

UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA



**APOPTOSIS AND miRNAs IN NON-ALCOHOLIC
FATTY LIVER DISEASE PATHOGENESIS**

Duarte Miguel Sacramento Ferreira

DOUTORAMENTO EM FARMÁCIA

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Duarte Miguel Sacramento Ferreira

Tese de Doutoramento orientada por:

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Always follow your dreams...

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ABBREVIATIONS

3'UTR	3'-Untranslated Region
Ac-p53	Acetylated p53 or Acetyl-p53
AGO	Argonats
AP-1	Activator Protein 1
APAF-1	Apoptotic Protease-activating Factor 1
BAD	BCL-2-Associated Death Promoter
BAX	BCL-2-Associated X Protein
BCL-2	B-Cell Lymphoma 2
BCL-x_L	B-Cell Lymphoma Extra-large
BID	BH3 Interacting-domain Death Agonist
BH3	BCL-2 Homology 3
BMI	Body Mass Index
CA	Cholic Acid
CYP7A1	Cholesterol 7 α -hydroxylase
DCA	Deoxycholic Acid
DIABLO	Direct Inhibitor of Apoptosis-binding Protein with Low pI
DISC	Death-Inducing Signalling Complex
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-related Kinase
FADD	Fas-associated Protein with Death Domain
FasL	Fas Ligand

FFA	Free Fatty Acids
FOXO	Forkhead Box Transcription Factor, subgroup O
FXR	Farnesoid X Receptor
GLUT-4	Glucose Transporter 4
GR	Glucocorticoid Receptor
HCC	Hepatocellular Carcinoma
HDL	High-Density Lipoprotein Cholesterol
HFD	High-fat Diet
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
IAP	Inhibitor of Apoptosis Protein
INSR	Insulin Receptor
IR	Insulin Resistance
IRS-1	Insulin Receptor Substrate 1
IRS-2	Insulin Receptor Substrate 2
IκB	Inhibitor of NF- κ B
JNK	c-Jun NH ₂ -terminal Kinase
LDH	Lactate Dehydrogenase
LDL	Low-density Lipoprotein Cholesterol
MAPK	Mitogen-activated Protein Kinase
MCL-1	Myeloid Cell Leukemia 1
MDM2	Murine Double Minute 2
miRISC	miRNA-induced Silencing Complex
miRNAs or miRs	microRNA

MMP	Mitochondrial Membrane Permeabilization
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt
NAFLD	Non-alcoholic Fatty Liver Disease
NAS	NAFLD Activity Score
NASH	Non-alcoholic Steatohepatitis
NEFAs	Non-esterified Fatty Acids
NEMO	NF- κ B Essential Modulator
NF-κB	Nuclear Factor κ B
norUDCA	24-Norursodeoxycholic Acid
OA	Oleic Acid
OGTT	Oral Glucose Tolerance Test
PA	Palmitic Acid
PGC-1α	PPAR- γ Co-activator 1 α
PI3K	Phosphoinositide-3-kinase
PIDD	p53-induced Protein with a Death Domain
PKA	Protein kinase A
PKB	Protein kinase B
PPAR	Peroxisome Proliferation Activator Receptor
pre-miRNAs	Precursor miRNA
pri-miRNAs	Primary miRNA
PTEN	Phosphatase and Tensin Homolog Detected on Chromosome Ten

PTPN1	Protein-Tyrosine Phosphatase 1B
PUMA	p53-upregulated Modulator of Apoptosis
RAIDD	RIPK-associated ICH-1 Homologous Protein with a Death Domain
RIPK1	Receptor Interacting Protein Kinase 1
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SIRT1	Sirtuin 1
Smac	Second Mitochondria-derived Activator of Caspases
SREBP	Sterol Regulatory Element-binding Protein
TC	Total Cholesterol
TGF-β1	Transforming Growth Factor β 1
TNF-α	Tumour Necrosis Factor α
TNF-R1	TNF Receptor 1
TRADD	TNF-R1 Associated Protein with a Death Domain
TRAIL	TNF-Related Apoptosis-inducing Ligand
TUDCA	Tauroursodeoxycholic Acid
TUNEL	Transferase Mediated dUTP-digoxigenin Nick-end Labeling
UDCA	Ursodeoxycholic Acid
UPR	Unfolded Protein Response
VLDL	Very Low Density Lipoprotein
XIAP	X-chromosome Linked Inhibitor of Apoptosis Protein

$\Delta\Psi_m$

Inner Mitochondrial Transmembrane Potential

PUBLICATIONS

The present thesis is based on work that has been published, or submitted for publication, in international peer-reviewed journals:

Ferreira DMS, Afonso MA, Rodrigues PM, Borralho PM, Rodrigues CMP, Castro RE. JNK1-activation of the p53/miRNA-34a/Sirtuin1 pathway contributes to apoptosis induced by DCA in primary rat hepatocytes. (under revision)

Castro RE*, **Ferreira DMS***, Afonso MB, Borralho PM, Machado MV, Cortez-Pinto H, Rodrigues CMP. miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in rat liver and activated by disease severity in human non-alcoholic fatty liver disease. *J Hepatol* 2013; 58: 119-125. *Equal contribution

Ferreira DMS, Castro RE, Machado MV, Evangelista T, Silvestre AR, Costa A, Coutinho J, Carepa F, Cortez-Pinto H, Rodrigues CMP. Apoptosis and insulin resistance in liver and peripheral tissues of morbid obese patients is associated with progression of non-alcoholic fatty liver disease. *Diabetologia* 2011; 54: 1788-1798.

The following manuscripts have also been published during the Ph.D. studies:

Machado MV, **Ferreira DMS**, Castro RE, Silvestre AR, Evangelista T, Coutinho J, Carepa F, Costa A, Rodrigues CMP, Cortez-Pinto H. Fatty muscle is associated with severity of non-alcoholic fatty liver and insulin resistance: liver and muscle interplay when facing energy surplus. *PLoS One* 2012; 7(2): e31738.

Borrvalho PM, Simões AES, Gomes SE, Lima RT, Carvalho T, **Ferreira DMS**, Vasconcelos MH, Castro RE, Rodrigues CMP. miR-143 overexpression impairs growth of human colon carcinoma xenografts in mice with induction of apoptosis and inhibition of proliferation. *PLoS One* 2011; 6(8): e23787.

Castro RE, Santos MM, Glória PM, Ribeiro CJ, **Ferreira DMS**, Xavier JM, Moreira R, Rodrigues CMP. Cell death targets and potential modulators in Alzheimer's disease. *Curr Pharm Des* 2010; 16(25): 2851-2864

Castro RE, **Ferreira DMS**, Zhang X, Borrvalho PM, Sarver AL, Zeng Y, Steer CJ, Kren BT, Rodrigues CMP. Identification of microRNAs during rat liver regeneration after partial hepatectomy and modulation by ursodeoxycholic acid. *Am J Physiol Gastrointest Liver Physiol*. 2010; 299: G887-897

ABSTRACT

The severity of non-alcoholic fatty liver disease (NAFLD) ranges from steatosis to non-alcoholic steatohepatitis (NASH). Although NAFLD correlates with insulin resistance (IR) and p53-mediated apoptosis, disease pathogenesis remains largely unknown. microRNAs (miRNAs or miRs) have recently been described to be altered in human NASH, and modulated by ursodeoxycholic acid (UDCA) in the rat liver. In turn, deoxycholic acid (DCA) modulates apoptosis-related proteins, including the c-Jun NH₂-terminal kinase (JNK), leading to hepatocyte apoptosis. Our aims were to investigate: 1) insulin signalling pathway at different stages of NAFLD, using muscle, liver and adipose tissues, and its correlation with apoptosis and JNK signalling; 2) miR-34a/Sirtuin1(SIRT1)/p53 pro-apoptotic pathway in human NAFLD and rat liver; and 3) potential modulation by bile acids. Our results showed that muscle and liver tissues display decreased activation of the insulin signalling pathway, in parallel with increased JNK phosphorylation, in more severe NASH. AKT phosphorylation decreased in all tissues during NASH. Similarly, caspase activation and DNA fragmentation were increased in the liver of NASH patients. In agreement, miR-34a and acetylated p53 increased with disease severity. UDCA inhibited the miR-34a/SIRT1/p53 pathway in primary rat hepatocytes and in rat liver, while DCA had opposite effects. miR-34a functional modulation confirmed its targeting by bile acids. In addition, in contrast with DCA, UDCA inhibited general p53 transcriptional activity, as well as p53 overexpression-mediated activation of miR-34a/SIRT1/p53. Finally, JNK1 arose as a key target of DCA in engaging the miR-34a pathway. In conclusion, this work suggests a link between NAFLD progression, IR, apoptosis, and miR-34a/SIRT1/p53. This pathway is specifically modulated by bile acids at the level of p53 transactivation, where JNK1-mediated activation of p53 is a key mechanism targeted of DCA. The JNK1/miR-34a/SIRT1/p53 pathway may represent an attractive pharmacological target for the development of new drugs to arrest progression of NAFLD and other metabolic, apoptosis-related liver pathologies.

