Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE E FARMACOLOGIA

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COORDINATORE Prof. Francesco Di Virgilio, MD.

Early metformin intervention prevents hepatocellular carcinoma in a carbon tetrachloride induced mouse model of cirrhosis.

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Dottorando
Dott. Ram Charan Shankaraiah, MD.

(firma)

Tutore
Prof. Massimo Negrini, PhD.

(firma)

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Early metformin intervention prevents hepatocellular carcinoma in a carbon tetrachloride induced mouse model of cirrhosis

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RAM CHARAN SHANKARAIAH

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PROGRAMME CO-ORDINATOR: PROF. FRANCESCO DI VIRGILIO, MD.

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To my Parents and Mentors

Early metformin intervention prevents hepatocellular carcinoma in a carbon tetrachloride induced mouse model of cirrhosis

ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and one of the most deadly cancers. Oncogenic transformation is generally ubiquitous in chronic liver disease, often as a consequence of liver fibrosis and cirrhosis. Routine and readily available laboratory investigations can identify at-risk for HCC cohorts. Because of abysmal prognosis of HCC, these patients are best candidates for chemoprevention. Metformin is an inexpensive, dimethylbiguanide hypoglycaemic agent used to treat type 2 diabetes mellitus patients (DM2) with a broad safety profile. Previous epidemiological studies have shown that DM2 is a major risk factor for development of HCC. However, in metformin treated diabetic cohorts, there has been significant reduction in cancer incidence and mortality. We hypothesized that early chemoprevention with metformin in non-diabetic liver fibrosis setting could decrease neoplastic transformation of hepatocytes.

Methods: Liver fibrosis and cirrhosis was induced in transgenic microRNA 221 overexpressing mice by chronic administration of carbon tetrachloride. Metformin intervention was initiated at the stage of early fibrosis in these mice. Using intravital imaging, molecular techniques and histological analysis we determined fibrosis, steatosis, incidence of tumours, apoptosis and proliferation in liver parenchyma, AMPK and AKT signalling. Data were analysed with one-way analysis of variance and unpaired student t-test. All statistical tests were two-sided.

Results: Early metformin intervention abrogated growth of tumours in TG221 mouse models of HCC with cirrhotic background. Metformin treated animals displayed 1) reduced liver fibrosis as evidenced by Masson's trichrome staining, 2) decreased steatosis and hepatic lipid content (Oil red O, $P < 0.01$ *vs* un-treated), 3) improved liver function and 4) reduced incidence of surface liver nodules (92% reduction $P < 0.0001$ *vs* un-treated). Metformin mediated AMPK activation, AKT pathway inhibition, global upregulation of microRNAs and induction of autophagy.

Conclusion: These data suggest that metformin interrupts more than one node in initiation of HCC, mitigates progression of chronic liver disease and as consequence confers hepatoprotective effects.

RIASSUNTO

Background: Il carcinoma epatocellulare (HCC) è il quinto tumore maligno più frequente al mondo ed uno dei più mortali. La trasformazione oncogenica è generalmente sempre presente nella malattia epatica cronica, spesso come conseguenza della fibrosi epatica e della cirrosi. Le indagini di laboratorio che si effettuano di routine e quelle normalmente disponibili possono identificare pazienti a rischio per HCC. A causa della prognosi infausto di HCC, questi pazienti sono i migliori candidati per la chemio prevenzione. La metformina è un agente ipoglicemico dimetilbiguanide economico, utilizzato per il trattamento di pazienti con diabete mellito di tipo 2 (DM2) e con un ampio profilo di sicurezza. Precedenti studi epidemiologici hanno dimostrato che il DM2 è un importante fattore di rischio per lo sviluppo dell'HCC. Tuttavia, in coorti diabetiche trattate con metformina, lì è stata una significativa riduzione dell'incidenza e della mortalità del cancro. Abbiamo ipotizzato che la precoce chemio prevenzione con metformina nella regolazione della fibrosi epatica non diabetica potrebbe ridurre la trasformazione neoplastica degli epatociti.

Metodi: La fibrosi epatica e la cirrosi sono state indotte in topi transgenici che sovra-esprimono il microRNA-221, mediante somministrazione cronica di tetracloruro di carbonio. L'utilizzo della metformina è stato introdotto nella fase precoce di sviluppo della fibrosi, in questi topi. Utilizzando tecniche di imaging intravitale, tecniche molecolari e analisi istologiche, abbiamo valutato la presenza di: fibrosi, steatosi, incidenza dei tumori, apoptosi , proliferazione nel parenchima epatico e la via di segnalazione di AMPK e AKT. I dati ottenuti sono stati analizzati tramite analisi della varianza e test t di Student per valori non appaiati. Tutti i test statistici utilizzati sono di tipo a due code.

Risultati: l'utilizzo precoce della metformina ha causato la soppressione della crescita dei tumori nei modelli di topo TG221 di carcinoma epatocellulare con cirrosi indotta. Gli animali trattati con metformina mostrano: 1) riduzione della fibrosi epatica, come evidenziato dalla colorazione tricromica di Masson; 2) diminuzione della steatosi e del contenuto lipidico epatico (olio rosso O, P <0,01 *vs* non trattato); 3) miglioramento della funzionalità epatica; 4) ridotta incidenza di noduli epatici superficiali (92%riduzione P <0,0001 *vs* non trattato). Inoltre la metformina induce attivazione di AMPK, inibizione del pathway AKT, sovra-regolazione globale dei microRNA e induzione di autofagia.

Conclusioni: questi dati suggeriscono che la metformina agisce interrompendo più di un nodo nella fase precoce del carcinoma epatocellulare, attenuando la progressione della malattia epatica cronica e quindi conferendo effetti epatoprotettivi.

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List of Abbreviations

18F-FDG 2-deoxy-2-fluorine-18fluoro-D-glucose
ACC Acetyl-CoA carboxylase
ATP Adenosine triphosphate
ALT Alanine transaminase
Aldh18a1 Aldehyde dehydrogenase 18 family member A1
AST Aspartate transaminase
Abcc1 ATP binding cassette subfamily C member 1
ATG Autophagy-related gene
BCLC Barcelona Clinic Liver Cancer
BC Bladder cancer
CAFs Cancer associated fibroblasts
Capg Capping actin protein-Gelsolin like
CCl4 Carbon tetrachloride
ICC Cholangiocarcinomas
CHB Chronic hepatitis B
CHC Chronic hepatitis C
CT Computed tomography
COX Cytochrome c oxidase
SCO2 Cytochrome c oxidase 2
DAA Direct-acting antiviral
DALY Disability-Adjusted Life Year
EGFR Epidermal growth factor receptor
EMT Epithelial to Mesenchymal Transition
ECM Extracellular matrix
GSEA Gene Set Enrichment Analysis
GBM Glioblastoma multiforme
GLUT, Glucose transporter
GS Glutamine synthetase

GPC3 Glypican-3
HMIT, H+/myo-inositol transporter
HSP70 Heat shock protein 70
HBeAg Hepatitis B e-antigen
HBsAg Hepatitis B surface antigen
HBV Hepatitis B virus
HCV Hepatitis C virus
HCC Hepatocellular carcinoma
HK Hexokinase
HOTAIR HOX transcript antisense RNA
Her2 Human epidermal growth factor receptor 2
HIF1 Hypoxia-inducible factor 1
IRS Insulin receptor substrate
IGFR Insulin-like growth factor receptor
LDH Lactate dehydrogenase
LI-RADS Liver Imaging Reporting and Data System
LKB1 Liver kinase B1
lncRNA Long non coding RNA
Ly6d Lymphocyte antigen 6 family member D
LPA Lysophosphatidic acid
mTOR Mammalian target of rapamycin
mTORC1 Mammalian target of rapamycin complex 1
mTORC2 Mammalian target of rapamycin complex 2
MEG3 Maternally expressed gene 3
mRNA Messenger RNA
miRNA MicroRNA
MCTS Multicellular tumor spheroid
NADPH Nicotinamide adenine dinucleotide phosphate
NADPH Nicotinamide adenine dinucleotide phosphate
NAFLD Non-alcoholic fatty liver disease
ncRNA Non-coding RNA
ORO Oil Red O
OS Osteosarcoma
OXPHOS, Oxidative phosphorylation
Panx1 Pannexin 1
PPP; Pentose phosphate pathway
PTEN Phosphatase and tensin homolog
PIP3 Phosphatidylinositol-3,4,5-triphosphate

PIP2 Phosphatidylinositol-4,5-biphosphate
PEP Phosphoenolpyruvate
PFK1 Phosphofructokinase 1
PFKFB Phosphofructokinase 2-F2,6Bpase
PGM Phosphoglycerate mutase
PI3K Phosphoinositide 3-kinase
PDPK1 Phosphoinositide-dependent protein kinase 1
piRNA, PIWI-interacting RNA
PET Positron emission tomography
PTENP1 PTEN pseudogene
Pkm Pyruvate kinase
PKM1 Pyruvate kinase M1
PKM2 Pyruvate kinase M2
RAS Rat Sarcoma viral oncogene
RTK Receptor tyrosine kinase
RR Relative risk
RCC. Renal cell carcinoma
STAT3 Signal transducer and activator pf transcription 3
siRNA, Small interfering RNA
snoRNA Small nucleolar RNA
SVR Sustained virological response
TIGAR TP53-induced glycolysis and apoptosis regulator gene
tiRNA Transcription initiation RNA
TG221 Transgenic microRNA 221 overexpressing mice
TP53 Tumour protein P53
DM2 Type 2 diabetes mellitus patients
UCA1/CUDR Urothelial cancer-associated 1
WRAP53 WD repeat containing antisense of p53
WHO World Health Organization
YLD Years Lost due to Disability
YLL Years of Life Lost

An investment in knowledge pays the best interest

Benjamin Franklin

1

Introduction and background

1.1 Hepatobiliary cancers

Hepatobiliary cancers, commonly termed as liver cancers, constitute malignant tumours originating from liver, bile ducts and gallbladder. Liver cancer is the sixth most common cause of cancer worldwide. Although globally, incidence of liver cancers has declined by 1.8% from 1990 to 2013 [1], yet nearly 746,000 deaths or 9% of all cancer related deaths as of 2012 is attributed to liver cancers (Figure 1.1.1) [2, 3]. Liver cancers are more common in men (1 in 38) than women (1 in 111) globally and burden of disease is predominant in developing and less developed countries like China, Mongolia, Southeast-Asia and Africa (Sub-Saharan, Western and Eastern), accounting for nearly 83% of global liver cancers diagnosis. The prevalence of liver cancer in developed countries is lower except for countries like Japan, France and Italy [4]. Global liver cancer associated mortality rates follow the same trends as incidence and prevalence of the disease (Figure 1.1.2) [5, 6].

Substantial morbidity is usually associated post diagnosis and during management of cancer across population. Disability-Adjusted Life Year (DALY) is one such measure to quantify burden of disease from morbidity and mortality. One DALY signifies, one year lost of general healthy

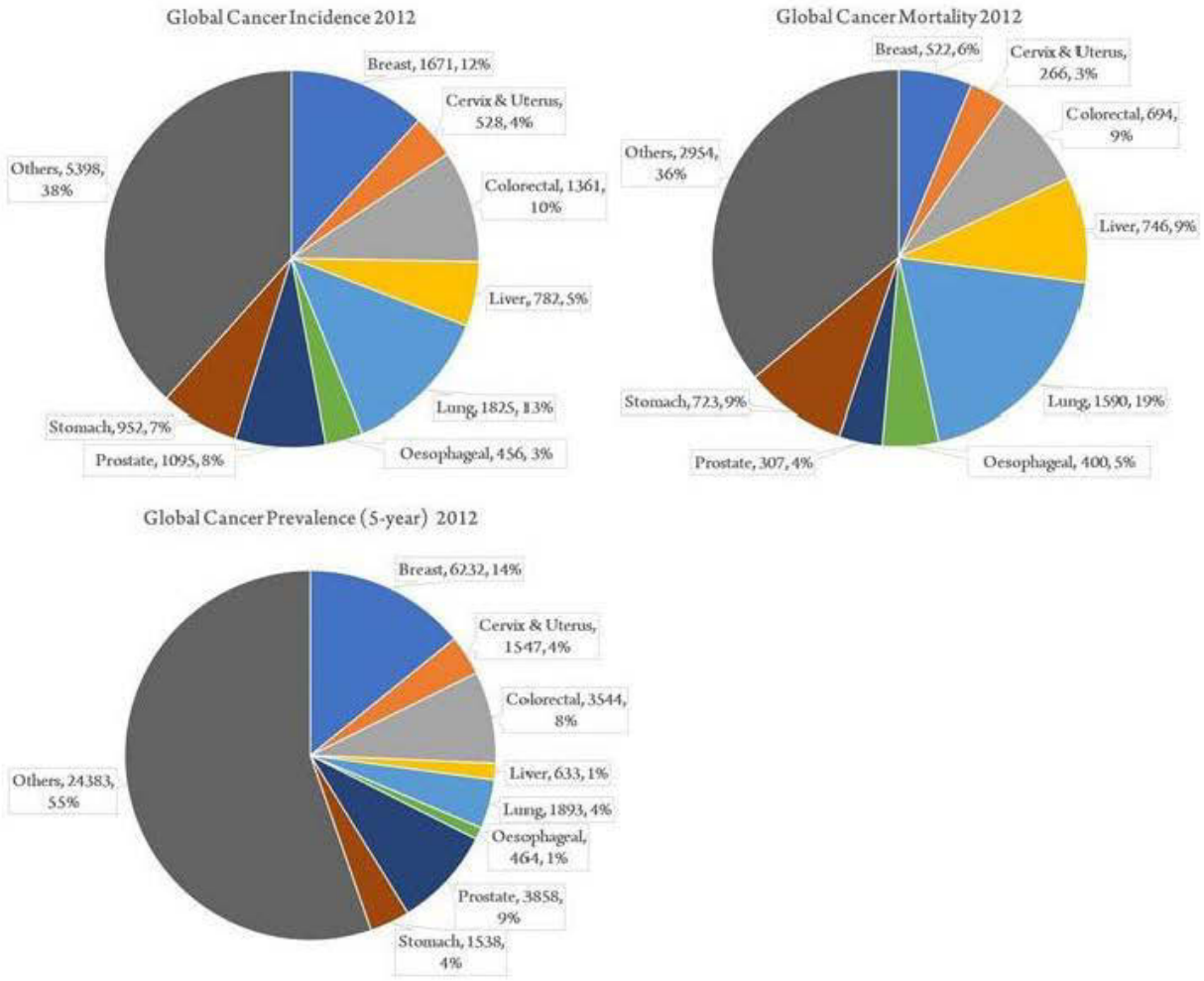


Figure 1.1.1: Pie charts showing incidence, mortality and 5-year prevalence of most common cancer types. Pie chart slice annotations are in the following order 'cancer type', 'number of events/100', '% of all cancer types'. Data source: GLOBOCAN 2012 (IARC) All cancers excluding non-melanoma skin cancer in both men and women global fact sheets.

life for any given disease globally. DALYs for any given disease is calculated as sum of Years of Life Lost (YLL) due to early mortality and Years Lost due to Disability (YLD) for patients diagnosed with the said disease in the population (DALY = YLL + YLD). Liver cancer cause 21million DALYs in 2016, with YLL accounting for 99% and YLD 1% [7]. Along with lung cancers, liver cancers amount to highest YLLs, which is a further testament to lethality of liver cancers in general population.

Primary malignant liver cancers include hepatocellular carcinomas (HCC), carcinomas of gallbladder, intrahepatic cholangiocarcinomas (ICC) and extrahepatic cholangiocarcinomas. Cholangicarcinomas originate in the epithelium of biliary tract. Vast majority of liver cancers are HCC with ICC accounting for most of the other subtypes. The two important risk factors for ICC include cirrhosis and chronic liver fluke parasite infestation. Endemicity of the latter in Thailand is one of the reasons for ICC being the most common subtype of liver cancer in that region [8].

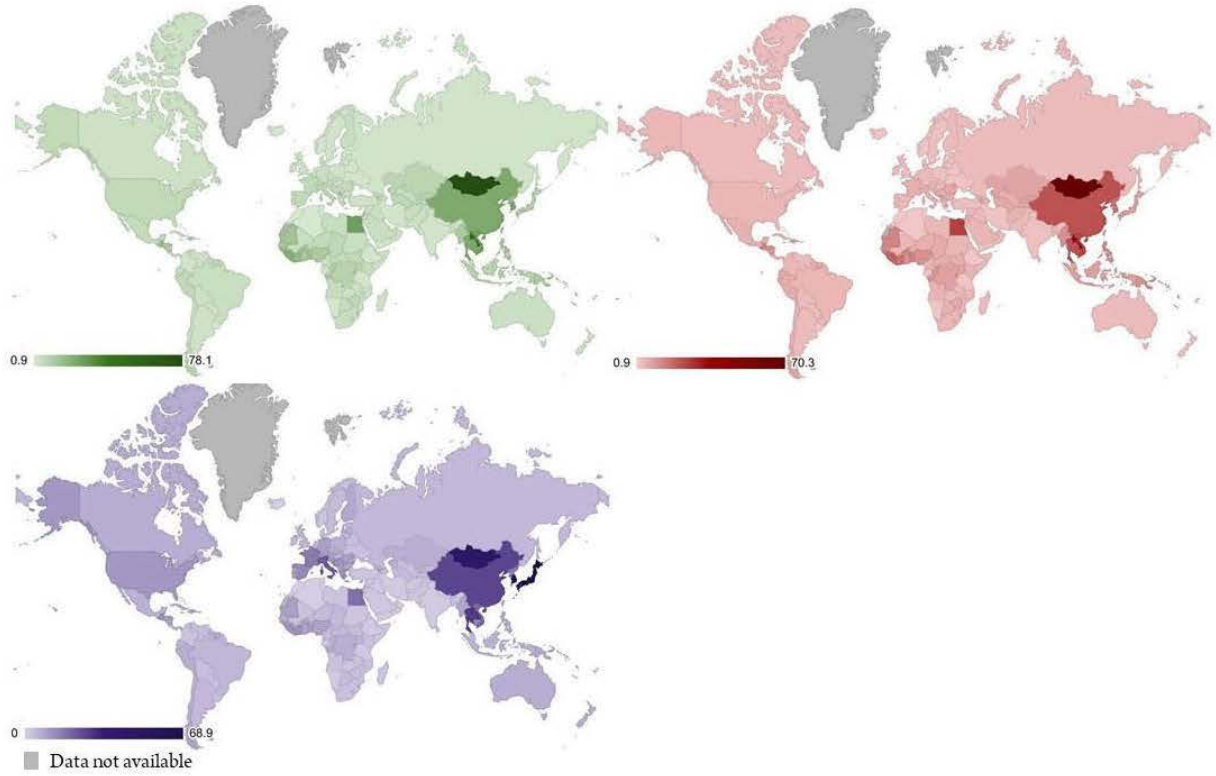


Figure 1.2.1: Heatmaps showing global liver cancer incidence, mortality and 5-year prevalence by geographic distribution. Green- incidence, red- mortality, lavender- prevalence, grey- data not available. Data source: GLOBOCAN 2012 (IARC)- Estimated age-standardized rates (World) of incident cases, both sexes, liver cancer, worldwide

1.2 Hepatocellular carcinoma

Hepatocellular carcinomas arise from hepatocytes in the liver and accounts for 85 to 90% of primary liver cancers. Burden of HCC is not evenly distributed worldwide. Majority of HCC cases (>80%) are documented in Eastern Asia and sub-Saharan Africa with China alone accounting for 50% of global HCC incidence [9]. This disparity in HCC occurrence not only depends on low sociodemographic indices of these regions but also on race and ethnicity. For example, in United States of America, a country with high sociodemographic index, incidence of HCC in Asians was 2 folds higher than white Hispanics and 4 folds higher than Caucasians [10]. The reasons for this ethnic disparity might include differences in acquisition time of risk factors for chronic liver disease leading to HCC.

1.2.1 Risk factors

About 80 to 90% of all HCC occurs within a background of chronic liver disease and cirrhosis. Broadly risk factors for cirrhosis in HCC patients can be dichotomised into viral and non-viral factors. Major viral risk factors include hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and are a global health problem resulting in acute and chronic hepatitis that can progress to liver cirrhosis and to HCC. Large population-based studies in chronic hepatitis B (CHB) and chronic hepatitis C (CHC) patients have identified high serum HBV DNA, HCV RNA viral load respectively, as independent risk factors for developing HCC [11, 12].

HBV, member of Hepadnaviridae family, is a double-stranded DNA virus that replicates via reverse transcription [13]. Unlike other retroviruses integration of viral DNA is not essential for HBV life cycle. Humans are the only natural hosts of HBV and its tissue tropism is limited to liver and particularly in hepatocytes, where they replicate via encoded proteins like polymerase, precore/core, envelope or surface proteins and X protein. Upon viral entry by endocytosis, double stranded viral DNA is converted to a covalently-closed-circular-DNA and transcribed into pregenomic RNAs and subgenomic messenger RNAs [14]. Three envelope proteins small, middle and large share identical C termini but vary in N-terminal extensions. The prcore/core open reading frame provides in-frame start codons encoding for HBV core protein and subsequently assembles into the nucleocapsid packaging of the HBV genome [15]. Exact function of HBV-X protein remains unclear and most studies suggest that it might be important in HBV replication in the host [16, 17]. The virus is transmitted through blood or other body fluids during sexual intercourse and injection-drug users and vertically from infected mother to the child. As few as 1 to 10 HBV virions are sufficient to cause infection. Once infected, manifestation of symptoms is age-dependent in acute stage. In children most infections are clinically silent. In adults, up to 30% of cases show transient jaundice and flu-like symptoms and in up to 70% of cases an increase in serum transaminases are documented as subclinical hepatitis [18]. Although in less than 1% of cases of acute HBV infection, fulminant hepatitis leading to liver failure occurs, yet these acute symptoms are usually short lived and self-limiting. Progression of CHB is often not apparent until cirrhosis or end-stage liver disease is diagnosed. Current clinical practice guidelines suggest utilizing a combination of hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) and serum HBV DNA levels to assess risk of HCC in CHB patients [19]. At the present time, there are no therapies to eliminate HBV infection. Management of chronic disease is aimed at slowing the process of liver decompensation and to decrease systemic viral load. Long term administration of nucleos(t)ide analogues like entecavir, tenofovir disoproxil and tenofovir alafenamide are treatment of choice since they inhibit reverse transcription and restrict HBV replication in majority of cases. Pegylated interferon-alfa might be considered in early stages of CHB and is associated with sever side affects. Typically, treatment is initiated at

HBV DNA >2,000 IU/ml and elevated levels of liver transaminases [20].

HCV, member of Flaviviridae family, is an encapsulated positive-sense single-stranded RNA virus. It has 5′ and 3′ nontranslated regions, a 5′ internal ribosome entry site for translation in cap-independent manner and is composed of a single open reading frame. This is translated to produce a large polyprotein of 3010 amino acids in length. The polyprotein is further cleaved by cellular and viral encoded proteases into mature structural and non-structural proteins. Structural proteins form the viral particle where as non-structural proteins (NS2, NS3, NS4A/B and NS5A/B) support viral genome replication [21]. Contrary to other flaviviruses that are arthropod borne, HCV spread is exclusively blood borne via sharing needles in drug users, needlestick injuries in healthcare providers and vertically from mother to the newborn. Less commonly HCV can be transmitted via unsafe sexual-intercourse. Replication of HCV occurs in the cytoplasm and the virus does not integrate into the host genome nor encodes for oncoproteins. In majority of cases, acute HCV infection is asymptomatic and only 15% of cases present with jaundice, elevated transaminases. In adults, 55 - 85% of cases develop CHC, with clinical course ranging from modest histopathological changes to fulminant hepatitis that progresses to liver fibrosis, cirrhosis and HCC. However, the progression of CHC to HCC takes several decades. Treatment regimens for HCV infection are aimed at curative outcome. Virological response means the virus is not detected upon treatment and efficacy of treatment is measured in terms of sustained virological response (SVR). With direct-acting antiviral (DAA) therapies becoming mainstay treatment options for HCV, outcome of HCV treatment has been optimistic. Several DAA agents (sofosbuvir, simeprevir, velpatasvir, glecaprevir, asabuvir, ombitasvir) have been approved and used alone or in combination, depending on HCV genotypes and other drug interactions with significant SVR rates of greater than 90% after eight to 12 weeks of treatment for almost all HCV genotypes [22]. It would be interesting to see long term effects of DAA therapies on HCC risk as high rates of SVR and eradication of HCV will tremendously impact on chronic liver disease progression and HCC incidence.

