to conserve the energy from glucose as ATP, three major metabolic pathways are involved in the mitochondria (Figure 11), directly linked to the oxidative phosphorylation outside in the cytosol. The glycolysis, the Krebs cycle and the β-oxidation needs to maintain their redox-balance which is on one hand performed lipid synthesis and by “reversed β oxidation” (fatty chain elongation) also leading to fat synthesis => so a vicious circle is observed during severe obesity [30, 31].

We stated earlier and depicted in Figure 4 that in “fatty liver” ischemic and/or hypoxic conditions may occur. Under these conditions it can be questioned how the Krebs cycle persist in its activity and the redox balance maintained. Since the early work at hypoxic conditions at fish clearly reviewed and outlined by [37] and recently in tumor research [38, 39] two hypothesis has been postulated related to these two research topics: fatty chain elongation and anaerobic endogenous reduction of unsaturated fatty acids [8, 31]. We observed in this research manuscript based on product-to-precursor ratios significant high (P<0.00023**) elongase activity (see Table 2) which increased significantly with 309% under High-Fat diet conditions.

In earlier work these Fatty chain elongation data are given an explanation mentioned following below mentioned described mechanism of reversed β-oxidation to maintain the redox balance and keep the Krebs cycle spinning resulting...
in anaerobic Fatty chain elongation and consequently fat (WAT) formation [25].

The concept of anaerobic fatty chain elongation involves the coupling of acetyl-CoA units to fatty acids via a reversal of the β-oxidation. The first three reactions are identical to the β-oxidation. However, the last reaction is catalyzed by the NAD-dependent enoyl-CoA reductase instead of the Co-Q dependent acetyl-Co-A dehydrogenase. In this way, the reaction is thermodynamically favored in the direction of chain elongation which corresponds to fat synthesis [40]. Since fatty chain elongation consumes 2 moles of NADH in each cycle, this pathway provides a suitable mechanism to maintain the mitochondrial redox balance [30, 31] see Figure 11. Indeed, fatty acid chain elongation (i.e. lipid synthesis) during anoxia could be stimulated in vitro by addition of Krebs-cycle intermediates such as glutamine [39] which is indicative for the redox coupling between Krebs-cycle activity and fatty acid chain elongation or “fat formation” (WAT) [40, 41, 42] (figure 12).

Fatty acid chain elongation (i.e. lipid synthesis) during anoxia/ischemia has been demonstrated in various organisms starting with invertebrate models and finding its way towards biomedicine [43]. But most important for cardiovascular diseases FA chain elongation has been observed in ischemic arterial tissue [45] and ischemic mammalian heart [46]. In the following section the lipid metabolism has been proposed as a suitable mechanism to maintain redox balance in anoxic tolerant invertebrates [43].

However, hypoxia may have also adverse effects on lipid metabolism, resulting in a decreased cellular functional integrity and eventually tissue damage. Especially in mammalian tissues being essentially hypoxia intolerant, these effects have received large attention [47].

When oxygen availability is decreased, the oxidative phosphorylation is inhibited and as a result of the accumulating reducing equivalents (NADH, FADH₂), the fluxes through the Krebs-cycle and β-oxidation are impaired. Since the flux through the β-oxidation is high during normoxic conditions [30, 31]. Fatty acids and their metabolites rapidly accumulate during anaerobiosis [47]. In addition, during hypoxia or ischemia, the cellular energy status may be affected. A number of ionic rearrangements occur due to the inhibition of ATP-dependent membrane associated ion pumps and intracellular acidosis. This may activate phospholipases which results in a marked hydrolysis of membrane lipids [29, 30, 37, 47].

In an earlier review related to cancer we stated that fatty acid synthesis is a paradigm of hepatic biosynthetic pathways because it requires the use of a Krebs cycle intermediate (citrate) that might otherwise be oxidized in the mitochondria. The shunting of metabolites from the Krebs cycle into other pathways (cataplerosis) is part of the fundamental biochemistry of cell growth, and it emphasizes the versatility of the Krebs cycle: rather than serving a purely oxidative function as a source of reducing equivalents for the Electron Transport Chain (ETC), it can also be used as a continuous source of precursor molecules for biosynthetic pathways [26].

Early characterization of cataplerosis in highly lipogenic hepatoma cells led to the concept of a “broken” or “truncated” Krebs cycle (figure 13) because of an apparent impairment in citrate oxidation and the demonstration that the rate of citrate export was directly proportional to the rate of cell proliferation [48]. In an earlier manuscript we demonstrated a “solution” in an obese C57 mouse model of reversed β-oxidation in order to maintain the redox balance and keep the Krebs cycle spinning [25]. This model –including fatty chain elongation- ultimately leads to fat (WAT) formation including –as we hypothesize- hepatic steatosis leading to Hepatocellular Carcinoma (HCC) or liver cancer.

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**Figure 12:** Schematic representation of the redox coupling between the Krebs cycle and fatty acid chain elongation. (1): Normal β-oxidation; (2): reversed β-oxidation (modified: [37]).
**Perspectives**

Screening for liver disease is the first step, including surveillance, or ongoing testing, of patients with HBV, particularly Asian men who are older than 40 years, women older than 50 years with chronic HBV infection, and patients with cirrhosis or HCV infection with cirrhosis. A rather new aspect of HCC is that it probably also evolves from metabolic syndrome/obesity/type 2 diabetes and surveillance and screening of these individuals with a BMI > 30 –especially in the US and Europe where obesity is a pandemic- with new “easy” blood biomarkers is an essence.

We gave in this research publication a biochemical model which supports our observations of hepatic steatosis. Still the transition from benign (hepatic steatosis) towards malign (HCC) is still a research area of speculation and the final prove for this transition has to be given in a mouse model.

More frequent surveillance for HCC may be warranted in obese patients with fatty liver and attempt should be made to interrupt the progression from simple hepatic steatosis to steatohepatitis, cirrhosis and ultimately HCC. Metabolomics—especially lipidomics—provides a functional read-out of the physiological status of an organism [49, 50] and when an appropriate biomarker for a complex human disease like a Hepatocarcinoom (HCC) is found it can lead to a “Personalized medical treatment” [49,50] were per individual the effects of nutritional intervention exercise protocol or drug therapy should be considered as part of a lifestyle strategy to prevent or manage some types of cancer [8].

Furthermore, efforts to target the Medical Systems Biology Lipidomics based approach in oncology must consider the influence of the hypoxic tumor microenvironment and the role of [51] cytokine release and of lactic acid [26] in the pathogenesis and sarcomatous change and metastasis.

**Conflicts of interest: None**

**References**


![Figure 13: Spinning Krebs cycle.](image-url)
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