Keywords: Apoptosis; Bile Acids; JNK; miRNAs; NAFLD; SIRT1

RESUMO

A apoptose é uma forma de morte celular tipicamente desencadeada pelos receptores de morte ou pela via mitocondrial. A via dos receptores de morte é ativada após interação de ligandos específicos com os seus receptores, iniciando, deste modo, a resposta intracelular. Por sua vez a via mitocondrial é ativada em resposta ao stresse genotóxico ou a alterações no ADN que conduzem à despolarização da membrana mitocondrial, de modo dependente ou independente do p53, com libertação de fatores apoptogénicos, como o citocromo c. As duas vias de morte celular acabam por conduzir à morte efetiva da célula devido à ativação de caspases que, por sua vez, clivam substratos específicos. A apoptose é um processo extremamente controlado, sendo regulado, por exemplo, a nível da transcrição e da tradução e, ainda, por modificações pós-transcricionais e pós-traducionais.

Os microRNAs (miRNAs ou miRs) são RNAs não codificantes com cerca de 22 nucleótidos que modulam a tradução. Tipicamente, os miRNAs ligam-se ao mRNA correspondente induzindo a sua desadenilação ou reprimindo a tradução. Para além disso, os miRNAs são também, eles próprios, alvos de uma regulação restrita. Por exemplo, estudos anteriores demonstraram que os ácidos biliares modulam a expressão de miRNAs no fígado de rato. O miR-34a é um miRNA pró-apoptótico que induz a apoptose de forma dependente e independente da p53. Curiosamente, a transcrição do miR-34a decorre de forma dependente da p53. Um dos alvos mais bem caracterizados do miR-34a é a Sirtuina 1 (SIRT1), uma desacetilase de histonas, que modula negativamente a apoptose, ao desacetilar a proteína p53. De facto, a SIRT1 inibe a transcrição de alvos pró-apoptóticos induzidos pela p53, como a proteína reguladora da apoptose PUMA.

A apoptose, além de ser regulada a nível endógeno pelos miRNAs, é regulada positivamente e negativamente pela ação dos ácidos biliares. Os ácidos biliares são sintetizados no fígado, a partir do colesterol, com o objetivo de auxiliarem na digestão e absorção de lípidos. Para além disso, são moléculas sinalizadoras que regulam inúmeras funções biológicas, entre

as quais a apoptose. Por exemplo, o ácido desoxicólico (DCA) é um ácido biliar tóxico que induz a apoptose, enquanto que o ácido ursodesoxicólico (UDCA) é um ácido biliar com propriedades anti-apoptóticas e citoprotetoras, sendo mesmo capaz de inibir a morte celular induzida pelo DCA.

O fígado gordo não alcoólico (FGNA) tem adquirido bastante relevo entre as patologias hepáticas. Estima-se que cerca de 26-40% dos doentes com doença hepática crónica apresentem FGNA. O FGNA encontra-se associado ao síndrome de resistência à insulina e à obesidade, sendo caracterizado por vários graus de lesão hepática, que vão desde a esteatose simples à esteato-hepatite não alcoólica (EHNA), podendo ainda progredir para cirrose e carcinoma hepatocelular. Por outro lado, as alterações no metabolismo dos ácidos gordos livres conduzem à acumulação de metabolitos tóxicos, derivados dos lípidos, que induzem stresse oxidativo, stresse do retículo endoplasmático, apoptose e resistência à insulina. Por este motivo, o FGNA encontra-se associado ao síndrome metabólico, que se caracteriza por um metabolismo lipídico disfuncional, obesidade e resistência à insulina. Para além disso, a apoptose das células hepáticas também está envolvida na patogénese do FGNA, estando mesmo correlacionada com a inflamação e a fibrose verificadas em estadios mais severos do FGNA. O próprio DCA foi recentemente descrito como estando associado à patogénese do FGNA, onde a apoptose dos hepatócitos parece ser, também, induzida pela p53, resultando num aumento de expressão da PUMA. Também a cinase do terminal amina da c-Jun (JNK) tem vindo a ser implicada na indução da apoptose durante a progressão do FGNA. Finalmente, muito recentemente verificou-se que a expressão de alguns miRNAs está alterada no FGNA. De facto, foi demonstrado que o miR-34a e a p53 encontram-se sobre-expressos no fígado de doentes com EHNA ativando a via mitocondrial da apoptose. De referir, ainda, que a SIRT1 é uma proteína chave na regulação do metabolismo lipídico hepático e que a sua inibição parece induzir esteatose e inflamação. Assim, uma desregulação na via pró-apoptótica miR-34a/SIRT1/p53 poderá levar à acumulação de lípidos, inflamação e indução da apoptose.

Com este trabalho pretendeu-se avaliar a ativação das cascatas da insulina e da apoptose em diferentes estadios do FGNA, a nível dos tecidos

hepático, muscular e adiposo. Também foi nosso objectivo avaliar a via pró-apoptótica miR-34a/SIRT1/p53 no fígado de doentes com FGNA, bem como a sua modulação pelos ácidos biliares UDCA e DCA no fígado de rato e em hepatócitos primários de rato.

Biópsias de tecido muscular, hepático e adiposo foram recolhidas, durante cirurgia bariátrica, em 28 doentes adultos, com idade média de 44 anos, com obesidade mórbida e FGNA. Os doentes foram agrupados, de acordo com a classificação de Kleiner/Brunt, em esteatose simples ($n = 15$), EHNA menos severa ($n = 5$) ou mais severa ($n = 8$). O fígado de rato foi extraído de animais sujeitos a dieta suplementada com UDCA a 0,4%. Hepatócitos primários de rato foram incubados com diferentes ácidos biliares ou com ácidos gordos livres e, em alguns casos, também transfectados com precursores ou inibidores específicos do miR-34a e/ou com um vetor de sobre-expressão da p53. Para além disso, os hepatócitos primários de rato foram ainda incubados com 10 e 50 μ M de resveratrol, para induzir a expressão da SIRT1 e com siRNAs para a JNK1 e JNK2, assim como com dominantes negativos para a atividade da JNK e da c-Jun. A fosforilação da cascata de sinalização da insulina incluindo o receptor da insulina, o substrato do receptor da insulina e a AKT, assim como a expressão da SIRT1, p53 total e acetilada e JNK total e fosforilada foram determinadas por Western Blot. A expressão dos miRNAs foi analisada por RT-PCR em Tempo Real. A atividade transcricional da p53 foi determinada em extratos nucleares de hepatócitos primários de rato, medindo a presença da p53 no núcleo, para exercer a sua atividade transcricional, ou a ligação da p53 à proteína MDM2 e por ensaios de luciferase recorrendo a plasmídeos com o elemento de ligação da p53 nos promotores de PUMA, p21 e do próprio miR-34a. Nos doentes, a apoptose foi avaliada através do ensaio de TUNEL, em cortes histológicos hepáticos e pela activação das caspase-2 e -3, em extratos de proteínas totais. Nos hepatócitos primários de rato a viabilidade celular e apoptose foram analisadas por LDH, MTS, coloração de *Hoechst* e ApoTox-Glo™, medindo viabilidade, citotoxicidade e atividade das caspase-3 e -7.