Non-viral risk factors for HCC include cirrhosis from varied cause (eg, alcoholic cirrhosis); obesity, type-2 diabetes mellitus; inherited errors of metabolism such as hereditary hemochromatosis, porphyria cutanea tarda, and alpha-1 antitrypsin deficiency; Wilson's disease [23]. Environmental exposure to aflatoxin, a natural product of Aspergillus fungus and excessive alcohol intake are other risk factors for HCC [24]. Studies evaluating incidence risk of HCC in alcoholic cirrhosis are confounded by the presence of other concomitant risk factors like CHB, CHC, obesity and type-2 diabetes mellitus (DM2) [25]. Furthermore, metabolic disorders [eg, obesity, DM2, impaired glucose metabolism, metabolic syndrome, non-alcoholic fatty liver disease (NAFLD)] are associated with increased risk of HCC [26].

Obesity is defined as abnormal or excessive accumulation of adipose tissue in the body that may

Cancer Type	BMI	RR
Liver	≥35	4.52
Pancreas	≥35	2.61
Stomach	≥35	1.94
Esophagus	≥30	1.91
Colon and rectum	≥35	1.84
Gallbladder	≥30	1.76
Multiple myeloma	≥35	1.71
Kidney	≥35	1.70
All other cancers	≥30	1.68
Non-Hodgkin's lymphoma	≥35	1.49
Prostate	≥35	1.34
All cancers	≥40	1.52

Table 1.2.1: Summary of cancer mortality type in men. Relative risk (RR) is compared between men in the highest BMI category with reference category (BMI 18.5 to 24.9). Results of the linear test for trend were significant ($P \leq 0.05$) for all cancer sites. Based on data from [31]. Reproduced with permission [298]

impair health. Incidence of obesity worldwide has nearly doubled since 1980. The increasing prevalence of obesity (body mass index, BMI ≥ 30 kg/m2) and overweight (BMI, ≥25 kg/m2) is a major public health concern. In 2008, The World Health Organization (WHO) estimated that

Cancer Type	BMI	RR
Uterus	≥40	6.25
Pancreas	≥35	2.61
Stomach	≥35	1.94
Esophagus	≥30	1.91
Colon and rectum	≥35	1.84
Gallbladder	≥30	1.76
Multiple myeloma	≥35	1.71
Kidney	≥35	1.70
All other cancers	≥30	1.68
Non-Hodgkin's lymphoma	≥35	1.49
Prostate	≥35	1.34
All cancers	≥40	1.52

Table 1.2.2: Summary of cancer mortality type in women. Relative risk (RR) is compared between women in the highest BMI category with reference category (BMI 18.5 to 24.9). Results of the linear test for trend were significant ($P \leq 0.05$) for all cancer sites. Based on data from [31].Reproduced with permission [298]

more than 1.4 billion adults, 20 years of age and older, were overweight. Of these over 500 million were obese [27]. In United States alone for example 34.9% (95% CI, 32-37.9%) of

adults (age-adjusted) aged 20 years or older were obese [28]. Association of excess BMI with risks of coronary heart disease, hypertension and type-2 diabetes (DM2) has long been known. In the past decade numerous epidemiological studies have shown obesity and DM2 to be a risk factor for cancer [29, 30]. In USA, about 20% of all cancer deaths in men and 14% in women were documented in individuals with BMI $\geq$ 30. The relative risk (RR) of dying due to liver cancer in individuals with BMI $\geq$35 was highest among all cancers in men (4.52) (Table 1.2.1, 1.2.2). Similar to obesity, epidemiological association studies have documented an increased risk for HCC in DM2 patients(Table 1.2.3). Although some studies suggest DM2 to be an independent risk factor, synergism of obesity and DM2 have the strongest association [32-34].

Cancer Type	RR	Reference
Endometrial	2.74	[35]
HCC	2.50	[36]
Pancreatic	2.1	[37]
Colorectal	1.30	[38]
Bladder	1.24	[39]
Breast	1.24	[40]
Non-Hodgkin's lymphoma	1.19	[41]
All cancer types	1.16	[42]
Prostate	0.91	[43]

Table 1.2.3: Summary of cancer mortality in diabetic men or women. Relative risk (RR) is compared between case control or cohorts of diabetics with non-diabetics.

1.2.2 Diagnosis of HCC

For much of its natural course, HCC presents without any symptoms warranting further diagnostic tests. Prodromal symptoms associated with HCC can include weight loss, malaise, jaundice and upper quadrant abdominal pain. Physical signs of HCC might only include hepatomegaly and ascites. Therefore, image guided screening procedures are invaluable for early diagnosis.

I. HCC Screening

Cancer screening and surveillance procedures are of paramount importance to identify the presence of a specific cancer type in at risk individuals irrespective of symptomatology. Universal screening programs facilitate early detection and thereby early means of intervention has the potential to improve patient outcome. In a large randomized controlled trial of 18,816 individuals with history of chronic hepatitis or HBV infection, screening every 6 months with AFP and liver ultrasound was beneficial in reduction of (37%) mortality due to HCC [44]. Additionally, in

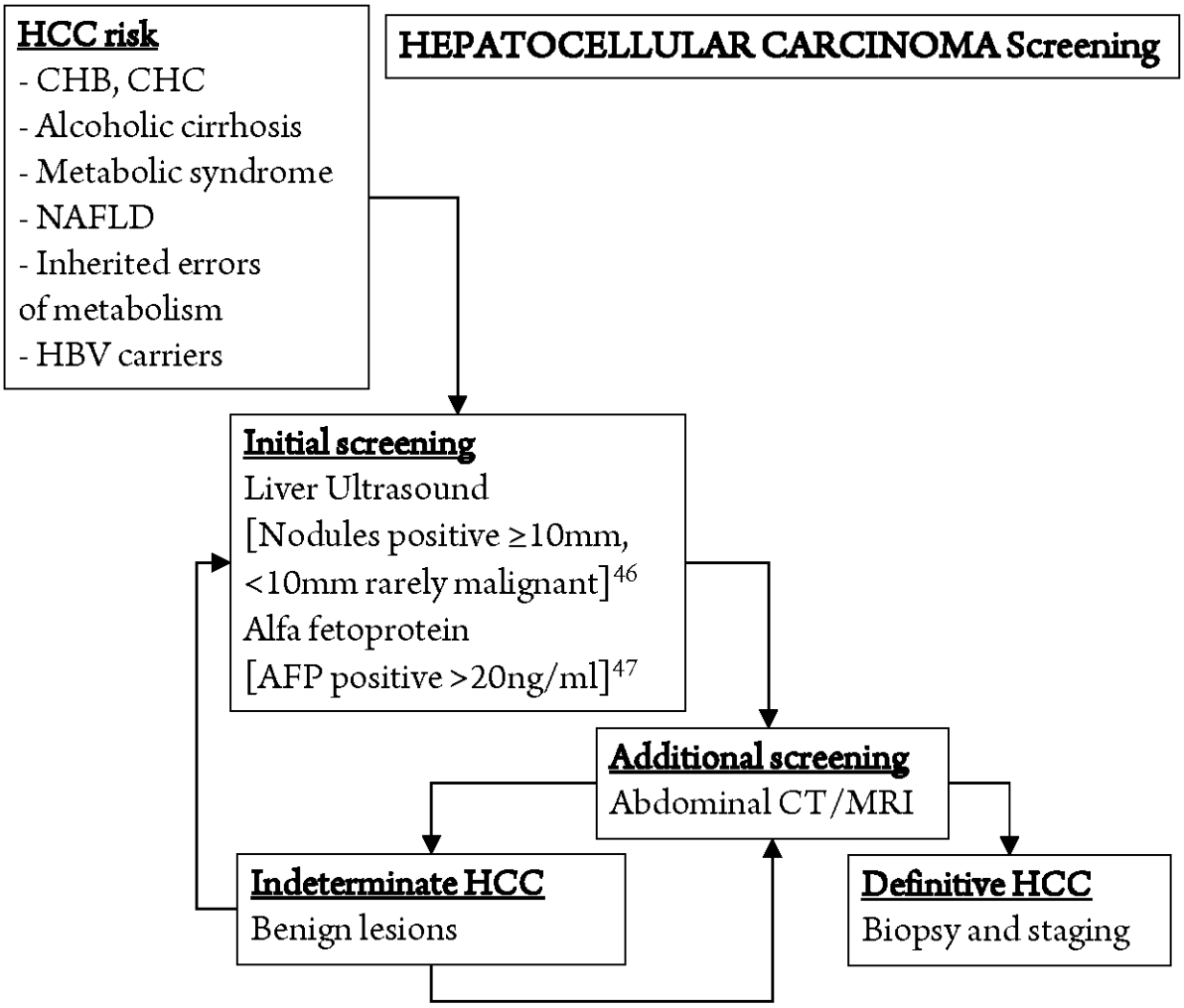


Figure 1.2.2: Typical screening and surveillance guidelines in HCC. Illustrations based on AASLD HCC management guidelines 2018 [48].

this study screening was not restricted to elderly individuals, which further reiterates necessity of screening programs based on presence of risk factors and not age alone [45].

II. Imaging

Unlike other solid tumours, imaging significantly contributes to establishing a diagnosis of HCC. Based on non-invasive imaging, treatment regimens are started. At-risk for HCC individuals after liver US surveillance are identified (as illustrated in Figure 1.2.3) and multiphase CT or MRI procedures are used for an initial diagnosis. Liver Imaging Reporting and Data System (LI-RADS) is used as most common diagnostic criteria to maintain consensus uniformity in imaging exam interpretation. LI-RADS categories are broadly based on several criteria (eg; diameter of liver mass, capsule appearance, threshold growth from previous imaging sessions,

etc.) that are integrated into an algorithm to define five categories [49]. Classically, on imaging, HCC lesions are characterised by intense arterial uptake or enhancement followed by contrast agent washout or hypo-intensity in the venous phase [50]. Most common benign lesions include hemangiomas, liver cysts, focal nodular hyperplasia that are most likely attributed to underlying cirrhosis. Non-definitive evaluation of liver nodules on CT or MRI warrants further biopsy or US surveillance for monitoring changes in nodule status. Contrast-enhanced ultrasound may be used for diagnosis of HCC; however, the procedure is limited by requirement of expert centers and operator dependent variability of US [51].

III. Biopsy

In the absence of cirrhosis, imaging protocols cannot be solely relied upon even when classical signs of HCC are present (intense arterial uptake and contrast washout) on CT or MRI. Biopsy is considered in such patients and standardized histological criteria for diagnosis of HCC have been developed [52]. Several immune-histochemistry biomarkers for delineating HCC from other dysplastic nodules have been proposed. Of these, a panel of three putative biomarkers, glypican-3 (GPC3), heat shock protein 70 (HSP70), and glutamine synthetase (GS) were found to have 72%, 100% sensitivity and specificity respectively when at least two of the markers were identified in surgically resected liver mass in differentiating HCC from non-malignant nodules [53]. Classical histological features of HCC include hepatocellular proliferation arranged in trabecular or acinar/psuedoglandular pattern with lack of portal tracts, increased arterialisation within the tumours, bile production, loss of reticulin network, deposition of fibrin and irregular liver architecture. Within the tumour cells Mallory-bodies (hyaline) can also be present [54].

IV. Serum biomarkers

Serum AFP has long been utilised as a marker for HCC. Although, it lacks sensitivity or specificity for HCC, combination of AFP and liver US for surveillance has been cost effective in a public health setting regardless of HCC incidence [55]. AFP > 20ng/ml is considered positive and negative if lower. Further, AFP can be elevated in other liver related malignancies like intrahepatic cholangiocarcinoma and liver metastases from colon cancer [12]. Other serum biomarkers have been previously studied but in multiple retrospective case control studies, they have not been found to be anymore effective than AFP (eg; des-gamma-carboxy prothrombin and an isoform of AFP- lens culinaris agglutinin-reactive AFP) [56-59]. Therefore, there is an urgent unmet medical need for blood-based biomarkers for HCC. To this end, cell-free DNA/microRNA based sequencing technologies might have considerable potential as biomarkers for both diag-

nosis and prognostication [60, 61].

1.2.3 HCC STAGING

Cancer staging systems are aimed at guiding treatment regimens based on prognostication, tumour burden and dissemination of tumour cells to other tissue or organs. Unlike other solid cancers, formulating an universally applicable staging system has been challenging cause underlying liver disease significantly affects therapeutic approach and prognosis [62]. Several staging systems have been in practise and are tailored to reflect geographically limited risk factors. Largely, classical American Joint Committee on Cancer-TNM classification (Edition-8) and Barcelona Clinic Liver Cancer (BCLC) are the most utilised staging systems [63, 64]. TNM classification relies on tumour size, presence or absence of lymph node tumour dissemination and distant metastasis. Further anatomic stages and prognostic groups are determined by histological grade and fibrosis score. BCLC incorporates Child-Pugh classification as a measure of liver function, tumour assessment depending on size and number of tumours, vascular invasion and evaluation of metastasis to categorize patients into five stages (0, A, B, C & D). Child-Pugh scoring takes into account clinical measure of liver disease like total bilirubin, serum albumin, prothrombin time and degree of ascites and hepatic encephalopathy to classify degree of cirrhosis into three classes (A, B & C) [65].

1.2.4 TREATMENT OPTIONS IN HCC

Treatment of HCC patients involves a multidisciplinary approach involving hepatologists, oncologists, transplant surgeons, radiologists and pathologists. Based on HCC staging and patients' liver status, treatment options can be generally divided into curative and non-curative management. Curative therapies in early stages of HCC (BCLC stage 0 to stage A) constitute surgical resection, orthotopic liver transplantation and tumour ablation techniques using microwave energy and radiofrequency. In advanced stages based on tumour burden and liver function status, non-curative therapies are undertaken to prolong survival and improve quality of life by slowing tumour progression. These therapy options include transarterial chemoembolization, transarterial radioembolization, stereotactic body radiation therapy, molecular targeted therapy, namely Sorafenib (Nexavar®) and Regorafenib (Stivarga®) and immunotherapeutic anti-PD-1 antibody Nivolumab (Opdivo®)[48].

Although termed curative, risk of recurrence in post surgical resection is about 70% at 5years [66]. There are currently no adjuvant therapies to prevent recurrence. A randomized phase III trial compared feasibility of adjuvant therapy with Sorafenib post complete resection or ablation.

However, Sorafenib had no impact on median resection free survival when compared to placebo (33.3 months- Sorafenib group, 33.7 months- placebo group) [67].

Sorafenib, an oral multi-kinase inhibitor, is the standard of care in advanced HCC patients with or without extrahepatic dissemination. However, a meagre 3 months overall survival benefit was demonstrated when compared to placebo [68]. Regorafenib, another oral multi-kinase inhibitor that has potent anti-angiogenic activity targets VEGFR2-TIE2 tyrosine kinases was FDA approved as second-line therapy in HCC patients. However, in randomised clinical trials, the overall benefit was about 3 months when compares to placebo group [69].

Nivolumab was approved by FDA for HCC treatment in patients who were previously treated with Sorafenib (second-line). In a recently concluded phase 1/2, Nivolumab treatment resulted in significant tumour reduction and objective response rates of 15-20% was achieved [70].

1.3 Pathophysiology of HCC

Pathophysiology of HCC initiation and progression are poorly understood. Undoubtedly, underlying chronic liver disease is a major disruptor of tissue homeostasis. Extrinsic risk factors play a significant role as hepatocytes undergo malignant transformation in altered tumour macroenvironment that, is conducive for tumour initiation and evades apoptosis that leads to a tumour proliferative environment. Added to that, angiogenesis and escape from immune surveillance further promotes the process of tumourigenesis. Pathogenesis of HCC in chronic infection of HBV and HCV is closely related to hepatitis that follows. Virus induced host immune response emanates inflammatory response, where in macrophages and neutrophils that infiltrate the liver, release radical oxygen species resulting in disruption of DNA repair mechanism in place and damage the mitochondrial apparatus. Since viruses themselves are not cytopathic, infiltration of viral specific CD8(+) T cells and natural killer cells have also been implicated in furthering live injury [71].

Alcohol and other toxins have similar inflammatory effects that potentiate activation of hepatic stellate cells and leads to deposition of collagen, hyaluronan resulting in liver fibrosis [72]. Sustained accumulation of previously mentioned extracellular matrix proteins subsequently leads to cirrhosis. Cirrhosis is characterized by distortion of the liver parenchyma associated with nodule formation, hypoxemia and liver failure at which stage decompensated liver is for significant morbidity and mortality unto itself [73]. Ensuing hypoxia further promotes 'fertile ground' for tumour cells [74].

1.3.1 Insulin resistance and insulin like growth factors

Insulin resistance is of paramount importance in diabetes mellitus and is considered a predictor of future development of DM2 [75]. Some degree of insulin resistance has been reported in obese subjects [76]. Relationship between insulin resistance and excess adipose tissue is intricate as one can be influenced by the other. Several clinical studies have demonstrated that patients with increased serum levels of IGF and insulin have an increased risk of HCC and several other cancer types like breast cancer, prostate and colorectal cancer [77-79]. In systemic insulin resistance, serum insulin levels are elevated to counteract hyperglycemic state [80]. As a consequence, hepatocytes produce excessive levels of IGF-1 via up-regulated growth hormone receptors. Moreover, obese individuals tend to have significantly higher levels of free IGF-1 when compared to lean subjects [81]. Insulin-IGF axis inhibits apoptosis and autophagy, stimulates cell proliferation and survival through down stream signaling namely phosphatidylinositol 3-kinase (PI3K)-AKT-mTOR system and Ras/Raf/MEK systems. Insulin binds to insulin receptors and IGF-1/IGF-2 mediate via specific IGF-receptors (IGF1R and IGF2R). IGF1 is the most potent activator of AKT pathway. Although there is no evidence that IGF1R mutations contribute to cancer, expression of IGF1R is increased in HCC [82]. In addition to a deregulated metabolic state, insulin has proliferative effects as IGF and acts through similar pathways. Experiments with HCC cell lines have demonstrated that insulin promotes DNA synthesis [83, 84]. Hence increased serum levels of insulin and IGF could facilitate accumulation of genetic mutations favouring carcinogenesis by creating a microenvironment that is mitogenic and anti-apoptotic and appears to be a major mechanism linking obesity to cancer (Figure 1.3.1).

1.3.2 Adipokines

Hepatic steatosis is a common initial finding in DM2 and obesity. Over the past decade several studies have demonstrated that adipocytes are not merely inert storage deposits but also secrete variety of biologically active proteins. These secreted proteins, collectively called adipokines, play a key role in endocrine, metabolic and inflammatory pathways that regulate cellular homeostasis [85]. More than 50 different hormone-like substances, cytokine and chemokines are grouped in the adipokine family. Most prominent of which are adiponectin and leptin.

ADIPONECTIN is a collegen like protein and gene product of the adipose most abundant gene transcript 1 (APM1) gene [86]. Exclusively derived from adipocytes, adiponectin has anti-inflammatory and insulin sensitizing effects. In obese patients, plasma concentrations of adiponectin are paradoxically reduced [87]. Several case control studies have established an inverse relationship between adiponectin plasma levels and increased risk of breast cancer pre- and post

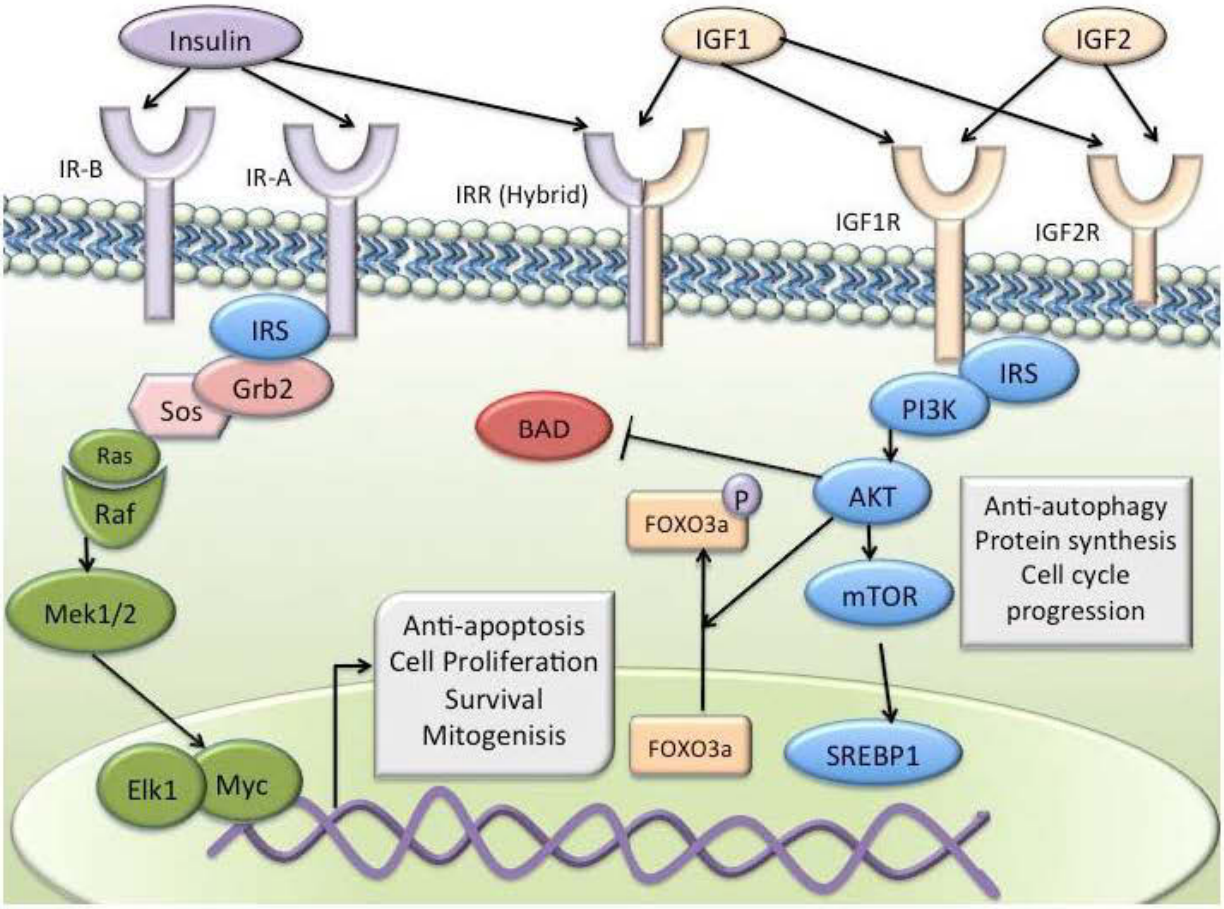


Figure 1.3.1: Insulin and IGF signaling: Hyperinsulinemia is a consequence of obesity, DM2 and circulating concentrations of IGF 1 and IGF 2 might be increased. Insulin acts via insulin receptors (IR-A and IR-B) and insulin related receptor (IRR), a heterodimer hybrid of IR and IGF-1 receptor (IGF1R). IGF mediates cell signaling via IGF1R and IGF2R. Upon binding of Insulin, IGF1 and IGF2 to their respective receptors, insulin receptor substrate proteins (IRS) are phosphorylated. Insulin signaling is mainly facilitated via Ras/Raf-Mek1/2 pathway involving transcription factors like Myc and Elk1. Whereas IGF1 and IGF2 signaling is mediated via PI3K-AKT pathways. Furthermore, AKT can activate mTOR to abrogate autophagy and promote protein synthesis and via transcription factor SREBP1. AKT inhibits BCL2-antagonist of cell death (BAD) protein to facilitate cell cycle progression. Forkhead box O (FOXO3A) is expelled from the nucleus unpon phosphorylation by AKT and propagate cell survival and mitogenesis.

menopause [odds ratio(OR) OR-3.62 (1.61–8.19)] [88], endometrial cancer [OR-2.75 (1.16–6.54)] [89] and pancreatic cancer [OR-2.81 (1.04–7.59)] [90]. Although direct etiological evidence is yet to be established, the role of adiponectin in cancer appears to be protective against carcinogenesis. The anti-carcinogenic effects are mediated through adenosine monophosphate-activated protein kinase (AMPK)/AKT system, activated mainly through two receptors, adiponectin-receptor 1 (AdipoR1) and AdipoR2. Activated AMPK system plays a vital role in the regulation of energy metabolism and cellular quiescence [91]. Furthermore, independent of signaling, adiponectin decreased production of reactive oxygen species and thereby inhibit proliferation

of cells [92]. Transgenic lipoatrophic diabetic mice (AZIP/F)1Vsn lack adipose tissue, are diabetic and do not have measurable levels of adiponectin. Several studies with AZIP/F1 mice have demonstrated that this particular strain of mice is more susceptible to carcinogen induced tumorigenesis when compared to wild type mice [93]. Therefore, decrease plasma levels of adiponectin in obesity may contribute to increased risk of cancer among obese individuals.