Os nossos resultados mostraram que a ativação das caspase-2 e -3 e

a fragmentação do ADN encontravam-se mais aumentadas no fígado dos doentes com EHNA mais severa, em comparação com os doentes com esteatose simples ($p < 0,01$). O tecido muscular e, em menor extensão, o tecido hepático, apresentavam uma maior diminuição na fosforilação da tirosina do receptor da insulina e do substrato do receptor da insulina nos doentes com EHNA mais severa, em comparação com os doentes com esteatose ($p < 0,01$, no músculo; $p < 0,05$, no fígado). Curiosamente, o tecido adiposo não apresentou qualquer variação na fosforilação da tirosina do receptor da insulina, ou do substrato do receptor da insulina, entre os diferentes grupos em estudo. De acordo com os resultados anteriores, a fosforilação da AKT diminuiu no tecido muscular, no tecido hepático e, curiosamente, no tecido adiposo, em doentes com EHNA mais severa, comparativamente aos doentes com esteatose simples (pelo menos, $p < 0,05$). Quando a fosforilação da JNK foi analisada, esta estava significativamente aumentada nos doentes com EHNA, em comparação com os doentes com esteatose, tanto no músculo ($p < 0,01$) como no fígado ($p < 0,05$). Os resultados indicaram, ainda, que existe uma expressão diferencial de miRNAs no fígado de doentes com diferentes estadios do FGNA. A expressão dos miR-122, -143 e -451, por exemplo, diminuiu progressivamente da esteatose para a EHNA ($p < 0,05$). De maior relevo, o miR-34a, a apoptose e a p53 acetilada aumentaram ($p < 0,01$), enquanto que a SIRT1 diminuiu ($p < 0,01$) com a gravidade do FGNA. De salientar que o UDCA diminuiu a expressão do miR-34a, tanto no fígado de rato, como em hepatócitos primários de rato ($p < 0,01$). A sobre-expressão do miR-34a confirmou que este miRNA seria um alvo do UDCA, dado que, mesmo nessas condições, o UDCA diminuiu a expressão do miR-34a ($p < 0,05$), aumentou a expressão da SIRT1 ($p < 0,01$) e inibiu a acetilação da p53 ($p < 0,05$) e a apoptose ($p < 0,05$). Curiosamente, a indução de apoptose por ácidos gordos livres em hepatócitos sobre-expressando o miR-34a foi, também, inibida pelo UDCA (pelo menos $p < 0,05$). A via pro-apoptótica miR-34a/SIRT1/p53 ($p < 0,05$) foi ativada, após sobre-expressão da p53, em hepatócitos primários de rato. Nesta situação, o UDCA foi, ainda, capaz de inibir a via do miR-34a, por diminuir a atividade transcricional da p53 (pelo menos, $p < 0,05$). Pelo contrário, o

DCA ativou a via pro-apoptótica miR-34a/SIRT1/p53, de uma forma dependente da dose e do tempo (pelo menos, $p < 0,05$). Também a ativação da via miR-34a/SIRT1/p53 pela p53 foi potenciada na presença do DCA (pelo menos, $p < 0,05$). Em concordância, o DCA aumentou a atividade transcricional da p53 e dos seus alvos transcricionais PUMA, p21 e o próprio miR-34a (pelo menos, $p < 0,05$), revelando um mecanismo funcional de ativação do miR-34a. A inibição do miR-34a e sobre-expressão da SIRT1 bloqueou significativamente os efeitos do DCA sobre o miR-34a e, consequentemente, a apoptose (pelo menos, $p < 0,05$). Por fim, verificámos que a JNK1 ($p < 0,05$), mas não a JNK2, era um alvo chave do DCA, ativando a p53 e induzindo a via pró-apoptótica miR-34a/SIRT1/p53.

No seu conjunto, estes resultados indicam que, no FGNA, existe uma forte correlação entre a resistência à insulina, a nível hepático e muscular e que o grau de lesão hepática está associado com um aumento da resistência à insulina e da apoptose. O aumento de apoptose verificado nos doentes com FGNA parece, ainda, estar associado ao aumento da atividade pró-apoptótica através da via miR-34a/SIRT1/p53. Esta via é alvo de inibição e ativação, respetivamente pelos ácidos biliares UDCA e DCA. Por fim, verificámos que a ativação da p53 é mediada pela JNK1, sendo este um mecanismo chave na indução do miR-34a pelo DCA em hepatócitos.

Uma melhor compreensão dos mecanismos responsáveis pela patogénese do FGNA poderá proporcionar novos alvos terapêuticos para impedir a progressão da doença, assim como para o tratamento de outras doenças hepáticas associadas a níveis exagerados de apoptose.

Palavras-chave: Ácidos biliares; Apoptose; FGNA; JNK; miRNAs; SIRT1

GENERAL INTRODUCTION

1.1. Apoptosis in the liver

In classical terms, apoptosis is defined as a pattern of molecular and morphological changes that result in the packaging and removal of dying cells. Cells committed to die are removed by macrophages or neighbouring cells without activation of the immune system. Morphological features such as nuclear pyknosis, chromatin condensation, membrane blebbing, and formation of apoptotic bodies can be used to identify and characterise apoptosis (Hengartner 2000). In the liver, apoptotic bodies are phagocytised by stellate cells and Kupffer cells, the resident macrophages (Canbay *et al.* 2003a; Canbay *et al.* 2003b). Engulfment of apoptotic bodies by Kupffer cells promotes the generation of death ligands, including Fas ligand (FasL), and tumour necrosis factor α (TNF- α). These death ligands then promote hepatocyte apoptosis in a feed-forward loop (Canbay *et al.* 2003a). In addition, apoptotic cells also produce profibrogenic factors, such as transforming growth factor β 1 (TGF- β 1) and type I collagen (Canbay *et al.* 2003b), and also release nucleotides that bind to purinergic receptors on macrophages and hepatic stellate cells to further activate them (Elliott *et al.* 2009). The continued and sustained induction of hepatocyte apoptosis culminates in hepatic inflammation and fibrosis. As such, hepatocyte apoptosis is considered to be a pivotal event in several types of liver injuries.

In hepatocytes, as well as in other cell types, apoptosis mainly occurs through two well-characterized pathways: the extrinsic and the intrinsic pathways (**Fig. 1.1.**). The extrinsic or death-receptor pathway is initiated by ligand-induced activation of death receptors at the plasma membrane (Hengartner 2000). The binding of a ligand to its receptor leads to the formation of a ligand-receptor complex that recruits further cytosolic factors, giving rise to the death-inducing signalling complex (DISC). DISC formation results in the activation of initiator caspases, which then cleave and activate effector caspases (Riedl *et al.* 2004). On the other hand, the intrinsic cell death or mitochondrial pathway is triggered by cellular stress signals like DNA damage (Hengartner 2000). This pathway is typically induced by pro-apoptotic members of the B-cell lymphoma 2 (BCL-2) family, in response to apoptotic stimuli, and results in the release of several proteins from the

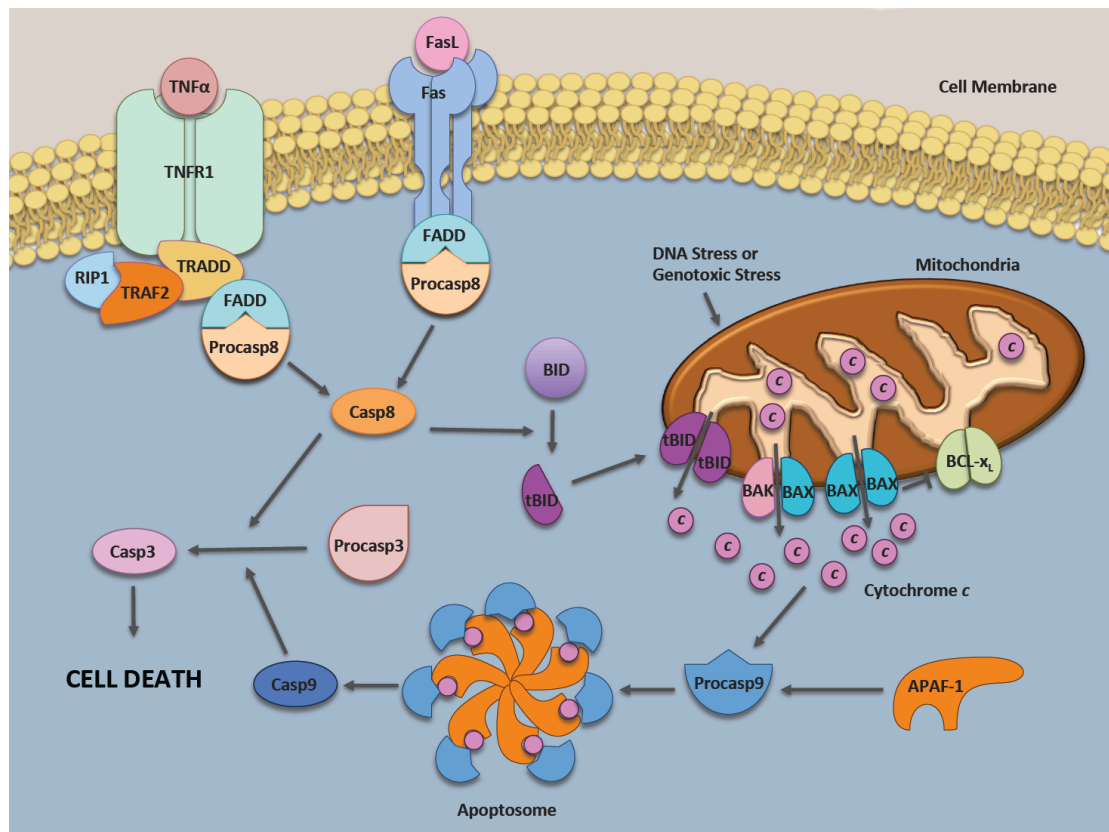


Figure 1.1. Schematic overview of death receptor and mitochondrial-mediated pathways of apoptosis. When cell death is triggered by extracellular signals, death receptors are activated after binding of its death ligands. This binding allows the formation of a ligand-receptor complex, which interacts with specific proteins. In hepatocytes, either FasL or TNF- α typically trigger the death receptor pathway. In particular, when FasL interacts with its receptor, it induces Fas oligomerization that recruits FADD protein to the oligomerized Fas receptor. FADD contains a death effector domain that enables the activation of initiator caspase-8 (Casp8). On the other hand, after binding of TNF- α to TNF-R1, the adaptor protein TRADD is recruited to the TNF-R1 death domain. TRADD interacts with the adaptor protein FADD and procaspase-8 (Procasp8), which can activate caspase-3 (Casp3). Alternatively, TRADD can interact with RIP1 and TRAF2 to induce the activation of pro-inflammatory and anti-apoptotic genes. Moreover, the mitochondrial pathway of apoptosis is triggered by stimuli such as DNA damage. When the mitochondrial pathway is triggered in hepatocytes, BH3-only proteins are activated and by-pass the inhibitory activity of anti-apoptotic BCL- x_L protein. This allows the oligomerization of BAX-BAX or BAX-BAK in the mitochondrial outer membrane leading to its permeabilization through a conformation change in the mitochondrial outer membrane, inducing the formation of large pores to release cytochrome c. When cytochrome c is released into cytoplasm, it interacts with APAF-1. This complex recruits and activates procaspase-9 (Procasp9) through autocatalytic cleavage, yielding active caspase-9 (Casp9) that can activate caspase-3. Interestingly, BID mediates the crosstalk between the death-receptor and mitochondrial pathways. Caspase-8-mediated

cleavage of BID greatly increases its pro-death activity, and results in BID translocation to mitochondria, where it promotes cytochrome c release. Procasp3, Procaspase-3; tBIB, truncated BID; TRAF2, TNF receptor associated protein 2.

intermembrane space of the mitochondria to the cytosol (Green 2005). Some of the already well-characterized proteins include cytochrome c, and second mitochondria-derived activator of caspases (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low pI (DIABLO). In the cytoplasm, cytochrome c binds to and activates the apoptotic-protease-activating factor 1 (APAF-1). The binding of cytochrome c to APAF-1 induces a conformational change in this complex, which allows the binding and activation of caspase-9, thereby triggering a cascade of caspase activation (Riedl *et al.* 2004). Moreover, the BCL-2 homology 3 (BH3)-only interacting domain death agonist (BID), a pro-apoptotic BCL-2 family member, mediates the crosstalk between death-receptor and mitochondrial pathways. Caspase-8-mediated cleavage of BID greatly increases its pro-death activity and translocation to mitochondria, where it promotes cytochrome c release (Li *et al.* 1998).

1.1.1. The death receptor pathway

Death receptors are transmembrane proteins characterised by the presence of two to five cysteine-rich repeats in both the extracellular and intracellular death domain, which are essential for protein-protein interactions (Wallach *et al.* 2008). When cell death is triggered by extracellular signals, death receptors are activated after binding of its death ligands (Rupinder *et al.* 2007). This binding allows the formation of a ligand-receptor complex, which interacts with specific proteins, such as the Fas-associated protein with death domain (FADD) and caspase-8. When all of these elements are assembled together, they form the DISC, which leads to the activation of caspase-8, and cleavage and activation of effector caspases, such as caspase-3. Finally, cleavage of crucial substrates of the cell takes place, originating the classical apoptotic phenotype (Riedl *et al.* 2004).

In hepatocytes, either FasL or TNF- α typically triggers the death receptor pathway. Hepatocytes constitutively express Fas on their cell surface and, therefore, are very susceptible to Fas-mediated signalling, which

plays an important role during viral and autoimmune hepatitis, alcoholic liver disease and endotoxin- or ischemia/reperfusion-induced liver damage (Galle *et al.* 1998; Schungel *et al.* 2009). In mechanistic terms, FasL interacts with its receptor, thus inducing Fas oligomerization, which then recruits FADD protein to the oligomerized Fas receptor. FADD contains a death effector domain that enables the activation of initiator caspase-8 (Reinehr *et al.* 2004). TNF- α overlaps with Fas signalling in many aspects, including the activation of caspase-8. After binding of TNF- α to TNF receptor-1 (TNF-R1), the main TNF- α receptor in hepatocytes, the adaptor protein TNF-R1 associated protein (TRADD) is recruited to the TNF-R1 death domain (Wajant *et al.* 2003). TRADD can then interact with the adaptor protein FADD and procaspase-8, resulting in the formation of DISC. DISC formation relies on internalization of the TNF-R1 complex and results in procaspase-8 cleavage and activation *via* an autoproteolytic process (Wajant *et al.* 2003). Alternatively, TRADD can interact with the receptor interacting protein kinase 1 (RIPK1) and the TNF receptor associated protein 2 to allow nuclear factor kappa B (NF- κ B) nuclear translocation and activation of pro-inflammatory and anti-apoptotic target genes (Micheau *et al.* 2003).

Of note, cells that undergo apoptosis through the death receptor pathway can be divided into two groups. In type I cells, receptors are associated with lipid rafts. After DISC formation, procaspase-8 is activated at very high levels, which leads to apoptosis very quickly. In type II cells, where hepatocytes are included, receptors are excluded from lipid rafts and this leads to lower activation of caspase-8. Therefore, signal amplification is required, which occurs through caspase-8 cleavage of BID, thus connecting and engaging the mitochondrial pathway of apoptosis (Yu *et al.* 2008).

1.1.2. The mitochondrial pathway

The mitochondrial pathway of apoptosis is triggered by stimuli that cause intracellular damage, such as DNA damage or cytotoxic drugs. The uncontrolled damage activates pro-apoptotic proteins from the BCL-2 family, which interact with the mitochondria, the crucial element in this pathway (Kroemer *et al.* 2007). The BCL-2 family is a group of proteins that regulate mitochondrial dysfunction during apoptosis. The family comprises both

pro- and anti-apoptotic members interacting with each other and/or with the mitochondria to control the integrity of the outer mitochondrial membrane (Youle *et al.* 2008). Interestingly, it was shown that, unlike in bile ducts and in the small bile duct epithelium, hepatocytes do not express anti-apoptotic protein BCL-2 (Charlotte *et al.* 1994). However, to resist to apoptosis induced by toxic bile acids in cholestasis, hepatocytes are able to turn on the expression of BCL-2 (Kurosawa *et al.* 1997). To compensate for the lack of BCL-2 under normal conditions, hepatocytes express B-Cell lymphoma extra-large (BCL-x_L), a critical hepatocyte apoptosis antagonist of the BCL-2 family. In fact, hepatocytes cannot survive without BCL-x_L, even at physiological conditions, suggesting that select apoptotic insults must always be antagonized by BCL-x_L to maintain hepatocyte integrity (Takehara *et al.* 2004). Myeloid cell leukemia-1 (MCL-1) is another anti-apoptotic BCL-2 family member, normally expressed in hepatocytes. Upon diverse stress signals, MCL-1 expression is rapidly induced and rescues cells from apoptosis (Fleischer *et al.* 2006; Sieghart *et al.* 2006). In contrast, deletion of MCL-1 in the liver causes a profound increase in hepatocyte apoptosis, transaminases levels and pericellular collagen deposition, a marker of fibrogenesis, while decreasing liver size (Vick *et al.* 2009).