Leptin is a 16 kDa protein secreted by adipocytes, which plays a key role in regulating body weight by inhibiting appetite and increasing energy consumption. Defects in leptin production leads to morbid obesity in humans and rodents. Systemic leptin levels are proportional to body adiposity [94] and clinical studies have suggested an association between systemic levels of leptin and colorectal [95], endometrial cancer risk [96]. Studies in leptin deficient (ob/ob) mice have suggested that transplanted tumor growth and overall tumor burden is reduced when compared to wild type mice. Further, tumors in ob/ob mice contain fewer cells that express phosphorylated Akt, a cell growth-promoting factor stimulated by leptin receptors (LepRb) [97]. In addition to Akt pathway, LepRb activated PI3 kinase, MAPK and STAT signaling pathways and plays a critical role in proliferation, survival and differentiation of cancer cells. However, studies are required to analyze clinical significance of elevated leptin levels in obese individuals and cancer risk.

1.3.3 Angiogenesis

Angiogenesis is a process by which new blood vessels are formed from pre-existing vessels. Tumor cells secrete several factors like vascular endothelial growth factor (VEGF), which induce capillary growth into the tumor, allowing for tumor growth and dissemination. Adipose tissue is also dependent on angiogenesis for its proliferation and a recent study suggests that anti-angiogenic therapy can be affective in treating obesity as well as in prevention of onset of obesity in transgenic and diet-induced obesity mouse models [98]. Another experimental study induced hypercholesterolemia in mouse models by feeding high calorie diet and concluded that hypercholesterolemia induces angiogenesis and accelerates orthotopically implanted breast tumor growth [99]. Furthermore, Leptin secreted by adipocytes induces proliferation and angiogenic differentiation of endothelial cells by upregulating VEGF and VEGF receptor 2 and transactivates VEGFR2 independent of VEGF. Increase in VEGF expression by leptin is mediated via interleukin-1 and NOTCH signaling [100]. Hence therapeutic targeting of angiogenesis with anti-VEGF or anti-VEGFR2 might be beneficial in cancer patients who are obese. However, a better molecular marker would ne necessary to individualize treatment regimens with anti-angiogenic therapy.

1.4 Role of non-coding RNAs in cancer glucose metabolism

Shankaraiah, R.C., et al., Non-coding RNAs in the reprogramming of glucose metabolism in cancer. Cancer Lett, 2018. 419: p. 167-174. [Reproduced with permission].

Neoplastic disease is characterised by uncontrolled cell proliferation. Cancer cells adapt their metabolism to meet the energy demands associated with increasing biomass. The unique reprogrammed metabolic phenotype exhibited by tumour cells known as the Warburg effect is characterised by high rates of aerobic glycolysis leading to the production of lactic acid and reduced mitochondrial oxidative phosphorylation (OXPHOS) even in the presence of oxygen [101-103]. Unlike in normal cells, glycolytic dependence in tumour cells generates anabolic macromolecules or intermediates that contribute to tumour growth, such as nicotinamide adenine dinucleotide phosphate (NADPH) and ribonucleotides via the pentose phosphate pathway (PPP); glycogen for glucose storage via the hexosamine pathway; and amino acids via the serine/glycine biosynthesis pathway, which fuels one-carbon metabolism to generate NADPH. Tumour cells compensate for the inefficiency in ATP production (two ATPs generated by glycolysis vs. 38 by OXPHOS) by upregulating the expression of glucose transporters (GLUTs), which increases glucose uptake into the cytoplasm [104, 105]. This property has been exploited for the non-invasive diagnostic imaging of human cancer in positron emission tomography with a radiolabelled glucose analogue as a reporter (2-deoxy-2-fluorine-18fluoro-D-glucose) combined with computed tomography [106, 107]. Processes that induce the glycolytic switch and promote cell proliferation are associated with activation of oncogenes (e.g., RAS and MYC), inactivation of tumour suppressor genes (e.g., tumour protein [TP]53 and phosphatase and tensin homolog [PTEN]), growth-factor mediated signal transduction (e.g., insulin-like growth factor receptor, epidermal growth factor receptor, and human epidermal growth factor receptor 2), and hypoxia-mediated signalling (hypoxia-inducible factor [HIF]-1α) [108-111]. Thus, changes in metabolism can be viewed as a hallmark of cancer [112], and targeting the relevant factors represents a potential strategy for cancer therapy [113]. However, our knowledge of the regulation of cancer cell metabolic reprogramming and the signalling pathways that are involved remains limited. Non-coding (nc)RNAs are a family of functional RNA molecules that are not translated into proteins but regulate the expression of target genes. NcRNAs can be classified based on their size: small interfering RNAs, PIWI-interacting RNAs, transcription initiation RNAs, and micro (mi)RNAs are smaller than 200 nucleotides; small nucleolar RNAs are of about 60–300bp in size; and long non-coding (lnc)RNAs are longer than 200 bp. NcRNAs have been implicated in a variety of cancers; miRNAs and lncRNAs are known to function as oncogenes and tumour suppressors, and act on receptor tyrosine kinases (RTKs) and hypoxia-mediated pathways [114, 115]. Recently, there has been increased interest in the role of miRNAs and lncRNAs in aerobic

glycolysis as it relates to cancer metabolism. Accumulating evidence has shown that ncRNAs can alter glucose metabolism either directly by targeting glucose trafficking and consumption, or indirectly by modulating cancer-associated signalling pathways.

1.4.1 Glucose trafficking

NcRNAs regulate glucose trafficking in cancer cells by altering glut levels. Glucose transport across the cell membrane along a concentration gradient is an energy-independent process. The passive transport of glucose is facilitated by solute carrier 2A (SLC2A) and GLUT family proteins. Among the 14 GLUT isoforms [116, 117], GLUT1 is known to be overexpressed in cancer. NcRNAs have been implicated in the regulation of GLUT1 expression. The lncRNA neighbour of BRCA1 gene (NBR)2 promotes cell survival by upregulating GLUT1 expression in response to phenformin treatment in renal cell carcinoma (786-O) and breast cancer cell lines (MDA-MB-231). Phenformin treatment increases glucose uptake via overexpressed GLUT1, while NBR2 knockdown abrogates GLUT1 expression, thereby rendering 786-O and MDA-MB-231 cells susceptible to phenformin-induced cell death [118]. Additionally, the GLUT1 gene is a target of miR-1291; transfection of miR-1291 mimic in human renal cell carcinoma (RCC) cells reduced SLC2A1 mRNA and GLUT1 protein expression [119]. Notably, miR-1291 was downregulated in RCC as compared to adjacent non-cancerous kidney tissue.

1.4.2 Glycolysis

NcRNAs regulate key glycolytic enzymes. There are three committed steps in glycolysis. The first is the phosphorylation of glucose by hexokinases (HKs) to generate glucose-6-phosphate (G6P). In addition to the ubiquitously expressed HK1, cancer cells overexpress HK2, which is critical for the Warburg effect since phosphorylated glucose is trapped in the cytoplasm. In human glioblastoma multiforme, HK2 expression was correlated with poor overall survival. Depletion of HK2 but not HK1 restored OXPHOS and increased oxygen consumption and susceptibility to cell death by radiation and chemotherapy [120]. The 3'-untranslated region (UTR) of HK2 mRNA has a predicted binding site for the miRNA miR-497. In osteosarcoma, HK2 is overexpressed whereas miR-497 is downregulated, which is likely caused by increased expression of the lncRNA plasmacytoma variant translocation (PVT)1, which acts as a molecular sponge that traps miR-497. Hence, the PVT1/miR-497 axis contributes to the Warburg effect through regulation of HK2 expression [121]. Additional regulators of HK2 are miR-143 and -145, which are clustered together on chromosome 5q32 and are downregulated in many cancer types [122, 123]. HK2 protein expression was suppressed by restoration of mature miR-143 and -145 in

RCC, head and neck squamous cell carcinoma, and lung cancer cell lines [124-126], making miR-143/145 an important regulator of glycolysis in cancer.

The second committed step of glycolysis is the conversion of F6P into F1,6P catalysed by phosphofructokinase (PFK)1. It was reported that TAT-activating regulatory DNA-binding protein (TARDBP), which is highly expressed in hepatocellular carcinoma (HCC), regulates glycolysis by inducing the expression of the platelet isoform of PFK1 (PFKP). TARDBP suppressed miR-520a/b/e expression by directly binding to its promoter region, preventing its binding to the 3'-UTR of PFKP and thereby inhibiting protein expression [127]. F6P is also converted into F2,6P by PFK2-F2,6Bpase (PFKFB), which allosterically activates PFK1. Of the four PFKFB isoforms that have been identified to date, PFKFB2 was found to be overexpressed in metastatic as compared to non-metastatic ovarian cancer and normal ovary. RNA immunoprecipitation studies have revealed that LINC00092 binds to PFKFB2 in ovarian cancer upon metastasis and induces the glycolytic phenotype. This induction of LINC00092 expression could partly be mediated by paracrine signalling by chemokine (C-X-C motif) ligand 14-positive cancer associated fibroblasts, highlighting the importance of the tumour microenvironment in the Warburg effect [128].

The final committed step of glycolysis is catalysed by pyruvate kinases (PKs) that convert phosphoenolpyruvate (PEP) into pyruvate. The first two steps consume ATP, whereas the last step generates ATP. As described above, cancer cells employ various gain-of-function strategies to increase glycolysis. While attenuation of PK function can be achieved by expressing a low-affinity dimeric form of pyruvate kinase (PK)M2, the tetrameric forms of PKM2 and PKM1 are functional at physiological PEP levels in normal cells [129, 130]. A number of miRNAs that are dysregulated in cancer have been found to modulate PKM2 expression. For instance, the PKM2 transcript has binding sites for miR-133a and -133b. In vitro studies have shown that miR-133a/b precursors could target PKM2 and block protein expression [131], although the effect on glycolysis remains to be investigated. Interestingly, miR-133a/b are downregulated in several types of cancer [132, 133]. MiR-326 was shown to directly target and inhibit the expression of PKM2. Moreover, miR-326 levels were inversely correlated with high levels of PKM2 in glioma cells, suggesting an endogenous mechanism for PKM2 regulation [134]. Liver-specific miR-122 expression was reduced in HCC [135]; restoring miR-122 expression prevented the development of HCC in mouse models [136]. One possible mechanism underlying this effect is the reversal of aerobic glycolysis, as suggested by the decrease in lactate production and increase in oxygen consumption observed in HCC cell lines (HepG2 and MHCC97H). This reversal of the glycolytic switch can be explained by the direct targeting of PKM2 by miR-122 [137]. Another study reported that miR-122-mediated inhibition of PKM2 protein expression also resulted in the downregulation of GLUT1 and a decrease in glucose uptake in breast cancer cells [138].

Accelerated glycolysis in cancer cells provides metabolites for branching anabolic pathways such as the PPP and serine biosynthesis. In cancer cells, gain of HK and PFK function and suppression of dimeric PKM2 lead to the accumulation of glycolytic intermediates that enhance anabolic processes by stimulating the synthesis of purine nucleotides to sustain the rapid growth of cancer cells [139, 140]. Although the above mechanisms suggest a decrease in pyruvate production, the conversion of pyruvate to lactate is increased to generate NAD+ required for the maintenance of energy flux in the branching pathways. Additionally, lactate accumulation results in acidification of the tumour microenvironment, leading to tumour invasion; secreted lactate can also be taken up by adjacent tumour-associated stromal cells to generate pyruvate [141, 142]. The reversible conversion of pyruvate to lactate is catalysed by lactate dehydrogenases (LDHs). One of the subunit that forms the LDH isoenzymes, LDHA, plays a critical role in tumour cells and its expression levels are correlated with survival outcome in various cancers [143, 144]. LDHA was one of the first MYC-induced glycolytic genes to be identified [145, 146]. It is known to be a direct target of miR-34a; the repression of LDHA by miR-34a inhibited glycolysis in breast cancer cells [147]. However, miR-34a has been reported to be a downstream effector of p53 protein and is therefore downregulated in cancers with dysfunctional p53 [148].

1.4.3 NcRNAs can influence glucose metabolism via regulation of cancer-related signalling pathways

Phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTor) pathway: The PI3K/Akt/mTOR signalling pathway is one of the most frequently altered pathways in cancer. PI3K is activated by extracellular stimuli such as growth factor via RTKs. Class I PI3Ks facilitate the production of the key lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidylinositol-4,5-biphosphate (PIP2). The lipid phosphatase PTEN negatively regulates this pathway by converting PIP3 to PIP2 [149]. PIP3 binds to the pleckstrin homology domain of Akt at the plasma membrane and phosphoinositide-dependent protein kinase 1 phosphorylates Akt at Thr308. Complete Akt activation is achieved by phosphorylation at Ser472 by the mTOR complex (mTORC)2 [150, 151]. Oncogenic activation of PI3K/Akt/mTOR signalling contributes to the Warburg effect by (i) increasing the expression of GLUTs and consequently, glucose uptake; (ii) increasing the expression of the glycolytic enzymes HK2 and PKM2; and (iii) increasing mTORC1 activity via Akt, leading to an excess of HIF-1α.

Many ncRNAs have been found to modulate PI3K/Akt/mTOR signalling [152]; we focus here on ncRNAs that are known to be associated with the Warburg effect. MiR-125a negatively regulates the PI3K/AKT/mTOR pathway in HCC and inhibits glycolysis by targeting HK2 [153, 154]. Let-7 family miRNAs are frequently downregulated in cancer and function as tumour suppressors

by negatively regulating RAS members, PI3K catalytic subunit δ polypeptide, AKT, and mTOR, thereby modulating cancer cell survival and growth [155].

The notion that pseudogenes are non-functional remnants of functional genes has been challenged by the finding that PTEN pseudogene (PTENP)1 can modulate PTEN protein expression. This mechanism is largely mediated by PTENP1 pseudo-mRNA acting as a sponge or decoy for miRNAs that suppress PTEN expression (e.g., miR-17, -21, -214, -19, and -26) [156]. Transgenic mouse models (super-PTEN) overexpressing PTEN have shown that PTEN can reverse the Warburg effect by PI3K-independent and -dependent mechanisms. PTEN abolished glycolytic dependence by inhibiting PFKFB3 and PI3K/Akt signalling, resulting in reduced mTORC1 activity and downregulation of PKM2 [157].

On the contrary, the lncRNA HOX transcript antisense RNA, which is overexpressed in cancer, can promote glycolysis by inducing the expression of GLUT1 via methylation of the PTEN gene promoter, leading to activation of PI3K/Akt/mTOR signalling in several cancer types [158, 159]. Similarly, in nasopharyngeal carcinoma, the lncRNA ANRIL increased the expression of GLUT1 and LDHA by promoting Akt phosphorylation and mTOR activation to increase glucose uptake, glycolysis, and cell proliferation [160]. The lncRNA urothelial cancer-associated (UCA)1 is highly expressed in bladder cancer (BC) and was shown to stimulate glycolysis and lactate production in BC cells. There are two possible mechanisms by which UCA1 enhances the Warburg effect: mTOR activation by UCA1 followed by activation of signal transducer and activator of transcription 3, and repression of miR-143. This dual-control process results in increased HK2 levels and a consequent increase in glycolysis [161].

P53 pathway: The best characterised functions of p53 are related to control of the cell cycle and cell death. However, p53 is also implicated in the Warburg effect since it can modulate the switch between glycolysis and OXPHOS. The cytochrome c oxidase (COX) complex is essential for mitochondrial respiration and oxygen consumption in energy production. Synthesis of cytochrome c oxidase (SCO)2, a transcriptional target of p53, is a key regulator of the COX complex. Under oxidative stress, cells undergo a shift from glycolysis to OXPHOS that depends on p53 and is mediated by SCO2 [162]. However, cells that lack functional p53 continue to carry out glycolytic metabolism. This example of the Warburg effect was also observed upon SCO2 gene disruption in wild-type p53 tumour cells and was reversed by overexpressing SCO2 in p53-null cell lines [163]. Another p53 target, TP53-induced glycolysis and apoptosis regulator (TIGAR), was shown to lower intracellular F2,6-BP levels, thereby inhibiting glycolysis and shunting substrates to the PPP pathway. Thus, induction of TIGAR expression resulting from the loss of p53 contributes to the Warburg effect. The regulation of glycolysis by p53 is not limited to its modulation of SCO2 and TIGAR expression; p53 also suppresses the expression of the

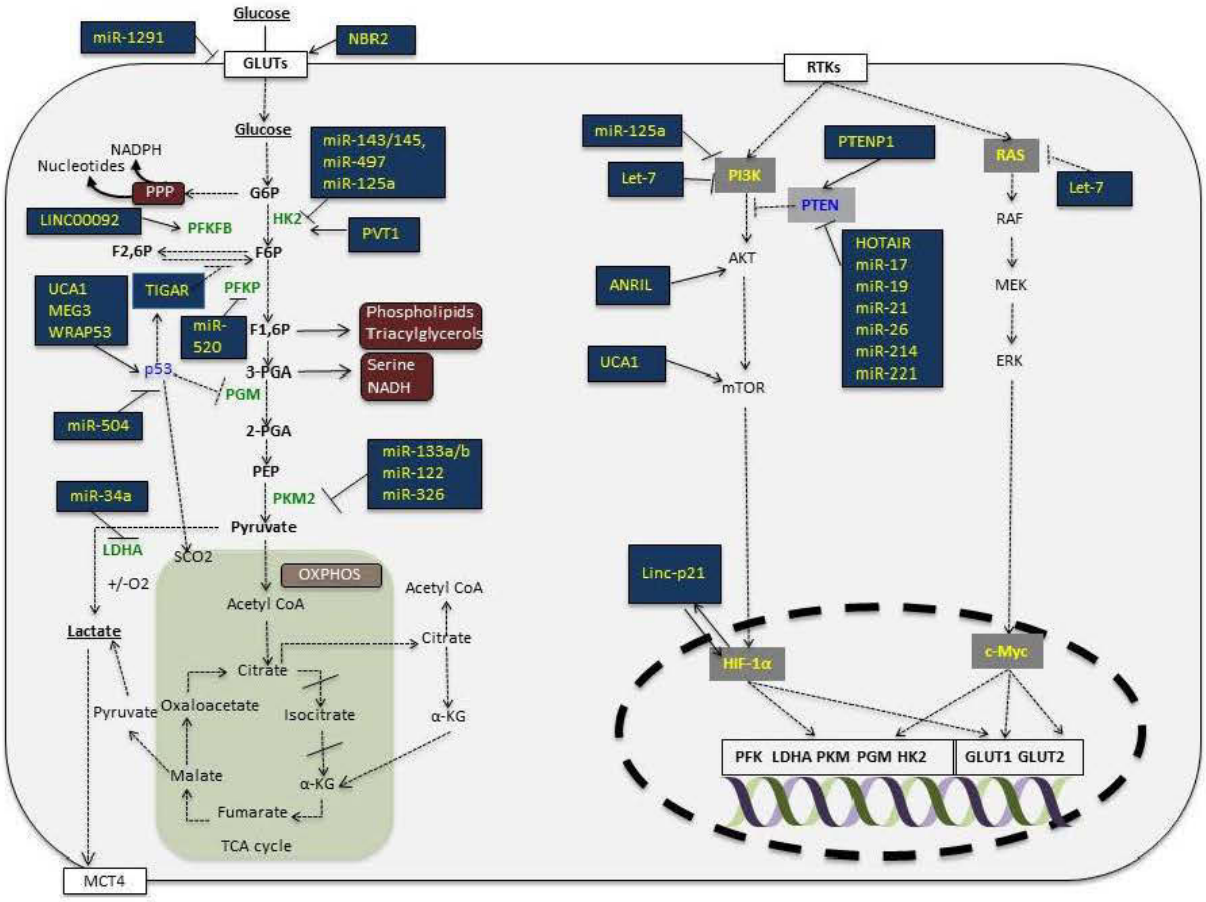


Figure 1.4.1: Glucose metabolism reprogramming in cancer cells by ncRNAs. The actions of ncRNAs, miRNAs, and lncRNAs promote the glycolytic switch either directly by altering the levels of the enzymes involved in glycolysis or indirectly by regulating various upstream cancer-associated signalling pathways. (Left) Effects of ncRNAs on the levels of various proteins (shown in green), leading to increased glucose uptake and glycolysis to ensure rapid ATP production and the shunting of metabolites to anabolic pathways. Pyruvate is ultimately transformed into lactate by LDHA induced by MYC and HIF-1 . (Right) NcRNAs that regulate oncogenic (PI3K, RAS, MYC, and HIF-1) and tumour suppressor (PTEN) pathways known to stimulate glycolysis. Reproduced with permission [300].

glycolytic enzyme phosphoglycerate mutase, thereby increasing glycolytic dependence [164].

The lncRNA maternally expressed gene (MEG)3 is expressed in various normal tissues and is silenced in several primary human tumours and cell lines. Ectopic expression of MEG3 in tumour cells resulted in the accumulation of p53 and alterations in the expression of p53 target genes, resulting in growth inhibition. These findings suggest that MEG3 can negatively regulate glycolysis via p53 and functions as a tumour suppressor [165, 166]. MiR-504 was identified as a direct negative regulator of p53 that binds to two sites in p53 3'-UTR. Ectopic expression of miR-504 decreased p53 protein levels and transcriptional activity, suppressing p53-mediated apoptosis and causing cell cycle arrest in etoposide- or gamma irradiation-induced stress responses. These results imply direct negative regulation of p53 and possibly of downstream glucose metabolism

reprograming by miR-504 [167]. Additionally, p53-inducible miR-34a was shown to directly inhibit the expression of HK2 and increase mitochondrial respiration [168].

P53 induces the expression of miR-145, which in turn promotes p53 cell death activity [169]. However, miR-483-3p expression is induced under conditions of high intracellular glucose [170]. MiR-483 is dependent on the activity of O-linked β-N-acetylglucosamine transferase, which acts as a sensor of cellular glucose levels [171]. Thus, in the presence of high glucose, miR-483-3p is upregulated and cells are protected from the pro-apoptotic action of the p53/miR-145 axis.

HIF-1α pathway. Oncoproteins and tumour suppressors contribute to the Warburg effect through various mechanisms that overlap with the hypoxia-mediated cellular response. It is widely accepted that the microenvironment of solid tumours is oxygen-deficient. The HIF1 transcriptional complex is composed of the oxygen-regulated subunit HIF-1α and constitutively expressed subunit HIF-1β. Under hypoxic stress and PI3K activation, HIF-1α dimerises with HIF-1β to form the active HIF-1 complex, which regulates the transcription of various genes by binding to hypoxia response elements [172]. HIF-1α stimulates glycolysis by (i) upregulating GLUT1, leading to increased glucose uptake; (ii) upregulating glycolytic enzymes to increase glucose consumption; and (iii) upregulating LDHA, which creates an acidic tumour microenvironment [173-175]. LincRNA-p21 and HIF-1α form a positive feedback loop that contributes to the Warburg effect. Under hypoxic stress, glucose uptake and lactate production are increased by upregulation of GLUT1 and LDHA. LincRNA-p21 is induced under hypoxia as a direct transcriptional target of HIF-1, and its knockdown abrogated hypoxia-enhanced glycolysis by decreasing GLUT1 and LDHA levels; this effect was reversed by ectopic expression of HIF-1α, suggesting that the function of lincRNA-p21 is HIF-1 dependent [176].

The high rate of aerobic glycolysis in cancer cells is beneficial to their proliferation. However, the current knowledge in the field of cancer metabolism has not yet been fully translated into clinically useful applications. It is likely that the high but inefficient rate of ATP production is not the main selective advantage of cancer cells. Instead, cells may benefit from the high levels of intermediate products shunted to pathways that generate nucleotides, amino acids, lipids, and NADPH [177]. Identifying the critical node(s) in the network of pathways that regulate aerobic glycolysis can reveal novel targets for anti-tumour therapies. Among the various enzymes involved in the glycolytic cascade, GLUT-1, HK2, LDHA, and PKM2 are known to be overexpressed in cancer and represent potential targets [120, 130, 178, 179]. However, there remain many outstanding challenges to this approach. Firstly, even if enzyme inhibitors have high specificity, cells may recruit other glycolytic enzyme isoforms in response to inhibition of the cancer-associated isoform. Secondly, the critical pathways to be targeted must be ascertained. This may be difficult to achieve given the phenotypic and functional heterogeneity among can-

Causal mechanism	Target	ncRNA	Cancer type	Ref.
Glucose uptake	GLUT1	lncRNA NBR2	Breast, Renal cell	[118]
		miR-1291	Renal cell	[119]
		miR-138,150,199a-3p, 532-5p,19a/b,130b,301a	Renal cell	[184]
Glycolytic enzymes	HK2	lncRNA-PVT1, miR-497	Osteasarcoma	[121]
		miR-143-145 cluster	Various	[124-126]
		miR-125a	Hepatocellular	[154]
	PFK	miR-520s	Various	[185]
		LINC00092	Ovarian	[128]
	PKM2	miR-133a/b	SCC	[131]
		miR-326	Glioma	[134]
		miR-122	Hepatocellular, Breast	[137, 138]
	LDHA	miR-34a	Breast	[147]
PI3K/Akt/ mTor pathway	PI3K	miR-125a	Hepatocellular	[153]
		let-7	Various	[186]
		H19	Various	[187]
	PTEN	PTENP1	Various	[156]
		HOTAIR	Various	[158, 159]
		miR-17,21,214,19,26	Various	[188]
	AKT	ANRIL	Nasopharyngeal	[160]
	mTOR	UCA1	Bladder	[189]
p53 pathway	p53	UCA1	Hepatocellular	[190]
		MEG3	Various	[165]
		WRAP53	Various	[191]
		miR-504	Various	[167]
		miR-34a	Various	[192]
HIF-1 pathway	HIF-1α	lincRNA-p21	Various	[176]

Table 1.4.1: Non-coding RNAs known to regulate glucose metabolism. Reproduced with permission [300]

cers and in individual tumours. Thirdly, the toxic effects of inhibiting glycolytic enzymes in normal cells must be clarified; in addition to cancer cells, aerobic glycolysis is a key process in immune and stem cells.

Treatment strategies tailored to an individual patient's genetic background is becoming a reality; a personalised view of the mechanisms underlying metabolic processes in a patient will pro-

vide additional useful information. Advances in systems biology, including the reconstruction of genome-scale metabolic models (GEMs), have enabled systematic evaluation of cancer cell type-specific metabolic profiles [180, 181]. GEMs integrate omics data to provide a broader understanding of metabolic aberrations and have revealed potential biomarkers and anti-metabolites specific to patients and cancer types. A population-based cancer-specific GEM could be used to stratify patients based on substrate utilisation and response to therapy [182].

The role of ncRNAs in the regulation of cell metabolism is well established. Targeting molecules involved in the Warburg effect may be an effective therapeutic strategy for cancer treatment [113, 183]. NcRNAs—specifically, miRNAs and lncRNAs—have multiple physiological targets that include not only enzymes and kinases, but also components of signalling pathways associated with aerobic glycolysis. Several pre-clinical studies mentioned in the review have demonstrated the effects of targeting ncRNAs on cancer cell survival and proliferation in the context of glucose metabolism reprogramming. With technological and computational advances in the synthesis of oligonucleotides, miRNA mimics, anti-miRNA oligonucleotides, or lncRNAs that modulate glycolysis and improvements in methods for in vivo delivery, ncRNAs can be used to render cancer cells more susceptible to existing cancer therapies.

1.5 Mouse models of HCC

Mouse models of cancer empower researchers to study complex and dynamic tumour pathophysiology systems. Particularly, in the area of precision cancer prevention, these mouse models are invaluable since in-vitro systems cannot be configured to reflect tissue or organ systems. Chronicity of tumourigenesis process in HCC further limits in-vitro models.

1.5.1 Genetically engineered mouse models of HCC

With the advent of genome-editing technologies, genetically engineered mouse (GEM) models of HCC have been developed. Most of these GEMs are engineered using concepts derived from reverse genetics, where in, frequent genetic mutations in HCC patients are introduced with the intension of generating spontaneous HCC development phenotypes (Table 1.5.1) [193]. The PTEN lipid phosphatase, acts as a tumour suppressor gene by regulating PI3-kinase signalling pathway. PTEN-null mice developed steatohepatitis at about 2 to 3 months of age, fibrosis at 10 months, and progression to HCC took about 18 months [194]. Class II germinal center kinases MST1/2 have been shown to act as tumour suppressors and knockout mice of these genes developed spontaneous HCC, underlying the importance of Hippo-Lats-Yorkie signalling in regulating

GEM model	Time to HCC development	References
$PTEN^{-/-}$	18 months	[194]
$Mst1^{-/-}Mst2^{F/-}$ (Adeno-cre)	7 to 15 months	[195]
$Atg5^{F/F}$ (CAG-Cre)	19 months	[202]
$Atg7^{F/F}$ (Alb-Cre)	12 to 18 months	
miRNA-122 LKO	12 to 17 months	[200]
c-MYC overexpression	15 months	[197]
TGFα- Overexpression	12 to 15 months	[197]
miRNA-221 Overexpression	9 to 12 months	[198]

Table 1.5.1: Genetically engineered mouse models of HCC.

tumourigenesis [195].

Deficiencies in autophagy, a physiological process by which cells degrade cytoplasmic components within lysosomes, are associated with increased tumourigenesis [196]. ATG5 and ATG7 are essential for autophagic process and knockout mice of these genes developed liver tumours spontaneously. More than three decades ago similar observations of liver tumourigenesis were observed by overexpressing c-myc and TGFα in mice [197, 198]. MiRNA dysregulation is a key factor that has long been studies in formation of tumours [199]. Understanding of the important differentially expressed miRNAs, could be harnessed to model transgenic mice that develop liver tumours. Similarly, miRNA-122 deficient and miR-221 overexpressed mice have been established and they spontaneously develop HCC with age [200, 201].

1.5.2 Chronic liver disease-mediated HCC mouse models.

Undeniably, most cases of HCC develop in the setting of chronic liver disease, characterised by cirrhosis, liver fibrosis and steatosis. GEMs described above cater to genetic perturbation in oncogene and tumour suppressor genes. Although these models are invaluable in studying molecular and pathway interactions during tumour initiation and progression, their utility is limited by long incubation times and inability to recapitulate underlying liver disease hierarchy. Several toxins, carcinogens and modified diets are combined with GEMs to accelerate tumourigenesis and closely resemble underlying human liver disease.

Carbon tetrachloride (CCl4) is the most widely used toxin for inducing reproducible and pre-

dictable hepatic fibrosis [203]. Oral gavage or intraperitoneal injection of diluted CCl4 for 12 to 14 weeks is routinely used to induce liver fibrosis in mice. CCl4 challenged mice are ideal tools for studying hepatic remodelling [204]. Thioacetamide and diethylnitrosamine (DEN) have also been used to promote liver damage. The later, a carcinogen, is usally administered i.p in neonatal mice. To model HCC with underlying metabolic syndromes, diet or nutrition-based models are combined with appropriate GEMs. Type of chow depends upon the type of metabolic perturbation required for a study. For example, methionine and choline-deficient diet can be used in mice to model nonalcohol dependent steatohepatitis (NASH), where as high-fat and choline-deficient diet combined with DEN has been shown to induce NASH-HCC phenotype [205].

1.6 Metformin

Metformin, an oral biguanide, is the most widely used anti-hyperglycaemic drug in the treatment of DM2. Biguanides are derivative of guanidine, a naturally occurring substance found in mushroom, turnips, cereals and mussels.

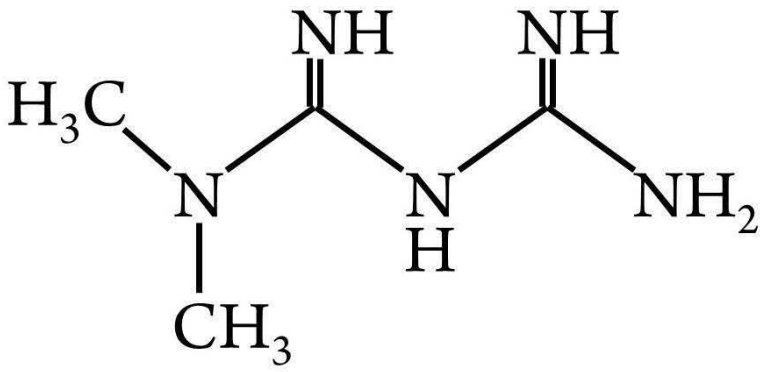


Figure 1.6.1: Chemical formula of metformin

1.6.1 Historical overview

Metformin (dimethylbiguanide) was derived from Galega officinalis (also known as Italian fitch or goat's rue), a traditional herbal medicine commonly used in Europe, in 1918. Guanidine derivates synthesized from galega included metformin, phenformin and buformin and were shown to have blood glucose lowering effects. Phenformin was mainly investigated and was discontinued due to toxicity.

Subsequently, in 1940s, metformin was rediscovered in the search for guanidine-based antimalar-

ial agent, proguanil [206]. Inadvertently, proguanil was modified to metformin hydrochloride salt and named, flumamine. Efficacy of flumamine as anti-influenza drug in an influenza outbreak in Philippines, in 1949 and subsequent tendency of flumamine to lower blood glucose levels in some patients, brought the limelight back on to metformin (Figure 1.6.1) [207]. However, metformin was considered weaker than other glucose-lowering biguanides and was not preferred. In 1970s, phenformin and other biguanides were discontinued due to their toxicity profile of Phenformin, mainly due to increased lactic acidosis observed. Metformin, received more credence at this point, appended by ability of metformin to counter insulin resistance in DM2 patients without the risk of hypoglycaemic adverse events or weight gain [208]. Metformin became the first-line anti-diabetic drug after FDA approval in 1994 following key clinical trials, that demonstrated improved glycemic control and lipid concentrations in DM2 patients with metformin therapy [209, 210].

1.6.2 Mechanism of action of metformin

The exact mechanism by which metformin lowers serum glucose in diabetes is still obscure. However, there is a growing consensus that inhibition of hepatic gluconeogenesis plays a central role along with increased glucose uptake by muscles and adipocytes resulting in decreased insulinemia [211, 212]. The preferential metabolic effect of metformin in liver is primarily due to high expression of organic cation transporter 1 (OCT1), a transporter protein that has been demonstrated to mediate cellular uptake of metformin [213]. Transgenic mice with hepatic deletion of the SLC22A1 gene (which encodes for OCT1) and SLC22A1 polymorphisms in humans have been shown to significantly reduce the effectiveness of metformin in lowering serum blood glucose levels [214].

At the cellular level metformin activates adenosine monophosphate kinase (AMPK) which is activated by increased intracellular levels of AMP and is considered as an energy sensor involved in regulating cellular metabolism. Inhibition of hepatic gluconeogenesis is secondary to metformin's inhibition of complex 1 of the mitochondrial respiratory chain and subsequent AMPK activation, which leads to decreased ATP synthesis and a rise in the cellular AMP: ATP ratio [215]. Additionally, metformin requires liver kinase B1 (LKB1) - tumor suppressor gene upstream of AMPK, in the liver to lower blood glucose levels. Deletion of LKB1 in the liver of adult mice resulted in a nearly complete loss of AMPK activity and resulted in hyperglycemia with increased gluconeogenic and lipogenic gene expression [216].

Activated AMPK phosphorylates numerous downstream targets such as fatty acid beta-oxidation and glycolysis, and suppresses several pathways such as gluconeogenesis, protein and fatty acid synthesis and cholesterol biosynthesis [217]. Metformin has also been shown to have beneficial

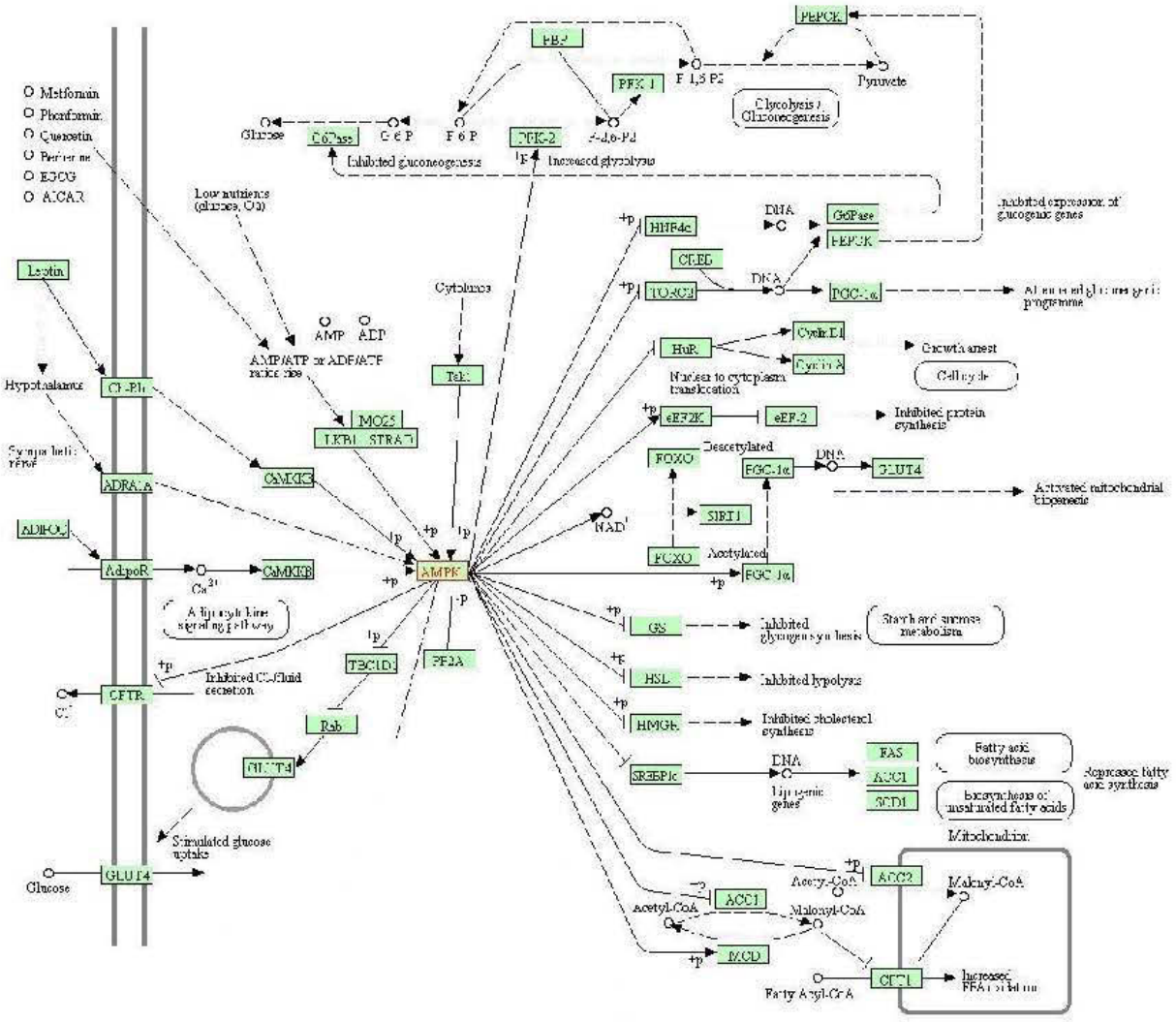


Figure 1.6.2: Metformin mechanism of action: AMPK signalling pathway (modified from Kegg hsa04152, https://www.kegg.jp/kegg/kegg1.html [299])

effects on lipid metabolism in diabetics. AMPK activation is also key to metformin's effects on lipid metabolism, at least in the liver, where increased ACC phosphorylation results in inactivation of ACC enzyme and subsequent reduction in triglyceride cellular content secondary to increased fatty acid oxidation and reduced de novo lipogenesis [218].

Furthermore, AMPK-independent metformin regulation of angiogenesis targets has been described; the advanced glycated end products (AGEs), the soluble intercellular cell-adhesion molecules (ICAMs) and the soluble vascular cell-adhesion molecules (VCAMs). AGE-induced inflammation and cellular oxidative stress are major contributors of diabetic complications. Metformin has been reported to reduce AGE synthesis and proinflammatory cytokines, plasma levels

of ICAM-1 and VCAM-1 in DM2 patients independent of its anti-hyperglycemic effects [219, 220]. These studies support the notion that metformin has a beneficial effect on endothelial function by suppressing oxidative stress in endothelial cells.

1.6.3 Epidemiological studies of metformin in cancer

Over the past decade, growing evidence suggests that there is the potential for metformin to reduce cancer risk and perhaps be used as cancer chemoprevention therapy. The interest in the potential of metformin as an anticancer agent was highlighted by a series of epidemiological studies, exclusively in diabetic patients, that suggested it had a protective effect against cancer [221-224].

In the largest study to date, which included 480,984 DM2 patients, the reduced risk for liver (HR = 0.06), bowel (HR = 0.36) and pancreatic (HR = 0.15) was of a similar magnitude to that of all cancers (HR = 0.12) in patients using metformin treatment [225]. Although majority of these epidemiological studies support cancer protective effect of metformin (Table 1.6.1), a few case control studies in lung and prostrate cancer did not find any beneficial effect of metformin use in DM2 patients towards protection from cancer [226, 227].

The precise relation between diabetes mellitus and chronic liver diseases still needs to be further evaluated. In a case-control study, association between HCC and diabetes mellitus in a large cohort of patients with HCC was explored. Temporal relationship between the onset of diabetes and the development of HCC was investigated in 465 HCC patients, 618 with cirrhosis and 490 control subjects. Results of which, described prevalence of diabetes mellitus was 31.2 % in HCC patients, 23.3% in cirrhotic patients and 12.7% in the control group. With univariate and multivariate analysis, the OR for HCC in diabetic patients were respectively 3.12 and 2.2. In 84.9% of cases, DM2 was present before the diagnosis of HCC. They further reported an OR for HCC of 2.99 (CI 1.34-6.65, P = 0.007) in diabetic patients treated with insulin or sulphonylureas, and an OR of 0.33 (CI 0.1-0.7, P = 0.006) in diabetic patients treated with metformin [233]. Therefore, DM2 is an independent risk factor for HCC and that it precedes HCC diagnosis. Moreover, these studies highlight that in patients with DM2, a direct association of HCC with insulin and sulphonylureas treatment and an inverse relationship with metformin therapy exists. Additionally, above mentioned epidemiological studies provide a strong rationale for metformin use in cancer chemoprevention.

Study type	Outcome	Comparison	Cancer type	Results (HR/OR/RR)	Ref.
Case control	Incidence	Metformin vs other	Various	OR 0.79	[221]
Retrospective	Mortality	SUs vs Metformin	Various	HR 1.3	[228]
Case control	Incidence	Metformin + Glibenclamide Glibenclamide monotherapy	Various	OR 0.28 OR 2.62	[229]
Retrospective	Incidence	Metformin vs other	Various	HR 0.63	[222]
Retrospective	Incidence	Metformin SUs monotherapy Metformin+SUs Insulin	Various	HR 1.0 HR 1.36 HR 1.08 HR 1.42	[230]
Prospective	Mortality	Metformin vs other	Various	HR 0.43	[224]
Case control	Incidence	Metformin vs other	Pancreas	OR 0.38	[231]
Case control	Incidence	Metformin vs other	Prostate	OR 0.56	[232]
Case control	Incidence	Insulin or SUs Metformin	HCC	OR 2.99 OR 0.33	[233]
Case control	Prevalence	Biguanides TZDs, SUs Insulin Dietary control	HCC	OR 0.3 OR0.3,7.1 OR 1.9 OR 7.8	[234]
Case control	Incidence	Metformin vs other	Breast	OR 0.44	[235]
Prospective	Incidence	Metformin vs other	Liver Various	HR 0.06 HR 0.12	[225]
Retrospective	Mortality	Metformin vs other	Various* Colorectal	HR 0.66 HR 0.66	[236]
Retrospective	Mortality	Metformin	Various	HR 0.56	[237]
Case control	Incidence	Metformin SUs Insulin	Ovarian	OR 0.61 OR 1.26 OR 2.29	[238]
Case control	Incidence	Metformin vs other	Prostate	RR 1.23♯	[227]
Prospective	Mortality	Metformin vs other	Various*	RR 0.73	[239]
Case control	Incidence	Metformin vs other	Breast	OR 0.81	[240]
Case control	Incidence	Metformin	Various	OR 0.46	[223]

Table 1.6.1: Epidemiological studies describing association of metformin use in DM2 patients with cancer incidence and mortality.Sulphonylureas (SUs), Thiazolidinediones (TZDs), * death from any cause (including cancer) and ♯ no association.

1.7 Aims of the study

Hypothesis:
In a non-diabetic setting, HCC chemoprevention with metformin at an early fibrosis stage, could be beneficial in abrogating tumours in livers with cirrhosis background.

Specific Aims:
I. Mouse model of HCC with cirrhosis background and determine time point that sufficiently recapitulates early fibrosis stage
II. Effects of early metformin intervention in the mouse model developed above will be investigated.
III. Plausible mechanism of action of metformin hepatoprotective effects.

Study design:
Aim I: To recapitulate HCC with cirrhosis background, we would utilize transgenic miRNA-221 over-expressing mice and induce chronic liver injury via carbon tetrachloride challenge. Various experimental end time-points will be researched to determine early fibrosis stage. Additionally, duration needed for liver tumours to appear in these mice will be determined by frequent abdomen ultrasonography surveillance.

Aim II: Metformin intervention would be undertaken at an early fibrosis stage and parameters needed to monitor liver function will be determined. At the experimental end points, morphological and histopathological changes in the liver between the experimental groups will be investigated.

Aim III: To determine effects of metformin intervention, microarray for gene expression and miRNAs will be investigated between experimental groups. Differential expression of genes and miRNAs would be analysed for important molecular pathways modulated, utilising publicly available and curated databases. Significantly affected pathways will be further validated by immunoblotting techniques.

Milestones
The studies described in this section are slated for completion within a period of three years. We expect to complete experiments with respect to Aim 1 in approximately sixteen months of which the longest time is dedicated to developing animal models. Aim 2 will be completed in approximately twelve months. Aim 3 would require nearly eight months and will be initiated soon after Aim 2 is achieved.

The methods and tools of science perennially breach barriers, granting me confidence that our epic march of insight into the operations of nature will continue without end.

Neil deGrasse Tyson

2

Materials and methods

2.1 CCl4 induced HCC mouse model with cirrhosis background and early metformin intervention

Male miR-221 transgenic (TG221) strain with B6D2F2 background mice (4-6 wks of age) were administered 150μl of olive oil (Arm 1) or 20% v/v CCl4 in olive oil (Arm 2) by oral gavage (p.o.) three times a week for a duration of 14 wks. Metformin intervention (Arm 3) was started after 3 wks of initiating CCl4 challenge at 300mg/kg body weight (BW) daily p.o., dissolved in distilled water. Dosage of metformin used in present study was calculated using Reagan-Shaw method [241]. Briefly mouse equivalent dose (mg/kg) = human dose (mg/kg) x human (k_m)/mouse (k_m). Where k_m factor, unique to each species is a constant used to normalize dosage based on body surface area. Metformin dosage in humans range from 1000mg to 2500mg, usually prescribed twice daily. For a 60kg human adult k_m equates to 37 and a 20g mouse k_m equals 3. Therefore, a dose of 1500mg per day in human adults translated to approximately 300mg/kg per day mouse dosage [242]. Mice were randomly assigned to the different experimental arms. Ellipse volume of surface nodules were measured ex-vivo as $V = (\pi/6) \times (\text{long axis}) \times (\text{short axis})^2$ [243].