Importantly, after activation of the mitochondrial pathway of apoptosis in hepatocytes, BH3-only proteins are activated and by-pass the inhibitory activities of anti-apoptotic BCL-x_L and MCL-1 proteins. This allows the oligomerization of BCL-2-associated X protein (BAX) or other pro-apoptotic BCL-2 family proteins in the mitochondrial outer membrane, leading to mitochondrial membrane permeabilization (MMP) (Youle *et al.* 2008). To do so, BAX cooperates with proteins from the permeability transition pore complex, including the adenine nucleotide translocator and the voltage-dependent anion channel, at the inner membrane. However, BAX can also induce MMP independently of permeability transition pore complex proteins, through a direct effect on the outer membrane (Kroemer *et al.* 2007).

Upon BAX-induced MMP, large pores are formed at the mitochondria to allow cytochrome c, Endo G, Smac/DIABLO, and apoptosis-inducing factor release (Oberst *et al.* 2008). Once release into the cytoplasm, cytochrome c interacts with APAF-1, along with dATP, to form the apoptosome. This

complex recruits and activates procaspase-9 through autocatalytic cleavage, yielding active caspase-9. Once activated, caspase-9 can activate caspase-3 and -7, the effector caspases. To further amplify the apoptotic cascade, Smac/DIABLO antagonizes the action of the IAP (Oberst *et al.* 2008). Importantly, MMP is also responsible for the alteration of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$), as well as the arrest of oxidative phosphorylation, leading to the accumulation of reactive oxygen species (ROS) (Kroemer *et al.* 2007).

p53 is a sequence-specific transcription factor that promotes cell cycle arrest or apoptosis in response to a variety of stress signals, such as DNA damage, hypoxia and aberrant proliferative signals like oncogene activation (Berube *et al.* 2005). Under these circumstances, p53 is stabilized and translocated to the nucleus, where it binds to DNA and transcriptionally regulates different genes, including pro-apoptotic genes p53 upregulated modulator of apoptosis (*puma*), *nox*a, and *bax* (Vazquez *et al.* 2008).

Under physiological conditions, p53 levels are maintained low by its negative regulator, the E3 ubiquitin ligase murine double minute 2 (MDM2), which targets p53 for ubiquitin-dependent degradation through the proteasome (Kubbutat *et al.* 1997) (**Fig. 1.2.**). Thus, the MDM2 protein acts as a negative regulator of p53, and p53 itself induces transcription of MDM2 (Lahav *et al.* 2004). The interaction between MDM2 and p53 is mediated *via* a well-defined hydrophobic cleft in MDM2, which is filled by only three side chains of the helical region of p53 (Phe19, Leu26 and Trp23) (Vazquez *et al.* 2008). When DNA damage occurs, the protein ataxia-telangiectasia mutated kinase is activated and phosphorylates p53 at a specific site, preventing the binding of MDM2 to p53, thus allowing p53-dependent apoptosis (Lahav *et al.* 2004). In addition, accumulated p53 is subject to extensive post-translational modifications including phosphorylation, acetylation, methylation, sumoylation, ubiquitination, neddylation and glycosylation. These modifications contribute to increase p53 protein stability, thus modulating its function as a transcription factor (Toledo *et al.* 2006). For example, p53 Ser46 phosphorylation is required for p53-dependent transcriptional activation of the pro-apoptotic factor p53AIP1 in response to high levels of DNA damage. It was shown that, under these conditions, p53AIP1 translocates to the mitochondria and

facilitates the release of cytochrome *c* during apoptosis (Oda et al. 2000). On the other hand, p53 acetylation in Lys320 is important for transcriptional activation of cell cycle arrest genes, such as *p21*, but not pro-apoptotic genes (Knights et al. 2006). In turn, Lys120 acetylation is critical for p53-mediated transcription of pro-apoptotic genes *bax* and *puma*

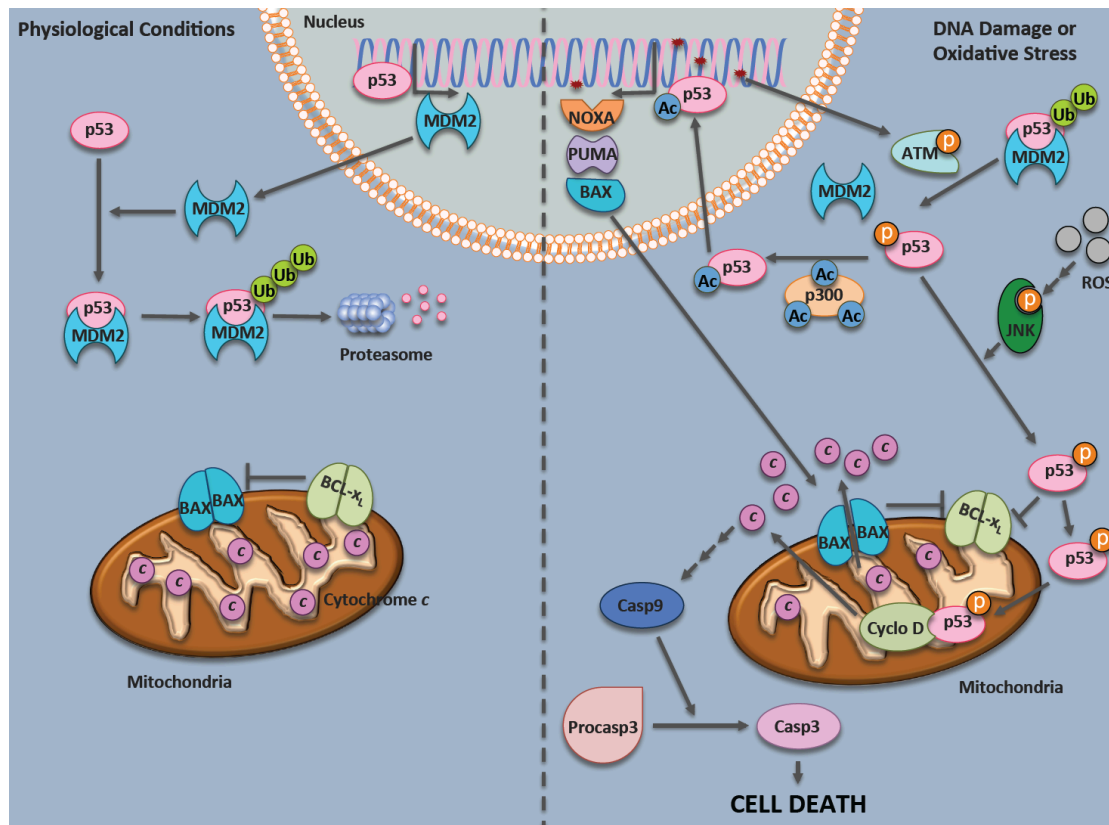


Figure 1.2. p53 signalling under physiological conditions and under DNA damage or oxidative stress. Under physiological conditions (left side), p53 levels are maintained low by MDM2, which targets p53 for ubiquitin-dependent degradation through the proteasome. Interestingly, p53 initiates the transcription of MDM2, which in turn targets p53 for degradation, so in normal cells the level of p53 is kept low. When DNA damage occurs (right side), ataxia-telangiectasia mutated (ATM) kinase is activated and phosphorylates p53 at a specific site, preventing the binding of MDM2 and p53, which allows p53-dependent apoptosis. In case of oxidative stress, there is an increase in ROS production, which activates JNK. JNK phosphorylates p53 that disrupts its binding to MDM2. To induce apoptosis, the acetyltransferase p300 acetylates p53 on Lys120, which is critical for p53 transcription of pro-apoptotic genes *bax*, *puma* and *noxa*. p53 can also interact with mitochondria by binding to BCL-x_L, which inhibits its anti-apoptotic functions. p53 also forms a complex with cyclophilin D (Cyclo D) leading to disruption of mitochondrial structure. Casp3, caspase-3; Casp9, caspase-9; Procasp3, procaspase-3.