2.2 Transaminase Assays

At time points of 0, 3, 6, 9, 14 wks blood sampling of 20μl was done by tail vein nick and post 24 wks by cardiac puncture after mice were euthanized by isoflurane inhalation anesthesia and subsequent cervical dislocation. Serum was collected from blood samples of corresponding experimental groups and AST and ALT levels assayed with respective colorimetric kits (Sigma; MAK055/MAK052) according to manufacturer's instructions and absorbance read on Tecan Infinite F200 Pro plate reader (TECAN, Switzerland).

2.3 Ultrasonography

Liver ultrasonography (Philips iU22 with a linear transducer) surveillance was performed at fortnightly intervals after 14wks to monitor growth of liver nodules. Mice were anesthetized with intraperitoneal (i.p.) cocktail of ketamine (90 mg/k BW) and xylazine (9 mg/kg BW) in 0.9% sodium chloride solution and were always placed on temperature-controlled heating pads during the entire imaging procedure. DICOM files were analyzed using an open source medical image viewer (Horos project v3.1.2).

2.4 Cell lines and reagents

Human hepatocellular carcinoma cell line HepG2 (ATCC HB-8065, Rockville, MD, USA) were cultured as monolayers in IMDM media (Sigma; I6529) supplemented with 10% fetal bovine serum (FBS, Sigma; F7524), 0.1% gentamicin (Gibco; 15750-037). Human hepatics stellate cell line LX-2 (generous gift from Vienna Hepatic Experimental Hemodynamic-HEPEX Lab at the Medical University of Vienna) were cultured as monolayer in DMEM media (Sigma; D5796) supplemented with 5% FBS, 0.1% gentamicin. All cell lines were maintained in a 37°C humidified incubator containing 5% CO2. Metformin (Sigma; D150959) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mol/L, stored at −20°C and subsequently diluted to appropriate concentrations in complete media without antibiotics for each assay. Multicellular tumour spheroids (MCTS) were cultured by hanging drop method as described previously [244]. Adherent cells were grown to 90% confluence, whereupon monolayers were be rinsed twice with PBS and drained. Cells were trypsinsed with 0.05% trypsin-1 mM EDTA and incubated at 37°C until cells detached. Trypsinization was arrested by adding complete medium and mixture of cells triturated to obtain single cell suspensions. After brief vortex and centrifuge at 1000 rpm for 5 minutes, cell pellet was washed twice with complete cell culture

medium. Cells were resuspended, and cell counts obtained using MUSE (Millipore) automated cell counter. Cell suspension was diluted so that 10μl would contain 5000 cells. On an inverted lid of 60 mm tissue culture dish several 10μ of cell suspension was deposited as drops making sure that the drops were placed sufficiently apart so as to not be in contact. PBS was placed in the 60 mm dish and the lid with cell drops was replaced onto the dish to form hanging drops and placed in cell culture incubator. Metformin was added to cell suspension media at varying concentrations before placing cell hanging drops. Control hanging drops did not contain any drugs. Images of MCTS were captured at different time point under a bright field microscope. All Cell culture experiments were performed in triplicated and repeated twice.

2.5 Western blot

Snap frozen tissues were homogenized in RIPA buffer (Sigma R0278) with protease (complete ULTRA tablets, Roche), phosphatase inhibitors (PhosSTOP, Roche) and sodium orthovanadate using a tissue homogenizer. Lysates were centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration of the supernatant was measured by modified lowry protein assay before boiling in 4x Laemmli buffer. Approximately 10µg of protein from lysates were loaded on precast PAGE, separated by electrophoresis and transferred to pvdf membranes with Trans-Blot Turbo system (Bio-Rad). Non-specific binding was blocked by 5% non-fat milk for 1hr. Membranes were incubated overnight at 4°C with primary antibodies. Primary antibodies against LKB1 (Cat- 3047), AMPKα (Cat- 2532), pAMPKα Thr-172 (Cat- 2535), AKT (Cat- 4691), pACC Ser-79 (Cat- 3661), ACC (Cat- 3662), S6 (Cat- 2217), p4E-BP1 (cat- 2855), 4E-BP1 (Cat- 9644), PARP (Cat- 9542), cleaved-PARP (Cat- 9544), Caspase 7 (Cat- 9492) 1:1000 dilution, pAKT Ser473 (Cat- 4060), pS6 Ser-235/236 (Cat- 4858) 1:2000 dilution were from Cell Signaling Technologies., α-SMA (Cat- A5228, Sigma) 1:500, p62/SQSTM1 (Cat- P0067, Sigma) 1:2000, LC3B (Cat- L7543, Sigma) 1:1000 and GAPDH as loading control (Cat- TA890003, Origene) at 1:5000 dilution. Following day, after a brief wash, membranes were incubated with anti-rabbit HRP (Cell Signaling 1:1000) or anti-mouse HRP (Cell Signaling 1:5000) secondary antibodies. Membranes were developed with Clarity western ECL (Bio-Rad) or high- sensitivity Westar Supernova Blotting Substrate (Cyanagen, Italy) and chemiluminescence visualized on Chemidoc XRS+ imaging system (Bio-Rad).

2.6 Histology and immunocytochemistry

Formalin-fixed paraffin embedded (FFPE) samples were sectioned into 5 μm-thick sections and stained with hematoxylin-eosin (H&E) and Masson's trichrome according to standard procedures. All slides were reviewed by the same pathologist. Formalin-fixed samples were further processed with 30% sucrose and embedded in optimal cutting temperature (OCT) compound and frozen to -80°C. OCT embedded samples were sectioned into 10 μm-thick sections and stained with Oil Red O (ORO) staining according to standard procedures. Lipid droplets were morphometrically quantified on ORO stained sections with image processing software (ImageJ, NIH) as described previously [245]. In total, 10 fields of visions were analysed for each section and lipid droplet size and number were averaged for comparison. HepG2 cells cultured with or without metformin were stained with ORO and images were captured. Amount of lipid droplets were quantified semi-quantitatively by extracting ORO from cells in 100% propanol and absorbance measured at OD450nm with Tecan Infinite F200 Pro plate reader (TECAN, Switzerland). Fixed LX-2 cells cultured on polylysine coated round coverslips were incubated with α-SMA antibody (1:100, Cat- A5228, Sigma), followed by incubation with Alex Fluor® 488 goat-anti mouse (1:1000, Cat- A11001, Thermo Fischer scientific) for 1hr at room temperature. Cells were then mounted with Vectashield mounting medium with DAPI, observed with a Nikon Eclipse TE2000-E confocal microscope (Nikon, Florence, Italy) and images acquired by Nikon DXM1200F digital camera.

2.7 Gene and microRNA expression microarray analysis

Gene expression profiling was done with an Agilent whole mouse gene expression 8x60K microarray (Cat- G4852A, Agilent Technologies). One-color microarray-based gene expression was analysed according to standard operating procedures from Agilent Technologies. Briefly, total RNA from snap frozen samples was extracted using Trizol Reagent (FS-881, FMB, Trevose, USA) according to manufacturer's instructions. Quantity and quality of RNA was assessed with RNA-600 nanochip (Agilent Technologies) on Agilent 2100 Bioanalyzer. Samples with RNA integrity number above 8 were utilized for microarray. Total RNA of 100ng from each sample was used to synthesize cyanine 3-CTP (Perkin-Elmer Life Sciences, Boston, USA) labelled cRNA with Low RNA Input Linear Amplification kit (Agilent Technologies). Labelled RNA was hybridized at 65°C for 17hrs at 10rpm in an incubator. Images of slides were captured by the Agilent scanner and raw microarray data was obtained by accompanying Agilent Feature Extraction Software (v10.5). MicroRNA expression profiling was done with Agilent SurePrint mouse miRNA microarray (Cat- G4872A, Agilent Technologies). Images of slides were captured by

the Agilent scanner and raw microarray data was obtained by accompanying Agilent Feature Extraction Software (v10.5). Quantile normalization of raw microarray expression and downstream analysis was performed with Qlucore omics explorer (v3.4, Qlucore AB, Sweden). Differentially expressed genes in multiple groups were sorted based on analysis of variance, F-test and <0.01 false discovery rate (FDR-q). Heat maps were generated based on hierarchical clustering of samples and genes. Genes that were differentially expressed >2-fold change and <0.01 FDR between two groups (Metformin+CCl4 vs CCl4 livers) on an unpaired t-test were considered for Gene Set Enrichment Analysis (GSEA). Differentially expressed genes were summarized into mouse Entrez gene IDs and mapped to human orthologs using mapping reports from Mouse Genome Informatics database (Jackson laboratories- www.informatics.jax.org). Differential genes between phenotypes were ranked according to t-statistics. Metformin mediated up-regulated genes were given a positive score and corresponding down-regulated genes a negative score compared to CCl4 only livers. Pre-ranked GSEA was applied with GSEA 3.0 software [246, 247]. The curated canonical pathways from MSigDB (Molecular Signature Database - Hallmark, KEGG and Reactome) were used. Statistical significance of normalized enrichment score was estimated using phenotype-based permutation (n = 1000) testing and FDR<0.25.

2.8 Statistical analyses

Statistical analysis was performed using data analysis software Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). An unpaired two-tailed t test was used to compare differences between two groups throughout the study and significance with a threshold of $p<0.05$ was considered. Summary data are expressed as mean ± standard deviation (SD).

Well begun is job half done.

Aristotle

3

Results

3.1 Mouse model of HCC with cirrhosis background

We utilised previously established transgenic mice strain (TG221), which are predisposed to the development of liver tumours [201]. Overexpression of miR-221 in these mice results in spontaneous liver tumours by the age of 9 to 12 months.

CCl4 is routinely used in mice to induce cirrhosis and accelerate tumourigenesis [248]. Hence, recurrent liver injury was induced in 4 to 6 weeks old TG221 male mice by oral gavage of 150 ul 20% CCl4 v/v in olive oil, three times a week for the duration of 14 weeks (Arm 2, n=7). In conjunction, TG221 mice in experimental group Arm 1 (n=6) were administered 150 ul of olive oil, at the same time points (Figure 3.1.1).

3.1.1 CCl4 induced Cirrhosis in TG221 mice

To verify liver injury caused by CCl4, liver enzymes AST and ALT were monitored in serum at 3, 6, 9, 14 and 24-week time points in both experimental arms and compared to base line enzyme

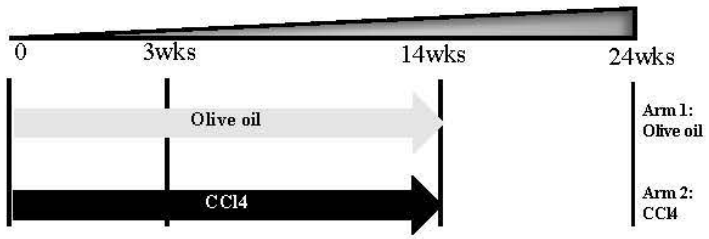


Figure 3.1.1: Experimental design: 4 to 6 weeks old mice were administered olive oil (Arm1, n=6) or 20% CCl4 in olive oil (Arm2, n=7) per os (p.o.) for 14 weeks. All mice in Arm2 developed liver nodules 10 weeks after cessation of CCl4 gavage. Experimental end-point was determined at 24 weeks after initiation of CCl4 challenge.

levels. AST and ALT are robust and commonly used markers of liver function [249]. Basal AST, ALT levels in mice before CCl4 challenge were determined. Subsequent liver enzyme levels at different time points with or without CCl4 challenge were determined as relative percentage change compared to basal levels. Liver enzymes in olive oil treated mice remained at mentioned basal levels at all measured time-points and at experimental end-point. However, CCl4 challenge resulted in incremental percentage increase in both serum AST and ALT levels, of 206% (±3.9), 273% (±5.2), 330% (±2.4) and 400% (±2.2) AST, 129% (±13.8), 166% (±22.7), 291% (±124.4) and 295% (±114.7) ALT, for 3, 6, 9 and 14 weeks respectively. At 24 weeks, liver enzymes remained increased (AST 492% ±4.8, ALT 347% ±114.4 increase from basal levels), thereby signifying a state of sustained chronic liver injury even cessation of CCl4 (Figure 3.1.2).

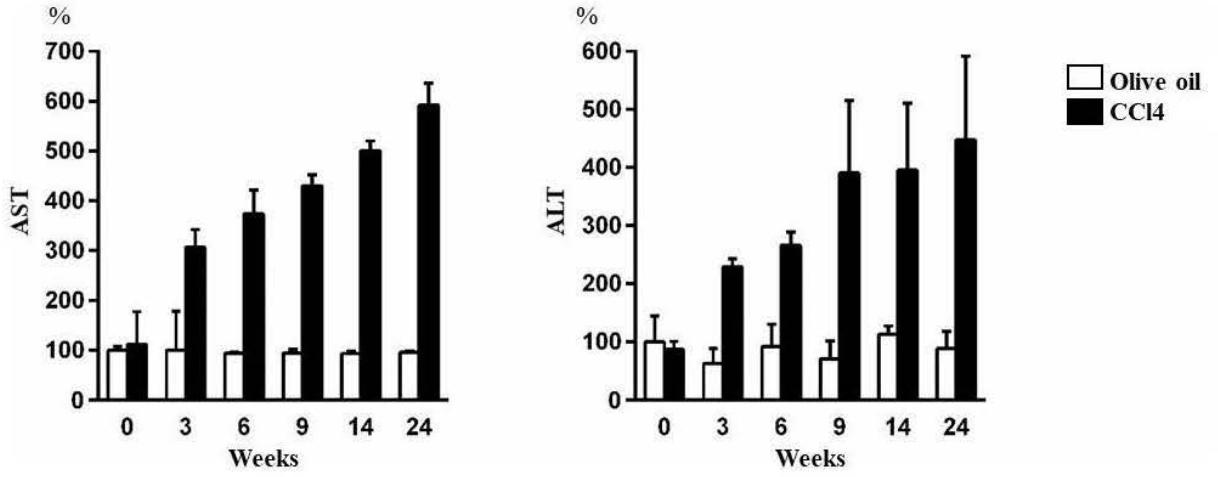


Figure 3.1.2: Liver function test: Liver damage was detected in CCl4 treated mice by the increase of liver Aspartate transaminase (AST) Alanine transaminase (ALT) over time, compared to olive oil treated mice. AST-ALT levels are depicted as percentage change between Olive oil and CCl4 experimental arms.

Ascites is associated with chronic liver disease and an important finding in cirrhosis. To eval-

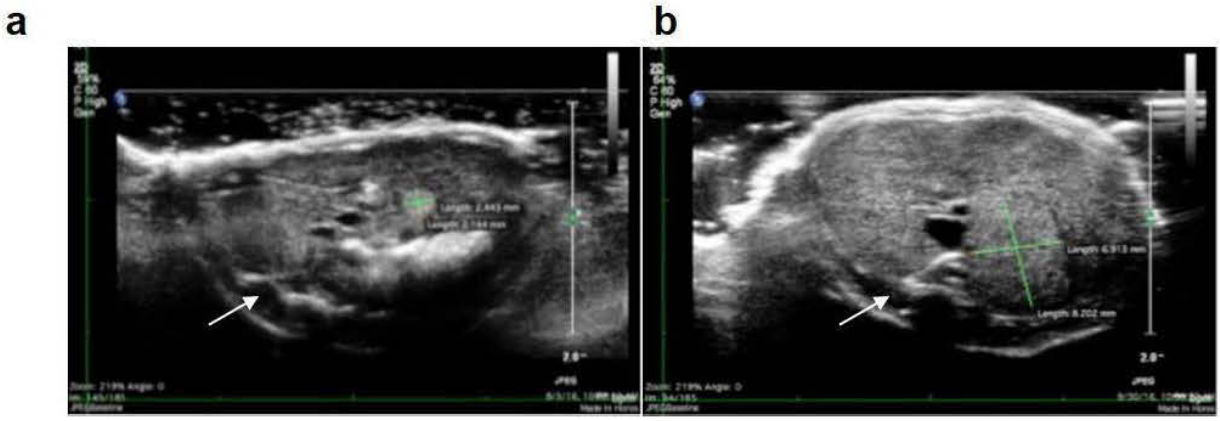


Figure 3.1.3: Transversal liver sonogram (a) after 14 and (b) at 24 weeks post initiating CCl4 challenge. Transverse diameters of hepatic nodules are marked in green lines and white arrow head represents anechoic ascites fluid

uate ascitic decompensation due to CCl4, we imaged mice livers with fortnightly abdominal ultrasound starting from 14 weeks to 24 weeks. anechoic ascitic fluid collection was observed in all CCl4 mice at 14 weeks and sustained presence of ascites was noticed at 24weeks (Figure 3.1.3).

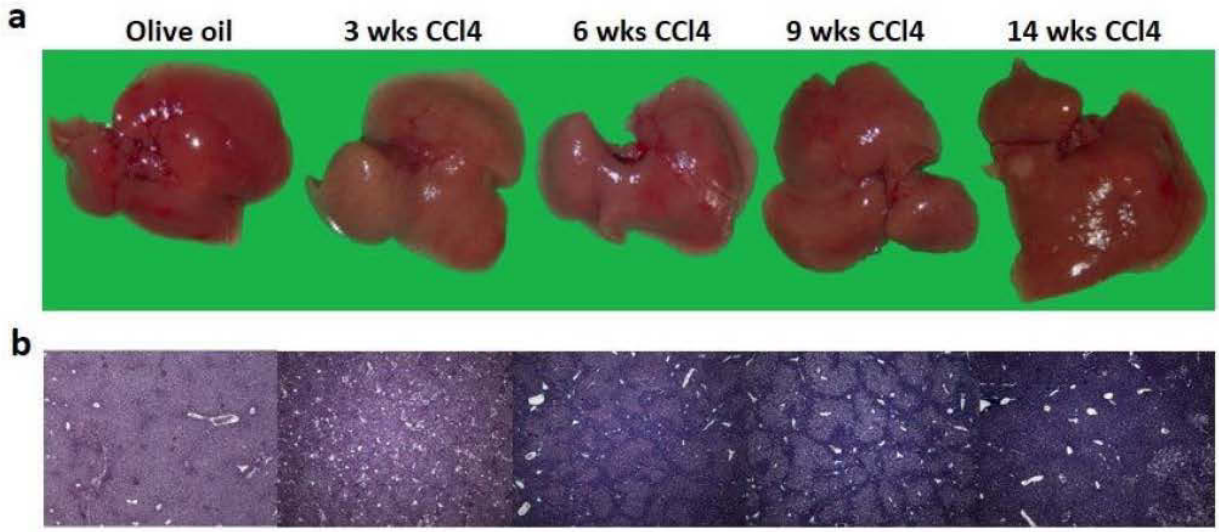


Figure 3.1.4: Representative images of mouse livers (a) at the indicated time of sacrifice. (b) Trichrome staining of FFPE livers at corresponding time points and treatments as of (a) (40x magnification).

The third most salient feature or cirrhosis is the underlying liver fibrosis. To evaluate the time-point at which early signs of fibrosis could be detected, we treated additional mice with CCl4 following the same protocol described above. However, at 3, 6, 9 and 14 weeks, mice (n = 2 at each time-point) were euthanised and livers examined. Macroscopically, livers showed signs of the CCl4-induced damage, characterized by a yellowish pale appearance rather than dark red,

a harder than normal consistency, and surface with nodules of varying sizes were evident by 14 weeks (Figure 3.1.4a). Trichrome staining revealed a sequential progression of liver fibrosis with sustained CCl4 challenge. Thin septal fibrosis was evident at 3wks, progressing to marked fibrous expansion of most postal areas with fibrous septae by 6 and 9wks but still maintaining liver architecture. However, 14wks of CCl4 challenge led to fibrous expansion with marked, distortion of liver architecture, nodule formation and ascites typical of cirrhosis (Figure 3.1.4b). Therefore, 3 weeks time-point was determined to be an early fibrosis stage.

3.1.2 Cirrhosis induced by CCl4 leads to tumours in TG221 mice

Given the extent of liver damage induced by chronic CCl4 challenge, we initiated liver nodule surveillance by abdominal USG starting at 14 weeks. Hyperechoic lesions could be detected at this time-point and fortnightly USG was continued until 24 weeks at which time, obvious liver mass could be identified. Experimental end-point was determined to be 24 weeks, in concordance with institutional humane animal care guidelines. Macroscopic examination of the livers revealed significantly large tumours in all mice treated with CCl4. Olive oil treated mice did not bear any visible liver lesions (Figure 3.1.5a,b). Histopathological examination of livers confirmed the presence of additional in-situ nodules that ranged from frank well-differentiated HCC to FNH-like nodules in CCl4 treated mice (Table 1). Additionally, occasional steatotic nodules could be detected (Figure 3.1.5b). Sections of large surface nodules conformed to typical histopathology of well-differentiated HCC (Figure 3.1.5c).

Next, we examined micro-array gene expression of livers in Arm 1 and 2 and tumours from Arm 2, to characterize molecular profile of HCC metamorphosis in our current mouse model. Interestingly a panel of recognized maker genes of progenitor/stemness hepatocytes were significantly increased in HCC when compared to livers of either Arm 1 and Arm2. Expression of liver progenitor cell markers lymphocyte antigen 6 family member D (Ly6d), Cd63, aldehyde dehydrogenase 18 family member A1 (Aldh18a1), four and a half lim domains 2 (Fhl2), CD133/promonin 1 (Prom1), pyruvate kinase (Pkm), epithelial cell adhesion molecule (Epcam), gamma-glutamyltransferase 1 (Ggt1), pannexin 1 (Panx1), ATP binding cassette subfamily C member 1 (Abcc1) and Capping actin protein-Gelsolin like (Capg) were increased in HCC compared to corresponding cirrhotic livers and olive oil treated liver tissue . The presence of these markers suggests that the tumours that arise in these mice have tumour initiating precursor or stemness cell phenotype [250, 251]. Furthermore, glypican-3 (Gpc3) is the most highly over-expressed membrane bound protein in HCC compare with non tumor liver. As expected, Gpc3 gene-expression in resected tumours of CCl4 treated livers was increased by 32.98 fold when compared to olive oil treated livers. (Figure 3.1.6)

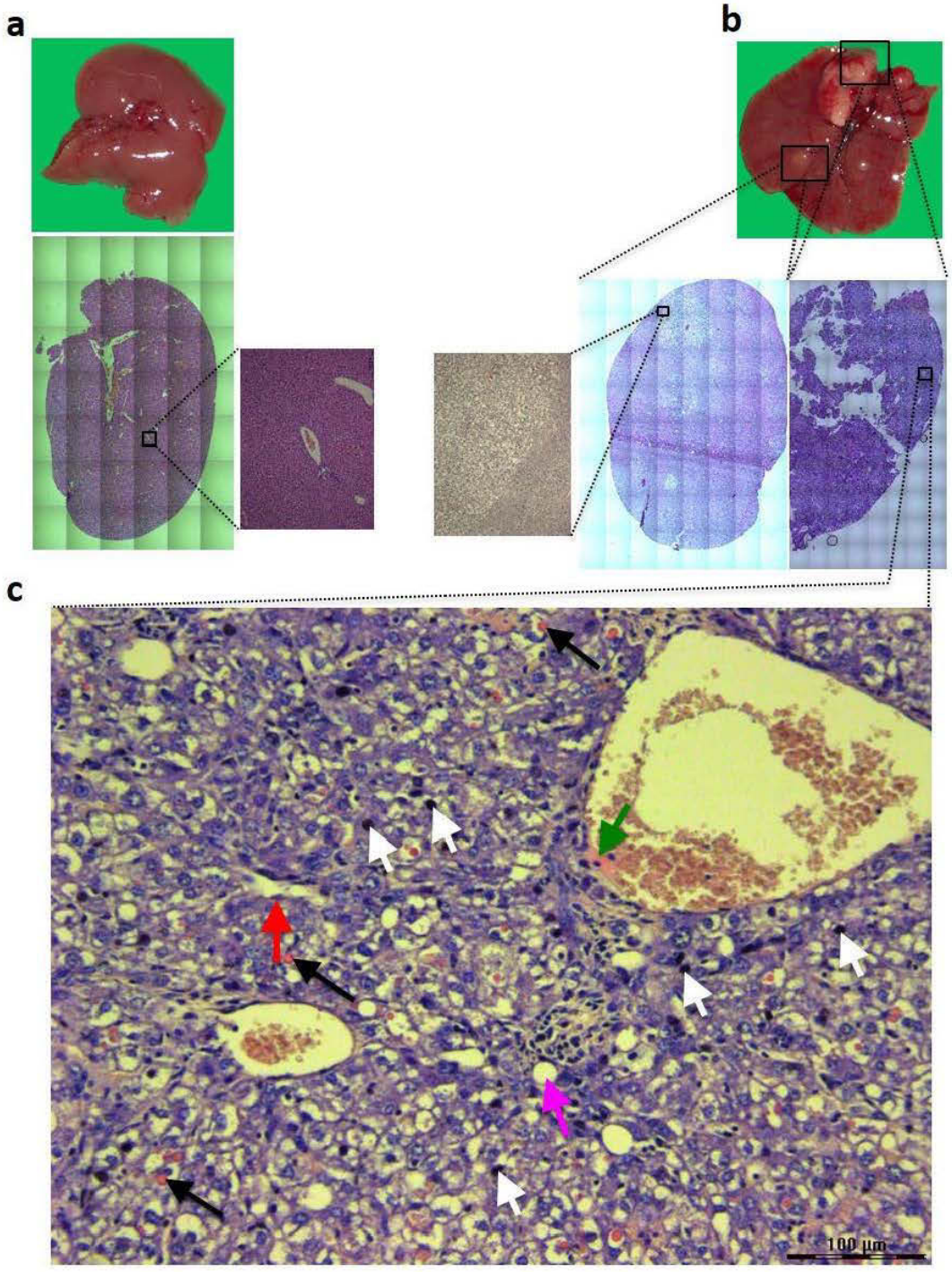
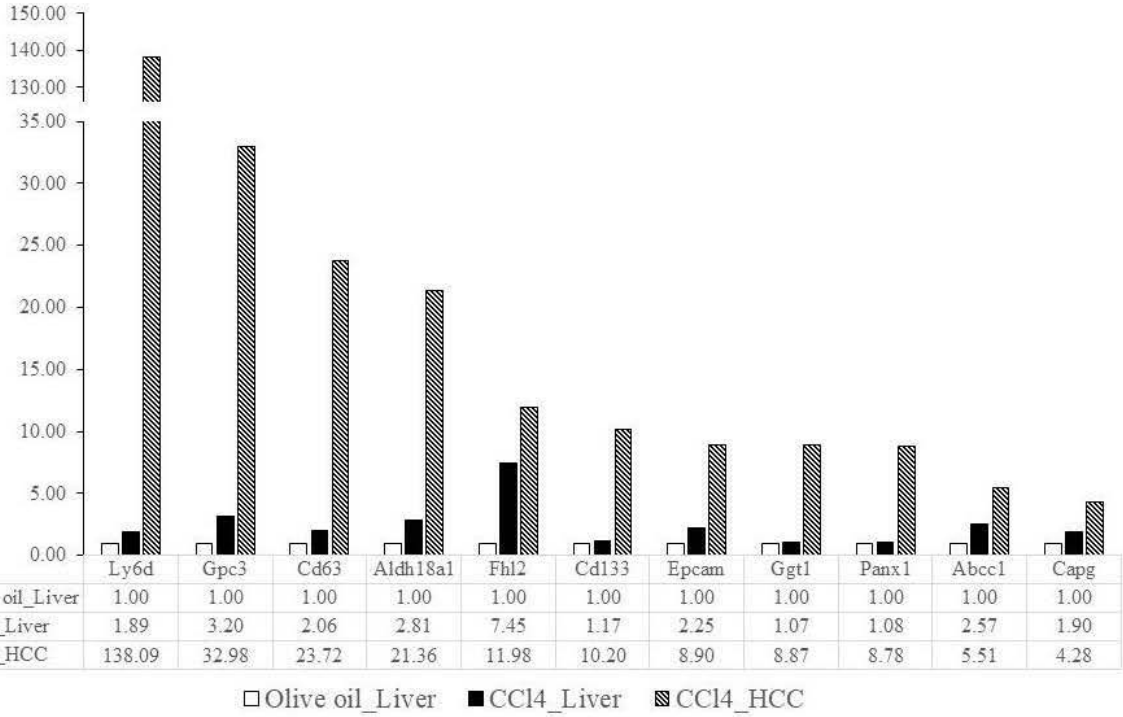


Figure 3.1.5: Representative images of livers and stained liver/tumour sections after 24weeks in (a) olive oil (b) CCl4 experimental arms. Mosaic images (40x magnification) show normal liver architecture in olive oil treated mice, whereas in CCl4 treated liver, intrahepatic steatotic nodule can be seen. Expanded areas of respective stained liver sections are shown at 100x magnification. (c) Representative H&E section of liver tumour shows salient histopathological findings of HCC; endothelial cells lining sinusoids (red arrow), rosette-like structures around blood vessels (green arrow), macrosteatosis- large fat vacuoles without nuclei (pink arrow), mitotic bodies-depict increased mitoses (white arrows), eaosinophilic globules (black arrows), absence of portal tracts and hepatic lobules and hepatocytes that appear to have prominent round nuclei with coarse chromatin and thickened nuclear membrane (200x magnification).



	Ly6d	Gpc3	Cd63	Aldh18a1	Fhl2	Cd133	Epcam	Ggt1	Panx1	Abcc1	Capg
Olive oil_Liver	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
CCl4_Liver	1.89	3.20	2.06	2.81	7.45	1.17	2.25	1.07	1.08	2.57	1.90
CCl4_HCC	138.09	32.98	23.72	21.36	11.98	10.20	8.90	8.87	8.78	5.51	4.28

Figure 3.1.6: Alterations in progenitor and stem cell genes in CCl4 induced cirrhosis TG221 mouse model. Gene expression was assayed by normalised microarray data from livers of Olive oil, CCl4 treated mice and tumours from CCl4 treated mice at 24 weeks. Data are represented as fold-change compared to olive oil treated livers.

3.2 Early metformin intervention abrogates fibrosis in CCl4 challenged TG221 mice

In patients, progression from asymptomatic liver fibrosis to decompensated cirrhosis takes several years. Furthermore, once end-stage liver disease is reached, liver transplantation represents the only option to improve quality of life in these patients [252]. Therefore, implementation of early intervention strategies are aimed at preventing deterioration of fibrotic disease [253]. Since in the TG221 mouse model, CCl4 elevated serum levels of AST, ALT and induced fibrosis as early as 3wks, we hypothesized that chemoprevention strategies at this stage could possibly prevent the progression of disease. TG221 mice (n=7) were treated daily by oral gavage with metformin at 300mg/kg dosage starting at 3wks post induction of CCl4-challenge (Figure 3.2.1, Arm 3). These mice were administered CCl4 up to14wks, similar to mice in Arm2 and metformin treatment was continued until the experimental end-point at 24wks. Three hours post final metformin gavage, mice were sacrificed, and livers harvested.

Metformin intervention significantly decreased CCl4 induced liver fibrosis as evidenced by

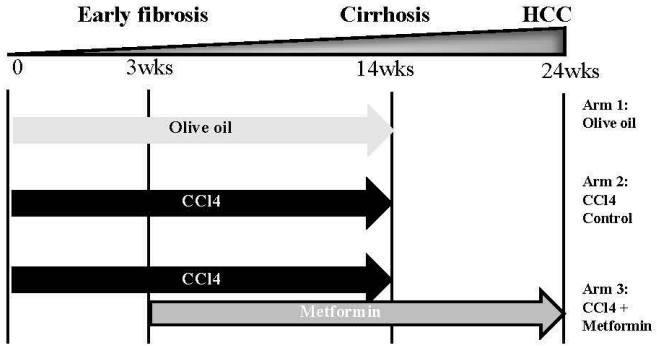


Figure 3.2.1: Experimental design of metformin treatments: (a) Metformin and control arms in CCl4-challenged TG221 mice.

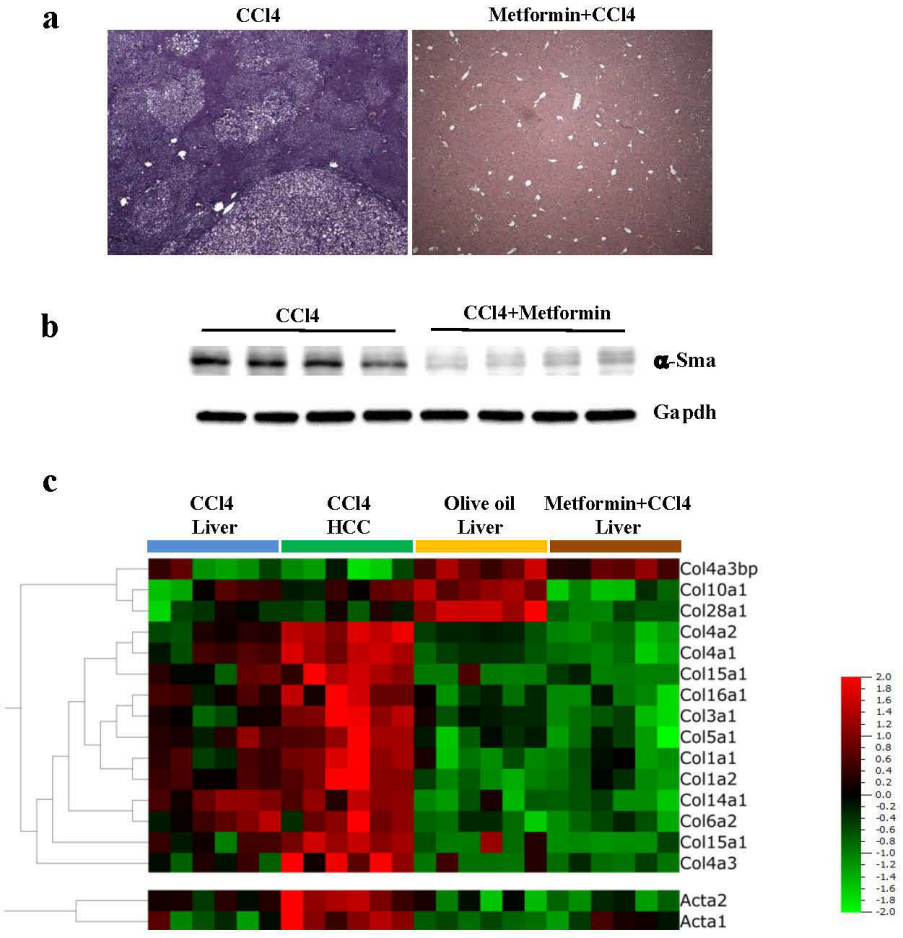


Figure 3.2.2: Metformin intervention reduces fibrosis in CCl4-challenged TG221 mice. (a) Representative trichrome staining of FFPE livers at 24 weeks without and with metformin intervention. (b) Western blot analysis of α-Sma / Acta2 protein in livers with and without metformin intervention. (c) Heat map for the expression of several collagen and α-SMA genes (Acta2 and Acta1) in CCl4 only livers, matched liver nodules, olive oil livers and metformin+CCl4 treated livers. Intense red means the highest expression; intense green the lowest.

collagen content, immunoblotting for α-SMA and gene expression analysis (Figure 3.2.2). Of note, significant reduction of Col1a1, Col3a1, Col4a1 expression and α-SMA protein levels in livers indicates an inhibitory effect on HSC activation by metformin. We further confirmed that metformin abrogated the expression of α-SMA in the human hepatic stellate cells LX-2 cells in vitro in a dose dependent manner (Figure 3.2.3).

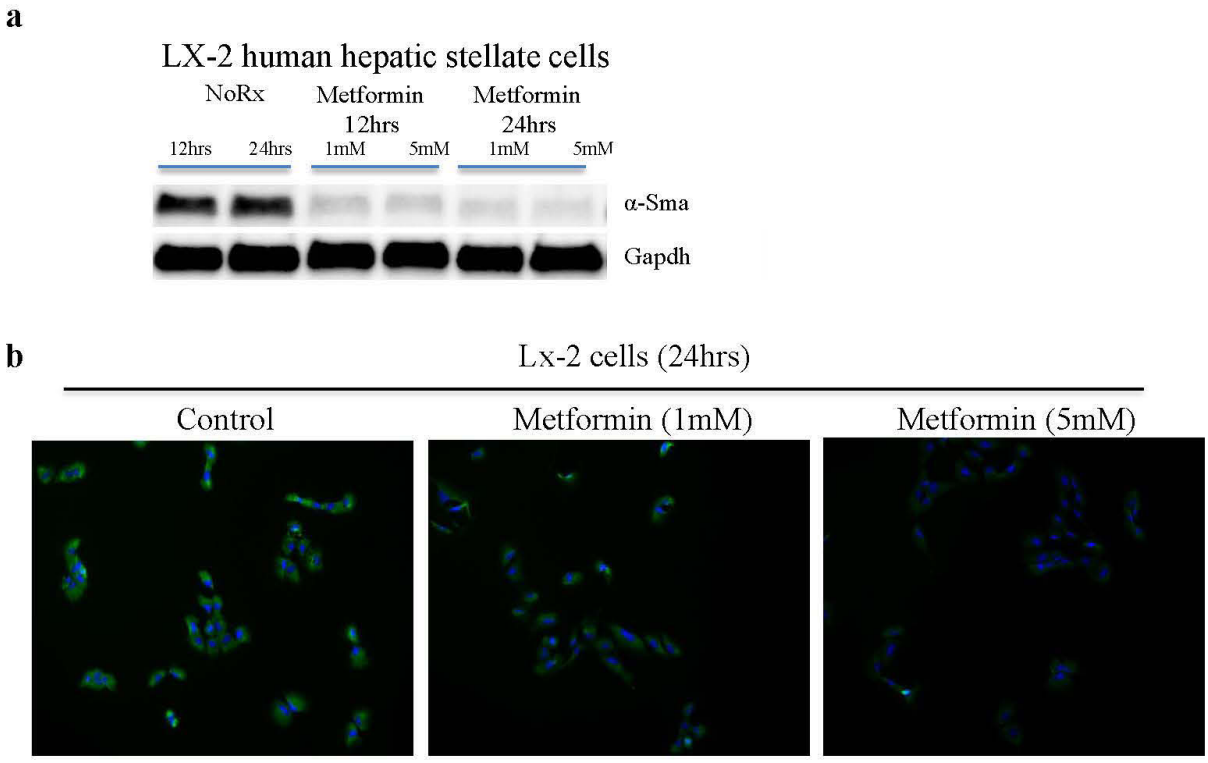


Figure 3.2.3: Metformin prevents hepatic stellate cell activation *in vitro*: (a) Western blots of LX-2 human hepatic stellate cell lysates show decreased α-SMA expression when treated with metformin in vitro in a dose and time dependent manner. Gapdh was used as loading control. LX-2 cells were incubated in vitro with metformin (1mM & 5mM) for 24hrs. (b) Representative immunocytochemistry merged images (100x) show decreased a-SMA (green) expression on metformin exposure. Nuclei are stained with DAPI (blue).

3.3 Early metformin intervention abrogates steatosis in CCl4 challenged TG221 mice

Microvesicular steatosis is another feature of cirrhosis where excess lipid droplets are observed in hepatocytes [254, 255]. Metformin significantly decreased accumulation of lipid droplets in livers when compared to untreated CCl4 challenged mice (Figure 3.3.1 a,b). One of the well-studied mechanism of actions of metformin, is its ability to activate AMPK [256]. AMPK has been attributed to be a master regulator of cellular metabolism and energy homeostasis [257].

Here, we confirmed by immunoblotting, that metformin increased the levels of total LKB1 leading to increased phosphorylation of AMPK at Thr-172.

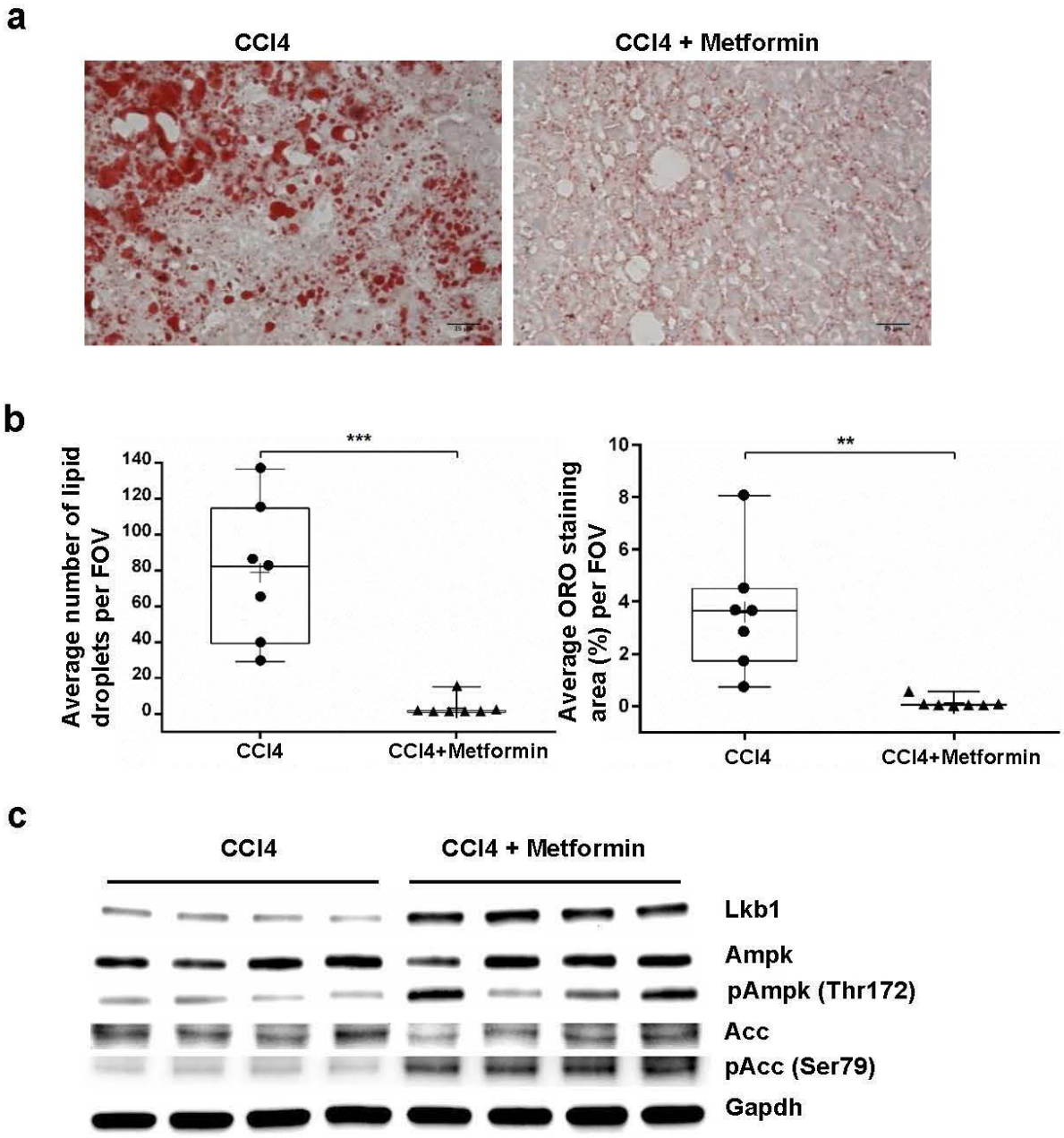


Figure 3.3.1: Metformin intervention reduces steatosis in CCl4-challenged TG221 mice. (a) Oil Red O (ORO) staining of sections from formalin fixed, OCT embedded and frozen livers at 24 weeks without and with metformin intervention. (b) Lipid droplets were morphometrically quantified after ORO staining for average number and percentage staining area per field of vision in CCl4 and metformin+CCl4 mice. $***P<0.001$ $**P<0.01$. Each data point represents a single mouse (c) Western blot analyses show that metformin induces AMPK activation and leads to the downstream inhibitory phosphorylation of ACC at Ser-79, a rate-limiting step in fatty acid synthesis.

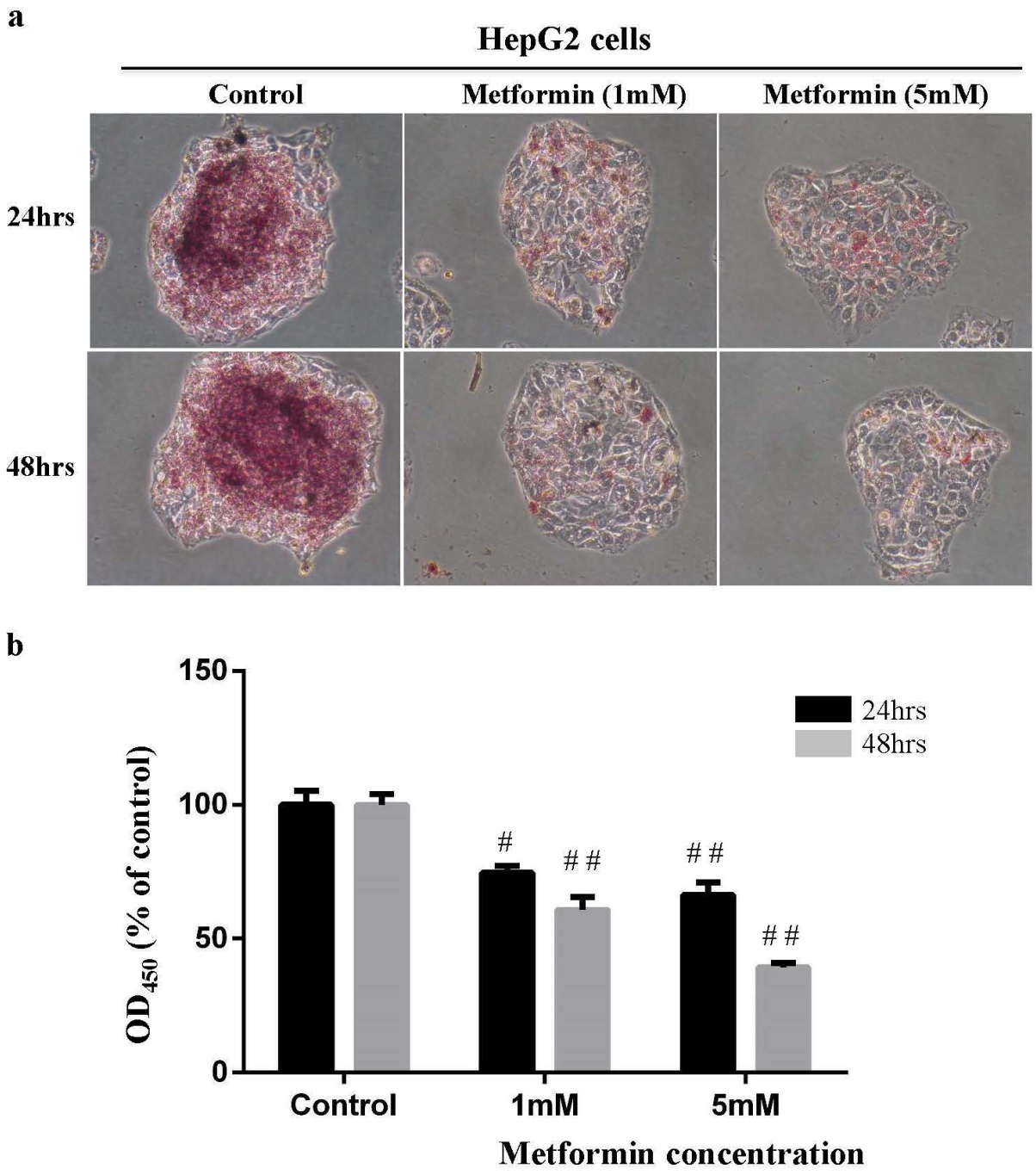


Figure 3.3.2: Metformin prevents intracellular lipid accumulation *in vitro*: HepG2 cells were incubated with metformin (1mM & 5mM) for 24hrs and 48hrs in vitro. (a) Representative images of cells fixed and stained with ORO showing effects of metformin on intracellular lipid droplets. Amount of lipids droplets was measured semi-quantitatively by retrieving ORO from cells and reading absorbance at 450nm. (b) Bar graph represents quantity of ORO measured (OD450) in metformin treated cells relative to untreated control cells (♯ $P<0.001$, ♯♯ $P<0.0001$ compared to no treatment controls of respective time points). Data presented as mean±SD of triplicates.