(Sykes *et al.* 2006). In addition, Lys120 acetylation is also required for efficient displacement of the MCL-1 protein from BCL-2 homologous antagonist killer, a pro-apoptotic member of the BCL-2 family. This displacement is critical for the induction of transcription-independent apoptosis by p53, presumably because it facilitates BCL-2 homologous antagonist killer oligomerization and permeabilization of the outer mitochondrial membrane (Sykes *et al.* 2009).

While p53 mostly acts as a nuclear transcriptional factor, it can also interact with the mitochondria by binding to BCL-x_L. Still at the cytosolic level, p53 may function as an activator of BH3-only proteins, such as truncated BID or BCL-2-interacting mediator of cell death, allowing the oligomerization and activation of pro-apoptotic proteins like BAX (Chipuk *et al.* 2006). Finally, p53 can also form a complex with cyclophilin D, leading to disruption of the mitochondrial structure (Wolff *et al.* 2008).

Although p53 is a strong inducer of apoptosis in many mammalian tissues, the liver is slightly more resistant to p53-mediated apoptosis, mostly due to the lower ability of liver p53 to translocate to mitochondria, following DNA damage (Erster *et al.* 2004). Nevertheless, p53-mediated hepatocyte apoptosis still plays a major role during, for instance, primary biliary cirrhosis and cholestasis. In fact, p53 expression is increased in primary hepatocytes exposed to bile acids, likely facilitating apoptosis (Zhang *et al.* 2008). In addition, oxidative stress-induced apoptosis of bile duct cells, during primary biliary cirrhosis, occurs in parallel with increased levels of p53, c-Jun NH₂-terminal kinase (JNK) and caspase-3 (Salunga *et al.* 2007). More recently, it has been shown that p53-induced apoptosis is critical in non-alcoholic steatohepatitis (NASH). In an animal model of NASH, insulin-like growth factor-1 was decreased with disease progression, resulting in increased p53 levels. p53 was then suggested to mediate mitochondrial cell death pathways, possibly being also responsible for increasing TNF-related apoptosis-inducing ligand (TRAIL) receptor expression, thereby linking intrinsic and exogenous apoptosis pathways during NASH (Farrell *et al.* 2009).

1.1.3. Caspase function

Caspases belong to a family of cysteinyl-aspartic-acid-proteases that are expressed as inactive zymogens, known as procaspases. Procaspases contain a p20 large subunit, a p10 small subunit, and a prodomain, which varies according to caspase function (Rupinder *et al.* 2007). The active caspase is structurally a homodimer with each monomer formed by a small and a large subunit; two monomers aligned in a head-to-tail configuration (Li *et al.* 2008). Because caspases are produced as enzymatic inert zymogens, activation occurs through proteolytic cleavage that separates the small and large subunits and removes the prodomain (Riedl *et al.* 2004). The catalytic site in the p20 subunit has Cys and His residues in the position 285 and 237, respectively, of the active site. In general, caspases recognise at least four contiguous amino acids in the substrates (P4-P3-P2-P1), and cleave after the C-terminal domain (P1), which normally is an Asp residue (Rupinder *et al.* 2007).

Caspases cleave a specific set of target proteins in just one or two positions, but have different abilities to do so; initiator caspases are more specific proteases that slice few substrates, such as their own precursors and other downstream caspases, whereas effector caspases are responsible for most of the proteolysis seen during apoptosis (Li *et al.* 2008). In general, when a caspase cuts its target protein, the final result is the inactivation of that same protein. This is the case during cleavage of the laminin network that causes nuclear shrinking (Rao *et al.* 1996). It is also possible that caspase cleavage results in the activation of the target protein, when a caspase acts on its substrate either by cleaving off a negative regulatory domain or, indirectly, by inactivating a regulatory subunit (Hengartner 2000).

1.1.3.1. Caspase-3

Caspase-3 is the major effector caspase and can be activated by multiple apoptosis-inducing stimuli. If the signal targeted the death receptor pathway, upstream caspases, like caspase-8, are first activated, independently of interactions with BCL-2 family members, and then directly activate procaspase-3. On the other hand, signals originated from inside the cell, such as DNA damage, cause mitochondrial outer membrane

permeabilization leading to the release of apoptotic factors, which activate procaspase-9 and then procaspase-3 (Mancini *et al.* 1998). Curiously, when Fas signalling activates the mitochondrial pathway through truncated BID, active caspase-3 can cleave initiator caspase-9 (Fujita *et al.* 2001). This is thought to be a positive feedback mechanism that further enhances apoptosis through mitochondria and caspase-3 activation. Furthermore, caspase-3 may also amplify Fas signalling through cleavage of BID and degradation of its own inhibitor X-chromosome-linked IAP (XIAP) (Ferreira *et al.* 2012), which is also neutralized by the release of Smac/DIABLO (Green *et al.* 2004). With XIAP inactivated, both caspases can be fully activated and the apoptotic signalling through mitochondria is accelerated. In fact, evidence shows that Smac/DIABLO not only cleaves and neutralizes XIAP, but also caspase-3. Structural analysis showed that caspase-3 binds to XIAP's BIR2 domain (Eyrisch *et al.* 2012) and both proteins co-immunoprecipitate in hepatocyte lysates (Jost *et al.* 2009).

1.1.3.2. Caspase-2

Similarly to initiator caspases, caspase-2 contains a long prodomain and a large and small catalytic subunit (Xue *et al.* 1996). The prodomain is a caspase recruitment domain, which interacts and binds to the caspase recruitment domain present in the RIPK-associated ICH-1 homologous protein with a death domain (RAIDD) (Duan *et al.* 1997). RAIDD is important to recruit caspase-2 to a signalling complex where the death domain of RAIDD interacts and binds to the death domain of p53-induced protein with a death domain (PIDD) to form the PIDDosome, detected in different cell extracts upon temperature shift (Tinel *et al.* 2004). The PIDDosome contains five PIDDs and seven RAIDDs, which form two stacked rings with a staggered hexagonal pattern. This complex allows the recruitment of seven caspases-2 molecules to activate it (Park *et al.* 2007). Caspase-2 has been shown to cleave BID, leading to the release of cytochrome *c* from the mitochondria, although low levels of active caspase-2 may not produce enough BID to commit a cell to apoptosis in the absence of another pro-apoptotic signal (Guo *et al.* 2002).

Interestingly, PIDD can also activate NF- κ B in response to DNA damage. In fact, in response to genotoxic stress, PIDD translocates to the nucleus to assemble with RIPK1 and NF- κ B essential modulator (NEMO), inducing NEMO sumoylation (Janssens *et al.* 2005). Upon sumoylation, NEMO translocates from the nucleus to the cytoplasm, where it stimulates the release of inhibitor of NF- κ B (I κ B) from NF- κ B. In turn, this allows NF- κ B to translocate to the nucleus and act as a transcription factor (Huang *et al.* 2003).

It is important to note that PIDD acts as a molecular switch between survival and apoptosis, since the recruitment of RAIDD and RIPK1 to the PIDDosome appears to be sequential. In fact, RAIDD and RIPK1 compete in their binding to PIDD, blocking the pro-survival or pro-apoptotic pathway, respectively. Interestingly, in cells deficient in RIPK1, caspase-2 expression increases in response to DNA damage. On the other hand, RAIDD knockout cells display stronger NEMO sumoylation and increased NF- κ B activity upon genotoxic stress (Janssens *et al.* 2005).

Caspase-2 activity can also be regulated through phosphorylation at critical residues. In human cancer cell lines, protein kinase CK2 was shown to phosphorylate caspase-2 at Ser157, which prevents its dimerization, processing and enzymatic activation. In fact, during TRAIL- and DNA damage-induced apoptosis, protein kinase CK2 activity is inhibited in parallel with increased caspase-2 activity (Shin *et al.* 2005). Moreover, caspase-2 enhances apoptotic functions of p53 through cleavage of MDM2. This increases p53 stability, which results in a positive feedback loop where p53 induces PIDD expression and PIDD stabilizes p53 through caspase-2 activation (Oliver *et al.* 2011).