As a consequence of activation of AMPK, downstream inhibitory phosphorylation of ACC at

Ser-79 was also determined (Figure 3.3.1c). The enzyme ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis [218]. Abrogation of ACC activity might account for metformin mediated reduction in de novo lipogenesis in hepatocytes. Further in vitro, metformin significantly reduced intracellular lipid droplets in HepG2 cells in a dose and time dependent manner (Figure 3.3.2).

3.4 Early metformin intervention prevents HCC in CCl4 challenged TG221 mice

Data on fibrosis and steatosis suggest that the change in the microenvironment could prevent tumour appearance. As predicted, metformin treatment significantly decreased the number of HCC tumours detectable at experimental end-point of 24 weeks (Figure 3.4.1 a,b) . Control animals harboured 11.14 ± 1.2 surface tumours, whereas metformin treated harboured 0.857 ± 0.26 tumours, that amounted to 92% reduction ($P<0.0001$ Figure 3.4.1 c). Since we took into account all surface nodules measuring greater than 1mm in diameter, we compared he total tumor burden between control and metformin treated groups. Total tumor burden was defined as sum of volume of nodules of each mice. Control mice had a tumor burden of 411.8 ± 102.8 mm3, whereas metformin treated mice had 1.024 ± 0.46 mm3 tumor burden that amounted to a dramatic 99.75 % reduction ($P<0.01$ Figure 3.4.1 d).

Metformin further improved serum AST and ALT levels in treated mice, with a significant decrease in both AST and ALT levels compared to CCl4 only treated mice. (Figure 3.4.1 e).

In addition, histopathology of livers did not reveal any detectable in-situ nodules and lobar architecture of the livers were intact with minimal hydropic changes of hepatocytes in metformin treated mice. Whereas, in control mice, abundant nodules varying from FNH-like to HCC were noticed. Liver tumors in control mice had steatotic features and arose in a background of diffuse liver lesions characterized by hydropic and fatty changes, focal and confluent necro-inflammation, fibrosis and stellate cell activation (Figure 3.4.2). These results suggest that metformin inhibits initiation of liver neoplasms when intervened at an early stage of fibrosis.

We further investigated impact of metformin on formation of tumour spheroids in vitro. To model solid tumors more accurately, several 3D culture systems are utilised, such as; tissue slice cultures, scaffold -based cultures and multicellular tumour spheroids (MCTS) [258]. Of these, the multicellular tumor spheroid (MCTS) model is the most well-characterized and the most widely used model. Spheroids mimic tumor behavior more effectively than conventional 2D culture systems as spheroids, similar to tumours, contain both surface-exposed and deeply buried cells,

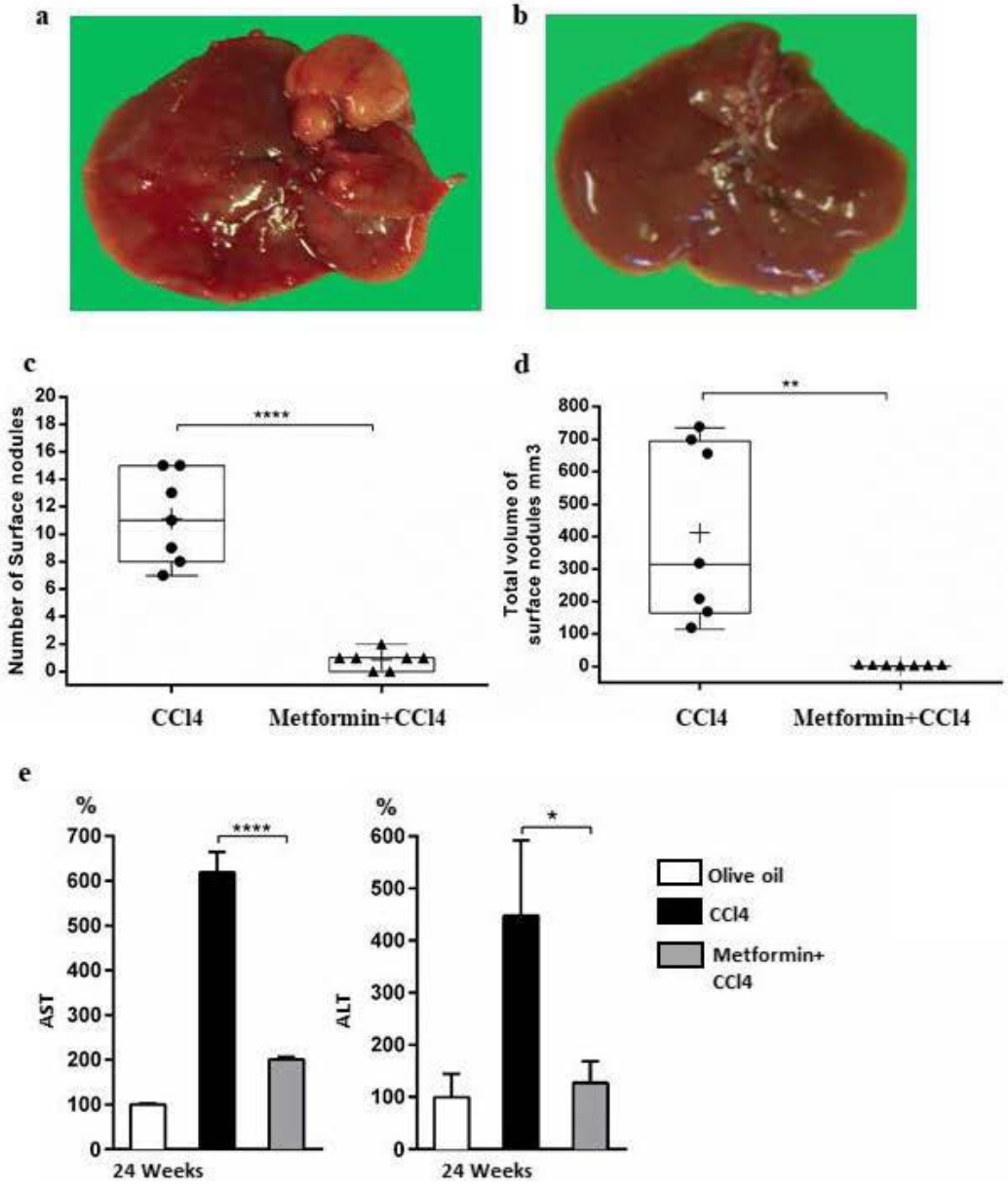


Figure 3.4.1: Metformin intervention prevents the appearance of tumor nodules in CCl4-challenged TG221 mice. (a) Representative liver from a CCl4-challenged mouse control. (b) Representative liver from a mouse treated with CCl4 and metformin. (c-d) Nodules number and volume in CCl4 mice in comparison with mice that received metformin+CCl4. The latter did not develop any tumor nodules. $^{****}P<0.0001$ $^{**}P<0.01$ Each data point represents a single mouse. (e) Metformin intervention improved serum levels of Aspartate transaminase (AST) Alanine transaminase (ALT). $^{****}P<0.0001$ $^{*}P<0.05$. AST-ALT levels are depicted as percentage change between experimental arms at 24 weeks time-point.

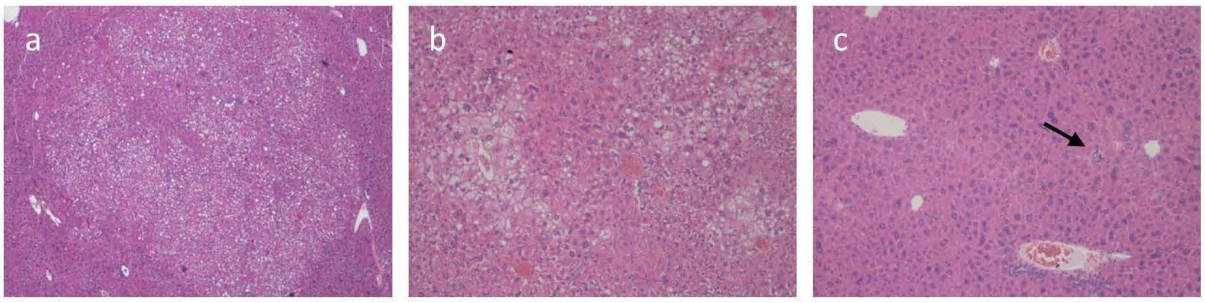


Figure 3.4.2: Histopathology of liver and liver nodules in CCl4-challenged TG221 mice: (a) Liver tumors in CCl4-challenged control mice had steatotic features and arose in a background of diffuse liver lesions characterized by hydropic and fatty changes, focal and confluent necro-inflammation, fibrosis and stellate cell activation (H&E, 40x magnification). (b) Prominent background steatosis in a control mice accentuated around terminal veins (H&E, 100x). (c) Metformin-treated mice had livers with intact lobular architecture, minimal hydropic changes of hepatocytes and 'spotty' cytolytic foci (arrow)(H&E, 100x magnification).

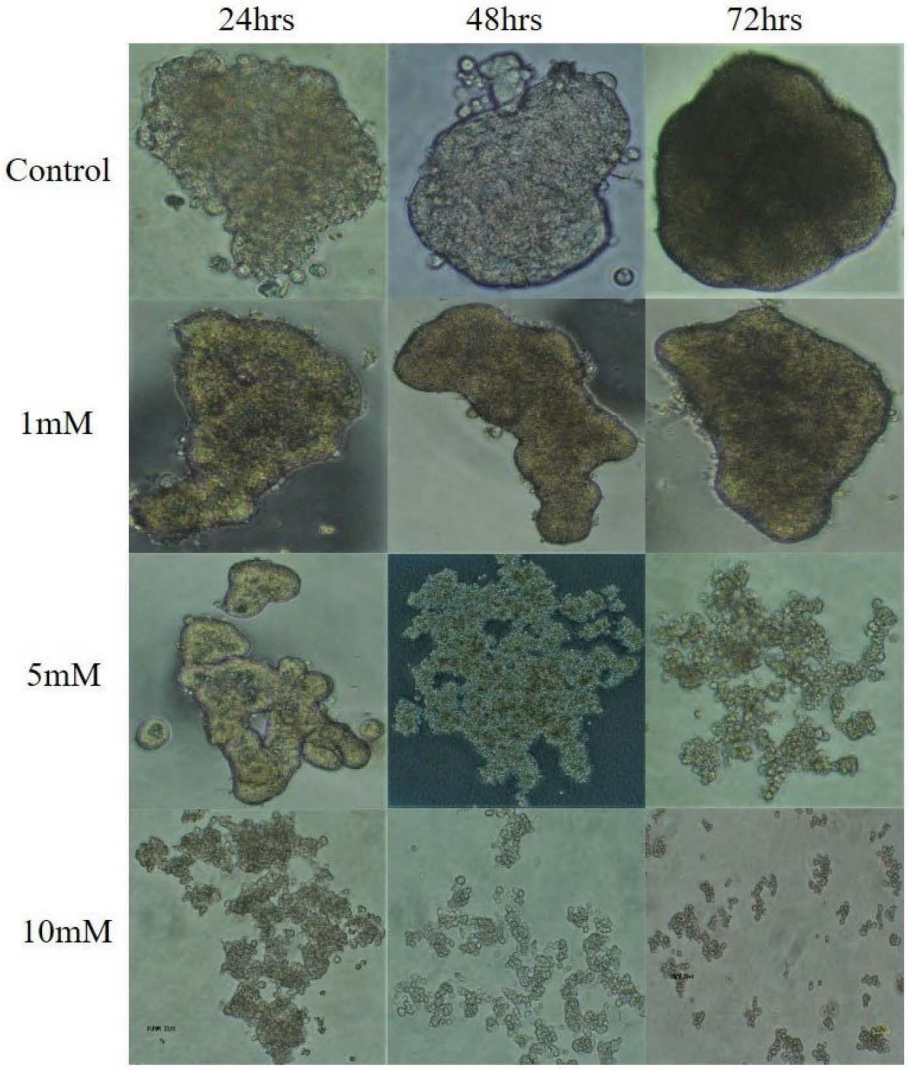


Figure 3.4.3: Metformin prevents initiation of multicellular tumour spheroids: Mouse Hep53.4 hepatoma cells were seeded without or with indicated concentration of metformin and representative images of MCTS at time-points 24, 48 and 72 hours are shown (magnification 200X).

well-oxygenated and hypoxic cells, and proliferating and non-proliferating cells. Furthermore, to model process of tumour initiation and propagation, MCTS are ideal to study the inherent nature of cancer cells to form closely packed cell clusters [259]. Mouse hepatoma Hep53.4 cells were seeded by hanging drop method to initiate MCTS in media without metformin and varying concentrations of metformin. Hep53.4 cells formed MCTS by 24hrs in culture and spheroids proliferated to become densely packed cells maintaining circularity by 72hrs. Metformin, interfered with formation of MCTS in a dose dependent manner, where in 1 millimolar (mM) concentration of metformin in media did not significantly affect spheroid formation, but decreased circularity of formed spheroids. At increasing metformin dosage, 5mM and 10mM formation of MCTS was greatly hindered beginning at 24hrs and cells could not recover to orient into 3D cell clusters at 72hrs (Figure 3.4.3). These finding further support the notion that metformin inhibits initiation of tumours.

3.5 Mechanisms of HCC chemoprevention by metformin

Microarrays are remarkable tools for high-throughput screening of two or more samples for side-by-side comparisons [260]. We first compared miRNA microarray expression profile of control and metformin treated livers. Interestingly, significant number of miRNAs were upregulated (fold change >2, $|R|^2 > 80$) by metformin (Figure 3.5.1). Further, we analysed pathways of upregulated miRNA-target genes miRPathDB [261]. Union of predicted miRNA target interactions extracted from miRPathDB pointed towards 33 key pathways. A detailed inspection of the targeted pathways highlights that the miRNAs jointly regulate several parts of the signaling cascades. However, PI3K/AKT, VEGF, type 2 diabetes mellitus and Proteoglycans in cancer signalling pathways curated in KEGG database, were targeted by more than 10 miRNAs that were upregulated by metformin (Figure 3.5.2a).

Similar metformin mediated global upregulation miRNAs has been reported in breast cancer xenograft mouse models [262]. In this study, metformin was effective in inhibiting implanted tumours and upregulated expression of miRNAs in tumour tissue on account of increased DICER1 expression. Therefore, we probed for DICER1 protein expression in control and metformin treated livers by western blot. Metformin significantly upregulated DICER1 expression in liver when compared to control mice (Figure 3.5.2b). These results could explain global upregulation of miRNA expression mediated by metformin.

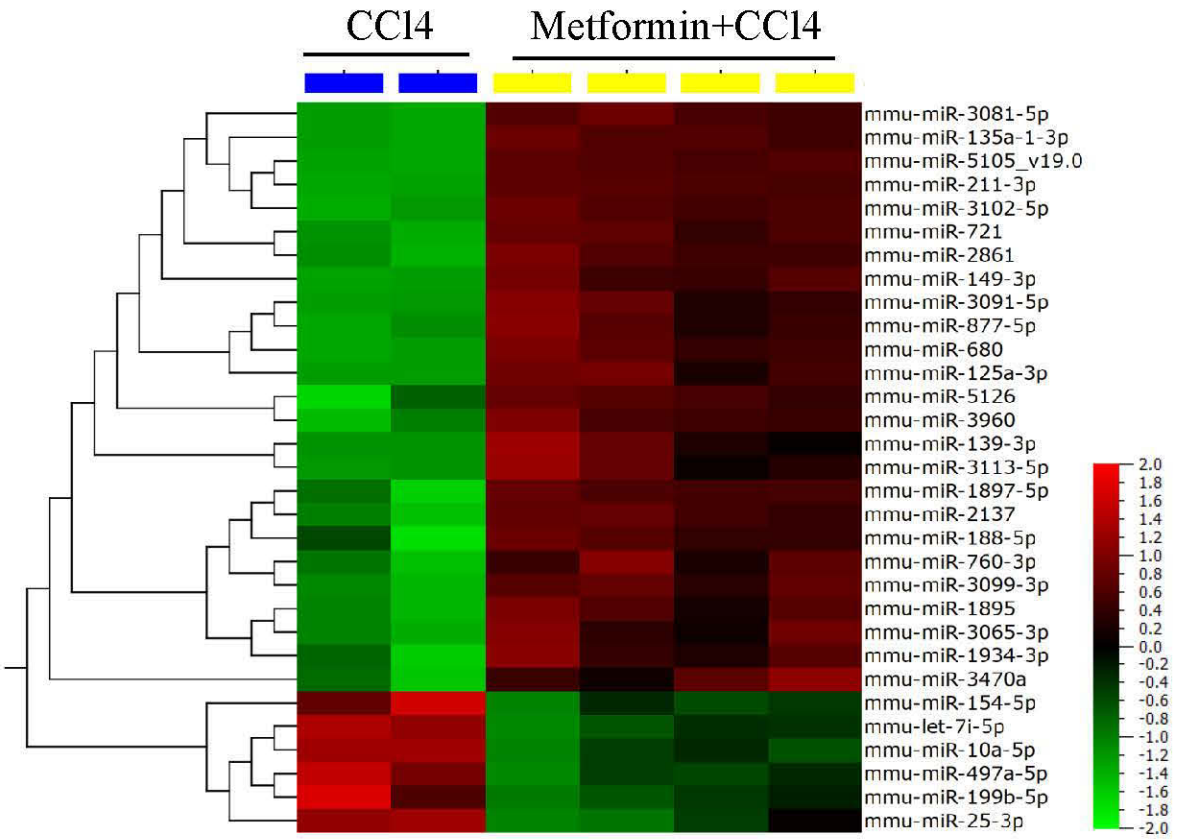


Figure 3.5.1: Metformin preferentially upregulates microRNAs in liver: miRNA expression is hierarchically clustered on the y axis, and liver samples from individual mice with and without metformin clustered on the x axis. The legend on the right indicates the miRNA represented in the corresponding row. The relative miRNA expression is depicted according to the color scale shown on the right. Red indicates upregulation; and green, downregulation.

Next, we explored the differences in gene expression profile of control and metformin treated liver tissue with microarray. Gene Set Enrichment Analysis (GSEA) was performed on microarray data from livers of untreated and metformin treated CCl4 exposed livers suggested direct effects on cancer-associated pathways. Hallmark gene sets enriched significantly for epithelial mesenchymal transition, adipogenesis, mTORC1, KRAS and PI3K/AKT/MTOR signaling pathways, with metformin negatively regulating these pathways (Figure 3.5.4). Confirming these indications, we observed that metformin inhibited AKT phosphorylation in liver tissue along with downstream key effectors S6 and 4EBP1 (Figure 3.5.3), albeit metformin had no apparent effect on total or phosphorylated status of mTOR. Since PI3K/AKT pathway is a key regulator of cell survival [263], metformin-mediated inhibition of AKT resulted in a higher level of apoptosis as demonstrated by the increased cleavage of caspase 7 and PARP.

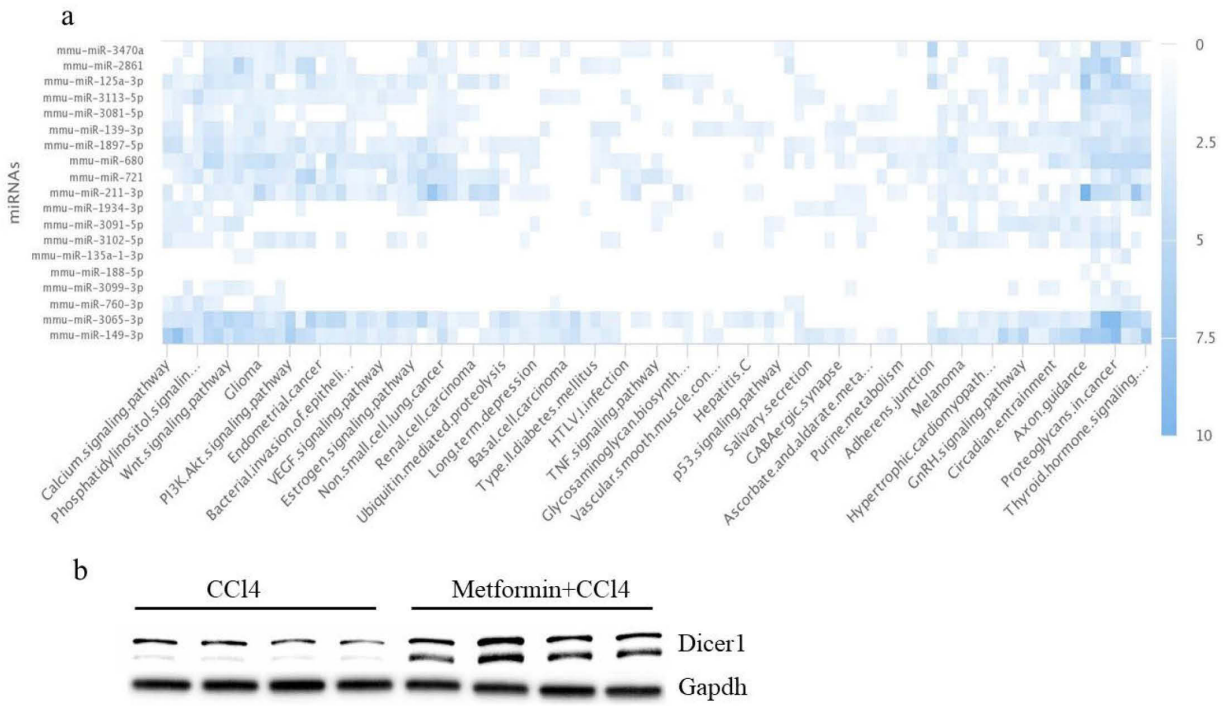


Figure 3.5.2: (a) Metformin up-regulated miRNAs union on predicted pathways: Custom heat map of pathway analysis for significant miRNAs and target genes supported by miRNA microarray data. For each miRNA in a row and each pathway in a column, the negative decadic logarithm of the P value is presented as color legend, indicating strong association of miRNAs targeting corresponding pathways. White spots (0) mean that miRNA does not regulates the pathway and vis-a-vis blue (10) spots mean strong regulation. (b) Western-blot depicting metformin mediated Dicer1 over-expression compared to untreated liver tissue lysate. Gapdh was used a loading control.