Caspase-2 also appears to be a major player in endoplasmic reticulum (ER) stress, although it is still unclear how ER stress translates into caspase-2 activation (Cheung *et al.* 2006). Once activated, caspase-2 leads to apoptosis through cleavage of BID; it was shown that silencing of caspase-2 protected SV40-transformed mouse embryo fibroblasts against ER stressors and significantly reduced BID cleavage, as well as cytochrome c release and cell death (Upton *et al.* 2008). In a hepatocellular carcinoma cell line, ER

stress-induced caspase-2 also led to BAD activation and down-regulation of MCL-1 (Yeh *et al.* 2007).

Finally, caspase-2 also regulates the oxidative stress response; livers of caspase-2 deficient mice display increased oxidized protein levels compared to age-matched wild-type mice, suggesting that caspase-2 deficiency compromised the animal's ability to clear oxidative stress-damaged cells (Zhang *et al.* 2007). In fact, caspase-2 deficient mice also show increased levels of lipid peroxidation and decreased levels of antioxidant enzymes involved in ROS removal (Shalini *et al.* 2012).

1.1.4. Kinase modulation

Protein phosphorylation by the mitogen-activated protein kinases (MAPK) or the AGC kinases is a conserved strategy that regulates cellular function in both prokaryotes and eukaryotes (Cuny 2009). Mammals express at least four distinctly regulated groups of MAPKs: the extracellular signal-related kinases (ERK)-1/2, JNK1/2/3, p38 proteins (p38 $\alpha/\beta/\gamma/\delta$) and ERK5. Despite being included in different groups, all MAPKs recognize similar sites for phosphorylation, consisting on a serine or a threonine followed by a proline. The specificity of each kinase is determined by the amino acids that surround the recognition sites. Through phosphorylation of several targets, MAPK signalling cascades regulate important cellular processes including gene expression, cell proliferation, cell survival and death, and cell motility (Chang *et al.* 2001).

On the other hand, the AGC family of protein kinases defines a group of serine/threonine protein kinases that share sequence similarity in their catalytic kinase domains with cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinase G, phospholipid-dependent protein kinase C, and membrane-bound protein kinase B (PKB, also known as AKT). In mammals, AGC kinases regulate signalling events that affect cell size, cell number, and cell death, thereby influencing growth and morphogenesis (Pearce *et al.* 2010).

1.1.4.1. JNK

JNK belongs to the MAPK superfamily and functions as an important regulator of cell proliferation, differentiation and apoptosis (Deng *et al.* 2003). JNK was first identified as the UV-induced factor responsible for phosphorylating and activating the proto-oncogene c-Jun (Hibi *et al.* 1993; Derijard *et al.* 1994). In fact, the classic function of JNK is to phosphorylate and activate c-Jun to increase activator protein 1 (AP-1) gene transcription; c-Jun is the transactivation component of the heterodimeric transcription factor AP-1 that, in parallel to JNK, has been linked to the regulation of proliferation and cell death (Shaulian *et al.* 2002).

Following activation, JNK may be translocated to mitochondria and inhibit the anti-apoptotic activity of BCL-2 proteins (**Fig. 1.3.**). JNK may also cleave and activate pro-apoptotic BID, and phosphorylate BCL-2-associated death promoter (BAD), which is then free to antagonize anti-apoptotic proteins such as BCL-2, and promote cell death (Dhanasekaran *et al.* 2008). Apart from these apoptotic and survival molecules, JNK was also shown to phosphorylate transcription factors like p53; migratory proteins such as paxillin and microtubule-associated protein kinases such as AKT; and E3 ligases (Bogoyevitch *et al.* 2006).

There are three highly related but distinct JNK gene products that can be expressed as a result of variable mRNA splicing (Gupta *et al.* 1996). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is expressed predominantly in neurons but also in cardiac smooth muscle and testis (Yang *et al.* 1997). In hepatocytes, very recent studies have suggested that JNK1 and JNK2 have opposing functions; while JNK1 usually correlates with cell death induction, JNK2 activation is associated with cell survival. In fact, siJNK1-treated or *jnk1* knockout hepatocytes are protected from cell death. On the other hand, siJNK2-treated or *jnk2* null hepatocytes are sensitized to cell death (Amir *et al.* 2012). In addition, treatment of hepatocytes with TNF- α activates JNK1, which promotes caspase-3 activation and apoptosis, although TNF- α -induced apoptosis is prevented when *jnk1* is disrupted (Chang *et al.* 2006).