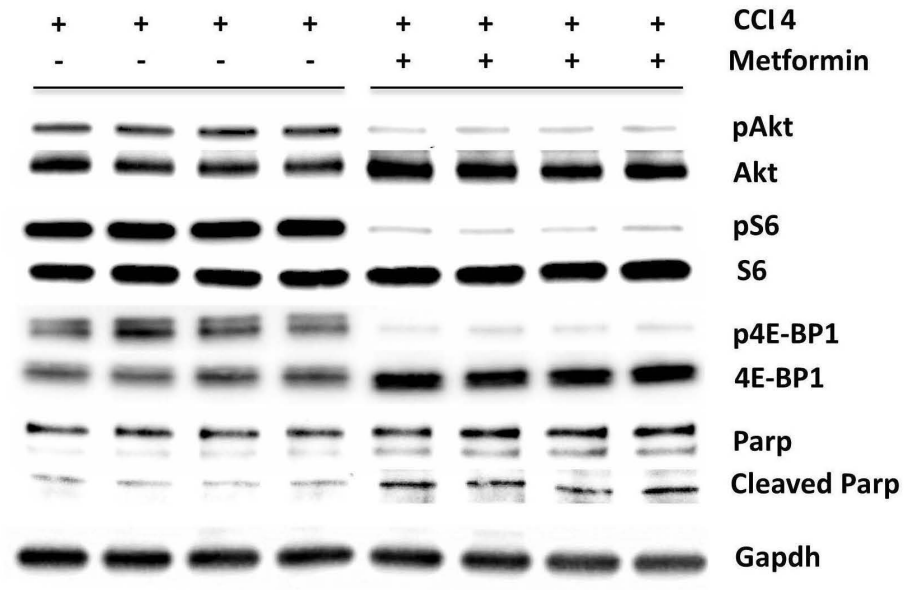


Figure 3.5.3: Western blot- AKT pathway: Analysis of AKT pathway and apoptosis in liver tissue lysates from CCl4 and CCl4+metformin treated mice. Gapdh was used as loading control

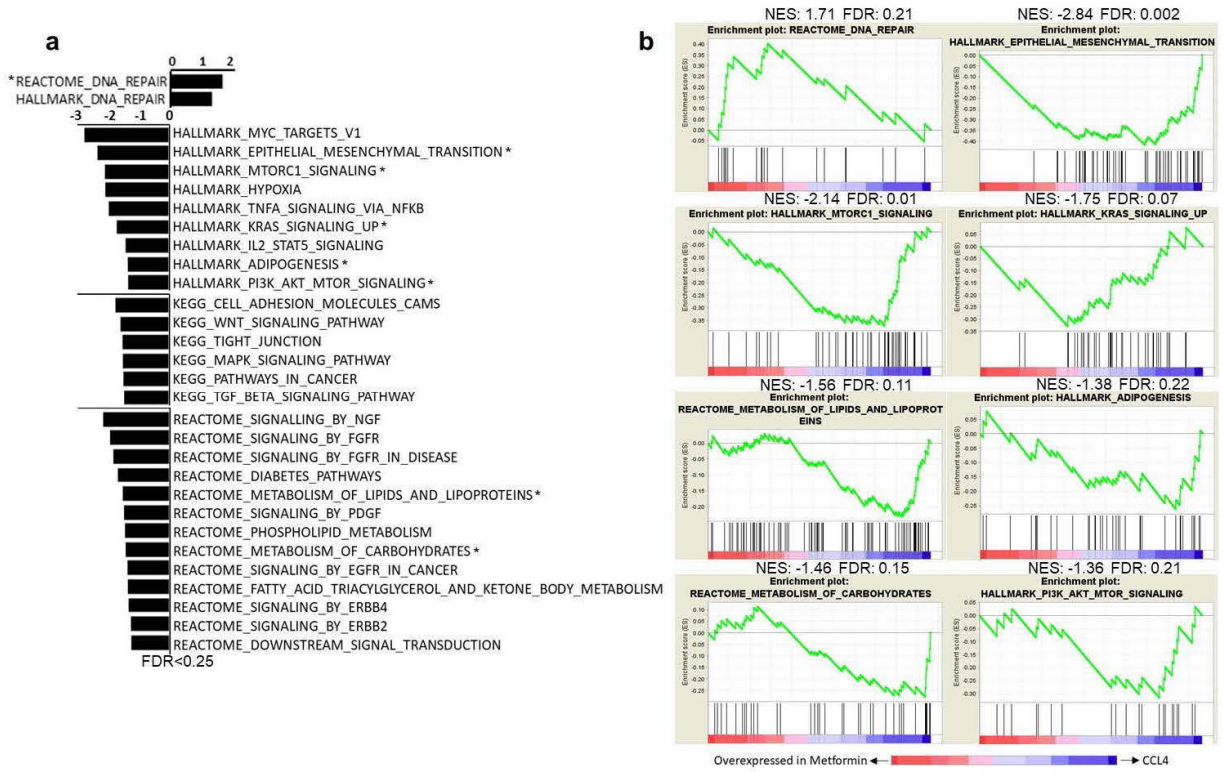


Figure 3.5.4: GSEA- microarray gene expression profile: (a) The bar graph shows normalized enrichment scores (NES) of gene set enrichment analysis (GSEA) on the Hallmark, KEGG and Reactome gene sets for the phenotype metformin+CCl4 vs CCl4 livers (FDR<0.25) with gene expression microarray analysis. Asterisks indicate the gene sets for which (b) enrichment plots are shown with corresponding NES and FDR.

Lastly, hepatomegaly was a salient feature in CCl4 treated mice. Metformin treatment reversed hepatomegaly in mice, which is consistent with similar findings observed in mouse models of obesity in previous studies [264]. Apart from anti-steatotic effects of metformin, other mechanisms such as regulation of autophagy are critical for maintenance of tissue homeostasis in liver [265]. Therefore, we investigated protein expression of autophagy markers p62, LC3B-I and LC3B-II in liver tissue lysates. Interestingly, CCl4 induced blockade of autophagy resulting in accumulation of p62 protein and decreased turnover of LC3B-I to LC3B-II isoform. Metformin activated autophagy in the liver and as consequence p62 expression was decreased and LC3B-II isoform was evidently augmented (Figure 3.5.5 a). Autophagy has usually been viewed as a major cell death mechanism in the mammalian organ systems. However, it is also considered as indispensable tissue repair process to maintain homeostasis of cells, tissues, and and organisms [266]. We further looked at expression of autophagy markers in liver of each of the mice in metformin treated and untreated groups (n=7). There was a definite trend of activation of autophagy in metformin treated mice and paradoxical inhibition in untreated livers of mice (Figure

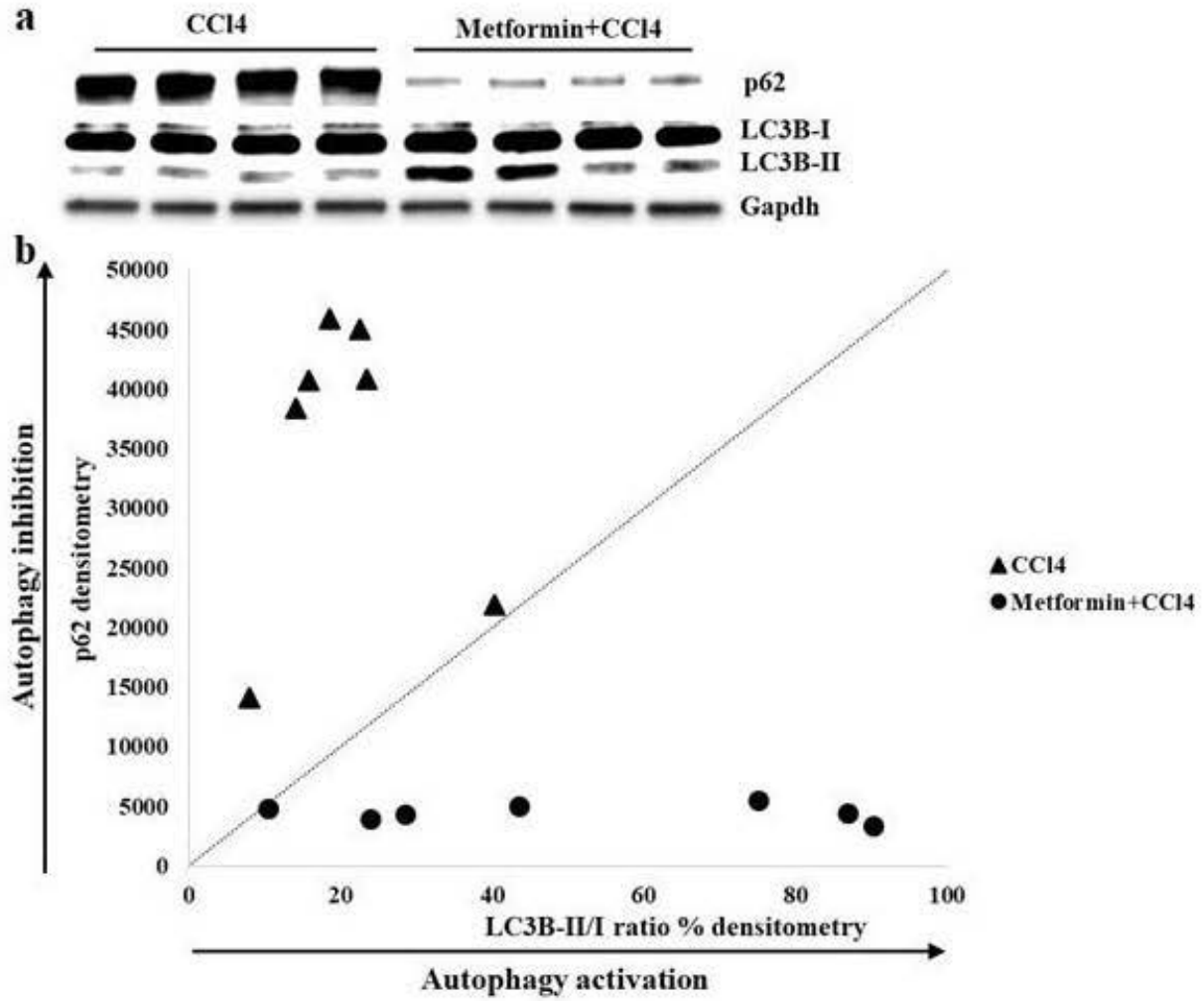


Figure 3.5.5: Metformin augments autophagy: (a) Western blot of representative liver lysates from mice in CCl4 and metformin+CCl4 groups. Gapdh was used as loading control. (b) Scatter plot representing densitometry values from western blot images of p62 protein on Y - axis and corresponding percentage ratio of LC3B-II to LC3B-I densitometry values on X - axis, normalised to respective Gapdh expression. Each data point on the graph represents single mouse (n = 7 for each group)

3.5.5b). These results might additionally explain inhibition of tumourigenesis mediated by metformin since, autophagy offers a protective tissue environment for tumor cells to survive during malignant progression by supporting cellular metabolic demands, decreasing metabolic damage, anoikis resistance and dormancy [267].

Je pense, donc je suis

René Descartes

4

Discussion

HCC and cirrhosis are the major life-limiting consequences of progressive chronic fibrotic liver disease, cause by any etiology. Although complete HCC tumour resection or ablation at early stages of disease is effective, underlying tumour conducive tissue microenvironment in the remnant liver could give rise to recurrence of tumours that progress to unresectable advanced-stage disease in majority of patients. Once tumours progress to advanced-stage, current approved medical therapeutics provide meagre survival benefit and are not cost effective [55, 227]. Thus, early detection and prevention of evolving HCC is, in principle, the most effective strategy to improve patient prognosis. However, a bi-annual universal HCC screening for early detection of tumours, as recommended by clinical practise guidelines, is less utilized mainly owing to under-recognition of cirrhosis and unexplored areas of etiology specific HCC risk prediction [228]. In such a context cancer prevention strategies become valuable means to decrease HCC burden in at-risk for HCC population.

Cancer prevention strategies encompasses diverse variety of interventions such as, lifestyle changes to reduce risk, aetiology specific environmental exposure interventions, preventive interventions aimed at eradicating risk factor affliction and actively interrupting the carcinogenic pathway. Primary prevention focusses on prophylactic elimination of HCC risk at an early stage before

onset of the disease. For example, universal new born immunization programs against HBV in Thailand in the early nineties and screening for HCV before blood transfusions during the same time-period have been effective in preventing respective viral hepatitis infection. As a consequence of these public health measures, hepatitis virus associated HCC risk has greatly decreased in general population [229, 230]. Secondary or tertiary prevention covers early detection and chemoprevention of HCC occurrence or recurrence, in patients already prone to aetiological agents [231]. The discovery and development of cancer chemoprevention strategies have been challenging. There has been scarce progress over the past decades owing to elusive mechanisms of human carcinogenesis [232]. It is not feasible to verify mechanisms of cancer initiation in patients that are inferred from preclinical studies, because it is ethically and logistically difficult to monitor cancer-free individuals with molecular assessment for long durations until a cancer develops. To overcome this challenge, a reverse-genetics approach has been proposed, in which clinically relevant targets are first identified in clinical cohorts with completed long-term follow-up, and subsequently validated in experimental systems [233]. Another factor limiting chemoprevention development is suboptimal animal models that may not resemble human disease, leading to false discovery of chemoprevention targets and biomarkers; these approaches may be improved by more sophisticated modelling strategies [234].

Transgenic mouse model (TG221) with induced cirrhosis background described in this study, could over-come these hurdles. In HCC, miR-221 is frequently upregulated and affects multiple cancer pathways [235]. Chronic exposure to hepatotoxin like CCl4 in these mice resulted in sequential degree of liver fibrosis that progressed to cirrhosis and HCC. Histopathological and molecular profile of cirrhosis and tumours of livers in these mice recapitulate human disease process and could be an ideal model to study generic chemoprevention strategies.

Given the elevated risk of HCC type 2 diabetes patients, anti-diabetic therapies may be rational HCC chemopreventive strategies. Metformin, a biguanide derivative, inhibits hepatocellular gluconeogenesis and lipogenesis [161, 236]. Metformin chemo-intervention at early fibrosis stage in TG221 cirrhosis mouse model, effectively abrogated formation of liver tumours. Metformin hepato-protective effects could be explained primarily by four mechanisms; 1) reduced epithelial to mesenchymal transition and liver fibrosis, 2) decreased lipogenesis, 3) induction of autophagy and 4) increased apoptosis and reduction of hepatocytes proliferation.

4.1 Metformin reduced EMT and liver fibrosis

The EMTs associated with wound healing, tissue regeneration, and organ fibrosis begins as part of a repair-associated process that normally generates cells like fibroblasts in order to recon-

struct tissues following trauma and inflammatory injury. In the setting of organ fibrosis, EMT can continue to respond to ongoing inflammation, leading eventually to organ destruction. Tissue fibrosis is in essence an unabated form of wound healing due to persistent inflammation. Inflammatory cells and fibroblasts secrete a variety of components of complex extracellular matrix products such as collagens, laminins and elastin [237]. In particular α-SMA, collagen I and collagen IV are considered to be reliable markers to characterize the mesenchymal products generated by the EMTs that occur during the development of liver fibrosis [238]. Furthermore, livers accommodate hepatic stellate cells that are localized in the subendothelial space of Disse, interposed between liver sinusoidal endothelial cells and hepatocytes. HSCs represent 10% of all resident liver cells. In normal physiology, HSCs maintain a non-proliferative, quiescent phenotype. Following liver injury or culture in vitro, HSCs become activated, transdifferentiating to myofibroblasts, which are proliferative and contractile cells and are characterized by enhanced ECM production [239]. Metformin significantly decreased EMT associated gene expression, activation of HSCs and deposition of extracellular matrix proteins, thereby improving CCl4 induced liver fibrosis.

4.2 LKB1/AMPK DEPENDENT ACC INACTIVATION BY METFORMIN LEADS TO REDUCED HEPATIC LIPOGENESIS

The liver plays a key role in regulating whole-body energy metabolism. AMPK is a highly conserved master regulator of metabolism, which restores energy balance during metabolic stress both at the cellular and physiological levels. Metformin-mediated AMPK activation, results in phosphorylation and inhibition of ACC. Depletion of ACC activity reduces conversion of acetyl-CoA into malonyl-CoA, leading to fatty acid oxidation and inhibition of fatty acid synthesis. AMPK is an energy sensor and master regulator of metabolism and functions as a fuel gauge that monitors systemic and cellular energy status. AMPK activity is induced via phosphorylation at Thr172 by LKB1, upon increase in intracellular AMP/ATP ratio leading to a metabolic switch from anabolism to catabolism. Further downstream, activated AMPK phosphorylates key metabolic enzymes, such as ACC, and transcription factors, thereby inhibiting synthesis of building blocks required for cellular growth like, glucose, lipids, and proteins. At the same time, activated AMPK stimulates catabolism of fatty acids and glucose uptake. previous studies have shown that regulation of ACC acetylation by AMPK is conserved in yeast and mammalian cells [218]. The mechanism by which metformin induces AMPK activation remains elusive, although consensus suggest that metformin mediated decrease in mitochondrial ATP production results in AMPK activation. An increased rate of fatty acid synthesis is essential for tumor progression. Blocking lipid biosynthesis by inhibiting lipogenic enzymes, such as ACC, fatty-acid synthase,

ATP-citrate, decreases proliferation and increases apoptosis of cancer cells. At the cellular level decrease hepatocytes displayed reduction in micro-steatosis both in vivo and in vitro.

4.3 Metformin induces autophagy

Autophagy is one of the major degradation pathways in the cell. Intracellular components are sequestered by autophagosomes and then degraded upon fusion with lysosomes. Genetic studies of yeast have identified more than 30 autophagy-related (ATG) genes that are essential for autophagy. Evidence has suggested that autophagy is also involved in tumor suppression. Possible tumor suppressive mechanisms thus far proposed in cell culture and allografted tumor models include suppression of tumorigenic inflammation, mitigation of metabolic stress and genomic damage and p62 degradation [240, 241]. Our results suggest that autophagy is inhibited and p62 is accumulated in cirrhotic livers, thereby creating a tumour conducive tissue environment. Metformin intervention reversed this effect by inducing autophagy and a consequence p62 was degraded in the liver tissue. Previous studies in autophagy deficient mice, in particular ATG5 and ATG7 deficient mice have shown that multiple benign tumors developed only in the liver, but not in other tissues. Swollen mitochondria and oxidative stress and genomic damage responses were detected in the hepatic tumor cells. Furthermore, strong p62 accumulation was documented in livers of these mice. Subsequently, concomitant p62 deletion in these mice, significantly reduced size and number of liver tumours that formed. Therefore, we could conclude that continuous autophagy is important for suppression of tumorigenesis in the liver, and metformin reversal of accumulation of p62 by autophagy resulted in induction of autophagy, thereby contributing to tumour suppression effects of metformin.

4.4 Metformin induces apoptosis by inhibiting AKT pathway

Pi3k/Akt pathway plays a critical role in controlling cell survival and apoptosis. Akt is known to be implicated in several types of cancer, including pancreatic, breast cancer, gynecological cancers and glioblastoma. In a transcriptome-based meta-analysis of 523 human fibrotic livers Akt was identified as a pan aetiology HCC risk driver [242]. However, surprisingly, liver specific Akt knockout and chemo-intervention with pan-Akt inhibitors in mice resulted in aggressive HCC tumours [243]. On the contrary, similar lung cancer pre-clinical studies targeting Akt have demonstrated protection against tumourigenesis [244-246]. This paradoxical observation might be due to pan-Akt inhibitor therapy being hepatotoxic unto itself, resulting liver injury and might exaggerate inflammation and consequently leading to carcinogenic tissue microenvironment. In

our study, metformin did not affect expression of Akt but abrogated phosphorylation of Akt. Subsequently, downstream signalling effectors of Akt were likewise down-regulated. Suggesting that upstream signalling of Akt, receptor tyrosine kinases and Pi3k should be down-regulated as well. This resulted in cleaved apoptosis proteins Parp and caspase-7, thereby enhancing apoptotic process. Thus, inhibition of Akt activation might be an essential strategy to suppress liver cancer progression via apoptosis and suppression of cell proliferation.

4.5 Conclusion

Epidemiological studies evaluating efficacy of metformin use in reducing HCC risk in DM2 patients have not been conclusive (Table 1.6.1). Chemo-prevention clinical trials in HCC are disadvantaged by the long latency period from fibrosis to development of cirrhosis or HCC. A select few HCC prevention clinical trials have been initiated. Phase I/II trial with erlotinib was initiated in cirrhosis patients to evaluate HCC prevention (NCT02273362). However, the study was suspended probably due to toxicity described in pre-clinical studies [247]. Statins are other class of drugs that are being evaluated for HCC prevention. Atorvastatin is being evaluated in a phase IV trial in early stage HCC post curative therapy for recurrence prevention effects (NCT03024684). Metformin phase III and phase II trials to evaluate secondary HCC prevention, change in liver fibrosis in HCV infected patients respectively were terminated based on decision of the investigator (NCT02319200) or lack of funding (NCT02306070). Further a phase III trial of metformin with or without celecoxib, a COX2 inhibitor for tertiary prevention in patients who underwent HCC resection is currently recruiting participants (NCT03184493). Our results suggest a definitive rationale for clinical translational metformin chemo-prevention in early stages of liver fibrosis to effectively reduce the risk of HCC occurrence and even prolong the chronicity of underlying liver disease. Currently there are no strategies or guidelines for detection of liver fibrosis at early stages in general population. However, effective non-invasive methods of accessing early stage liver fibrosis have been proposed in at risk for cirrhosis population cohorts, that essentially support the concept of liver fibrosis screening in general population [248-251]. This approach could better equip clinicians and further studies in prevention of chronic liver disease and HCC.

The future rewards those who press on. I don't have time to feel sorry for myself. I don't have time to complain. I'm going to press on.

Barack Obama

5

Future perspectives

In summary, our current pre-clinical study has generated a great deal of data on primarily two fronts. 1) Reliable mouse model of hepatocellular carcinoma with cirrhosis background, and 2) Rationale for metformin early intervention in non-diabetic clinical setting for prevention of hepatocellular carcinoma.

We described in detail progression of liver fibrosis from early fibrosis (Isahk score 1) to cirrhosis (Ishak score 4) and tumourigenesis (chapter 3.1.1). These time points delineated were uniform and could provide a road map to future chemoprevention studies aimed at studying liver cancer prophylaxis at different stages of liver fibrosis leading up to HCC progression. Along those lines, our lab group has investigated HCC prophylaxis potential of miR-199a-3p mimics and anti-miR-221 at Ishak score 3 stage in the described mouse model of HCC. The results so far have been exciting as these molecular therapies were able to curtail the growth of tumours in these mice, thereby providing valuable insight into role of small non-coding RNAs in tumour progression (unpublished- manuscript under consideration).

Based on the observations in chapter 3.2 that reduction of fibrosis and steatosis in liver was beneficial and prevents tumourigenesis, we could postulate a few potential hypotheses towards HCC chemoprevention.

Fibrosis, characterized by excess accumulation of extracellular matrix (ECM) contributes to development of HCC. Continuous intense researches on drugs targeting fibrosis have resulted in several class of drugs targeting extracellular factors such as growth factors, cytokines, matrix metalloproteinases, endothelin-axis and angiotensin. A new class of drugs antagonizing type 1 lysophosphatidic acid (LPA) receptor has been developed and is under investigation in clinical trials for the treatment of systemic fibrosis [288]. Recently AKT and LPA pathway were identified as potential targets for HCC chemoprevention by retrospective studies in a large cohort of cirrhosis and HCC patients [278].

Intrahepatic lipid lowering effect of metformin could also contribute to its hepatoprotective effect. These observations open up avenues to explore use of statins in HCC chemoprevention. For millions of people, taking a statin represents an integral part of everyday life. According to data from CDC 26% of Americans use some form of lipid-lowering drugs [289]. Statins account for majority of lipid-lowering drugs used to decrease cholesterol levels and reduce the risk for cardiovascular disease, myocardial infarction and stroke. Several preclinical studies have shown anti-neoplastic and anti-inflammatory properties of statins, and multiple clinical observational studies have shown a significant protective effect from statin use in certain cancers with metabolic aberrations [290]. However, discord exists among researchers as to the anti-neoplastic effects of statins. Clinicians also may discontinue statin use specifically in chronic liver disease due to fear of hepatotoxicity solely based of select few case reports [291].

Our mouse model of HCC might be an appropriate pre-clinical model to test these hypotheses at several stages of HCC development and characterize preventive cellular and molecular mechanism at play.

As pointed out in chapter 1.6.3 several observational studies in type 2 diabetes mellitus patients have associated metformin use with reduced cancer risk and mortality. However, our pre-clinical study suggests that metformin could be beneficial in non-diabetic population as well. Metformin has a broad safety profile with negligible risk of hypoglycaemia in monotherapy and relatively few drug interactions of clinical relevance. The most serious clinical risk associated with metformin is the development of lactic acidosis. Typically, this occurs in patients with compromised renal function, where in systemic drug clearance in severely affected.

Hepatotoxicity of metformin in patients with impaired liver function has not been reported in observational studies or clinical trials, but a few select case reports in compromised chronic liver disease [292]. Therefore, there is minimal evidence to withhold prescribing metformin in patients with elevated liver transaminases. The most common side effects of metformin are gastrointestinal and include diarrhea, abdominal bloating, nausea and anorexia. A few clinical trials (NCT02319200, NCT02306070) were initiated for metformin use in chronic liver disease with

aims to prevent development of HCC. These trials could not be completed due to no scientific reasons. Since, the chronicity of liver disease has a long latency period before a diagnosis of HCC, funding, recruitment of patients and their follow-up might be a challenge. Albeit, it would be worthwhile to study HCC prevention and metformin use in both diabetic and non-diabetic settings.

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