Interestingly, mice subjected to a high-fat diet (HFD) display obesity in

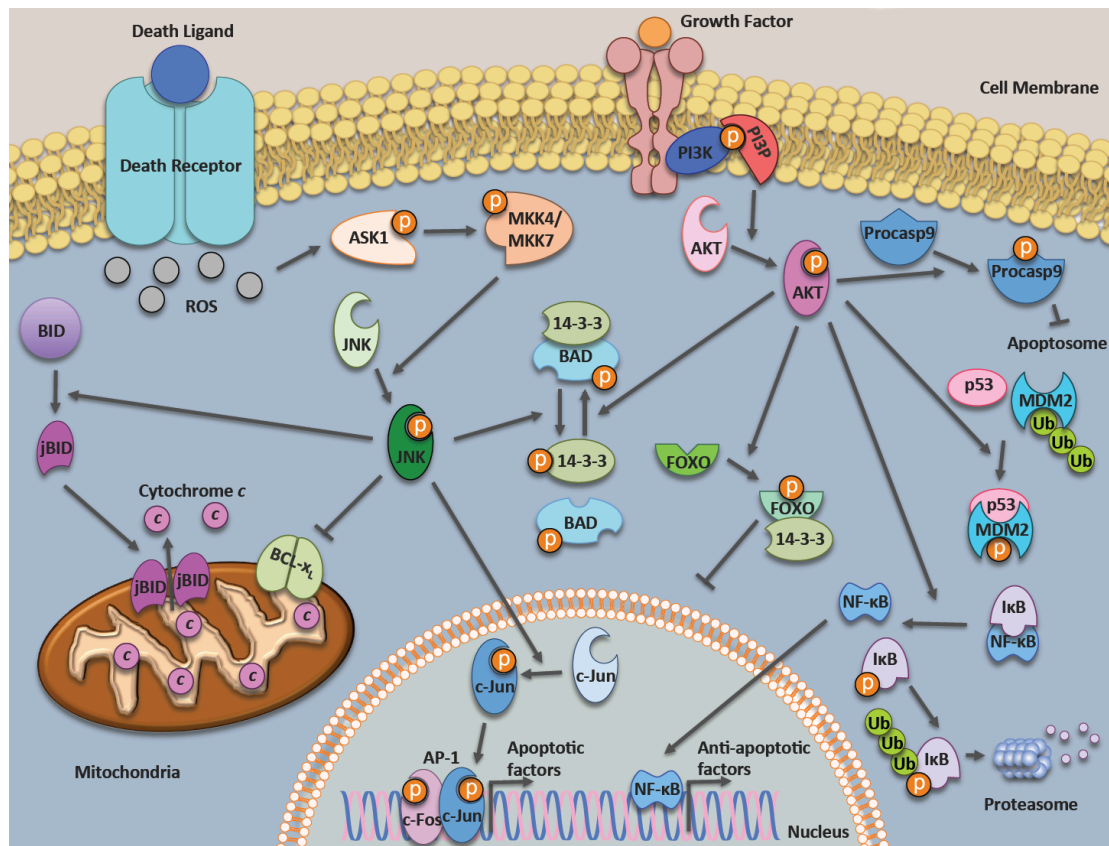


Figure 1.3. JNK and AKT phosphorylation targets and pathways. When a ligand binds to its death receptor, ROS production increases activating ASK-1 through TRAF2 and finally leading to the phosphorylation of JNK by upstream MKK4/MKK7. Phosphorylated JNK translocates to the nucleus, where it phosphorylates and transactivates c-Jun. Phosphorylation of c-Jun leads to the formation of AP-1, which is involved in the transcription of pro-apoptotic genes. Following activation, JNK can also translocate to mitochondria and inhibit the anti-apoptotic activity of BCL-x_L. Other functions of JNK include the cleavage of BID, which results in a 20-kDa protein (jBID) that translocates to mitochondria and allows cytochrome c release. Finally, JNK phosphorylates Ser128 of BAD, which inhibits its interaction with 14-3-3 protein and releases BAD to antagonize anti-apoptotic proteins, such as BCL-x_L, and promote cell death. JNK also phosphorylates 14-3-3 Ser184 that helps the release of BAD from the 14-3-3 protein. On the other hand, AKT activation occurs when a survival stimulus binds to transmembrane receptors. These receptors recruit PI3K isoforms to the cytoplasmic surface of the plasma membrane. PI3K catalyzes the transfer of phosphate from ATP to PI3,4P and PI3,4,5P. The binding of PI3K-generated phospholipids to AKT is crucial for its activation. Then AKT can phosphorylate BAD at Ser136, which sequesters BAD at 14-3-3 protein leading BAD away from mitochondria. AKT may also participate in the phosphorylation of Forkhead transcription factors (FOXO). Phosphorylation retains FOXO in the cytoplasm, in contrast with the constitutively localization in the nucleus, and allows its binding to 14-3-3 protein that anchors phosphorylated FOXO within the cytoplasm. Moreover, AKT can phosphorylate IκB causing its ubiquitination and degradation. When that happens, NF-κB is free to undergo nuclear translocation and activate its

anti-apoptotic target genes. In addition, AKT also phosphorylates MDM2, which decreases MDM2 self-ubiquitination and renders the protein more stable. As a consequence, p53-mediated apoptosis is inhibited. Finally, caspase-9 is another pro-apoptotic member that is inactivated by AKT. AKT phosphorylates procaspase-9 (Procasp9), which will cause procaspase-9 inactivation through impairment of the intrinsic catalytic activity of caspase-9.

wild-type animals but not in *jnk1* knockout mice (Hirosumi *et al.* 2002; Solinas *et al.* 2006). In fact, *jnk1* null mice show decreased insulin receptor substrate 1 (IRS-1) phosphorylation at the inhibitory Ser307 site and increased phosphorylation at the tyrosine activator site, thus resulting in lower insulin resistance (IR) (**Fig. 1.3.**). Moreover, HFD-induced hepatocyte injury and steatosis are suppressed in *jnk1* null mice (Hirosumi *et al.* 2002). Interestingly, despite showing a similar degree of hepatic steatosis as wild-type mice, *jnk2* knockout mice under HFD presented increased levels of hepatocyte injury, obesity, and IR. The authors then showed that these mice had higher JNK1 activity, suggesting that JNK1 was overcompensating the loss of JNK2 and, by doing so, promoting liver damage and IR (Singh *et al.* 2009).

1.1.4.2. AKT

AKT constitutes an important node in many signalling cascades. In fact, AKT plays key roles in cell survival (Hsieh *et al.* 2011), proliferation (Sale *et al.* 2008), insulin-dependent glucose transport (Welsh *et al.* 2005), and glucose and lipid metabolism (Gottlob *et al.* 2001; Berwick *et al.* 2002). Therefore, impairment in AKT activity has been associated not only with cancer (Chiarini *et al.* 2009; De Luca *et al.* 2012), but also with other disorders, including type 2 diabetes mellitus, cardiovascular diseases, and muscle hypotrophy (Chappell *et al.* 2011; Hers *et al.* 2011).

AKT is a member of the AGC serine/threonine protein kinase family (Bellacosa *et al.* 1991). In mammals, there are three genes encoding for AKT - AKT1/PKB α , AKT2/PKB β , and AKT3/PKB γ . While AKT1 and AKT2 are ubiquitously expressed (Hanada *et al.* 2004), AKT3, similar to JNK3, is found predominantly in the brain, kidney, and heart (Masure *et al.* 1999). Despite sharing a high degree of sequence homology in their catalytic domains, AKT

isoforms diverge in the remaining regions of the protein (Hanada *et al.* 2004).

To undergo activation, all three AKT isoforms require activation of particular transmembrane receptors that recruit phosphoinositide-3 kinase (PI3K) isoforms to the cytoplasmic surface of the plasma membrane. Once there, PI3K catalyzes the transfer of phosphate from ATP to the D-3 position of the inositol ring of membrane-localized phospholipids, generating 3'-phosphorylated phosphoinositides (PI3,4P and PI3,4,5P) (**Fig.1.3.**) (Datta *et al.* 1999). The activity of AKT is dependent on phosphorylation at Ser124, Thr308, Thr450 and Ser473. Constitutively, AKT is phosphorylated at Ser124 and Thr450, independently of cell stimulation. When PI3K is activated and levels of PI3,4P and PI3,4,5P increase, AKT translocates to the cytoplasm surface of the plasma membrane. Here, AKT can undergo a conformational change, exposing Thr308 and Ser473 sites for phosphorylation by 3-phosphoinositide-dependent protein kinase 1 (Bayascas 2010; Raimondi *et al.* 2011).

Once activated, AKT phosphorylates different elements of the apoptotic machinery, particularly BAD. Phosphorylation of BAD at Ser112 and Ser136 causes BAD-BCL-x_L dissociation, followed by sequestration of BAD with 14-3-3 protein, thus inhibiting its function (**Fig. 1.3.**) (Datta *et al.* 2002). Caspase-9 can be phosphorylated by AKT at Ser196, causing procaspase-9 inactivation through impairment of its intrinsic catalytic activity (Datta *et al.* 1999). AKT can also communicate with the NF-κB pathway. In fact, AKT can phosphorylate IκB at Thr23, causing its ubiquitination and degradation, thus freeing NF-κB to undergo nuclear translocation and activate its target genes (Romashkova *et al.* 1999). AKT is also capable to phosphorylate MDM2 at Ser166 and Ser186, which decreases MDM2 self-ubiquitination and renders the protein more stable. As a consequence, p53-mediated apoptosis is inhibited (Feng *et al.* 2004).

Finally, AKT may modulate the activity of different nuclear transcription factors, including the Forkhead box transcription factor, subgroup O (FOXO). These transcription factors share a core domain of 100 amino acids, known as Forkhead domain, which allows their interaction with DNA. Since the Forkhead domain is the most important one for protein activation, the major

site of phosphorylation by AKT appears to be the DNA binding domain (Arden *et al.* 2002).

1.1.5. microRNAs

microRNAs (miRNAs or miRs) are a large family of ~21-nucleotides in length RNAs that act as post-transcriptional regulators of gene expression and control several developmental and cellular processes in eukaryotic organisms. As such, several studies have shown that abnormal changes in miRNA expression are often associated with human pathologies (Bartel 2009).

miRNAs are first transcribed as long precursor molecules termed primary miRNAs (pri-miRNAs) (**Fig. 1.4.**). Pri-miRNAs are produced either by RNA polymerase II transcription or as the result from the cleavage of introns in protein-coding genes (Carthew *et al.* 2009). Pri-miRNAs are then processed in a two-step sequence through the actions of Drosha and Dicer, members of the RNase III family of enzymes. First, nuclear Drosha processes the pri-miRNA into a ~70-nucleotide precursor hairpin (precursor miRNA or pre-miRNA), which is then exported to the cytoplasm *via* Exportin 5. In the cytoplasm, the pre-miRNA is further processed by Dicer into a ~21-nucleotide miRNA/miRNA* duplex. One strand of this duplex is the mature miRNA, which will form a complex with the Argonaunts (AGO) proteins to form the miRNA-induced silencing complex (miRISC). The other strand (passenger strand or miRNA*) is released and degraded. Still, recent studies have shown that the miRNA* strand is not always degraded and may be loaded into miRISC to function as a normal mature miRNA (Okamura *et al.* 2009; Ghildiyal *et al.* 2010).

Efficient mRNA targeting by miRNAs requires continuous base-pairing of miRNA nucleotides 2 to 8 (the seed region) (Bartel 2009). However, most miRNAs base-pair match imperfectly with the sequences in the 3'-untranslated region (UTR) of target mRNAs, inhibiting protein synthesis by either repressing translation or by promoting mRNA deadenylation and decay. The initial effect of miRNAs consists on the inhibition of mRNA translation at the initiation step, without mRNA decay. This is followed by increased mRNA

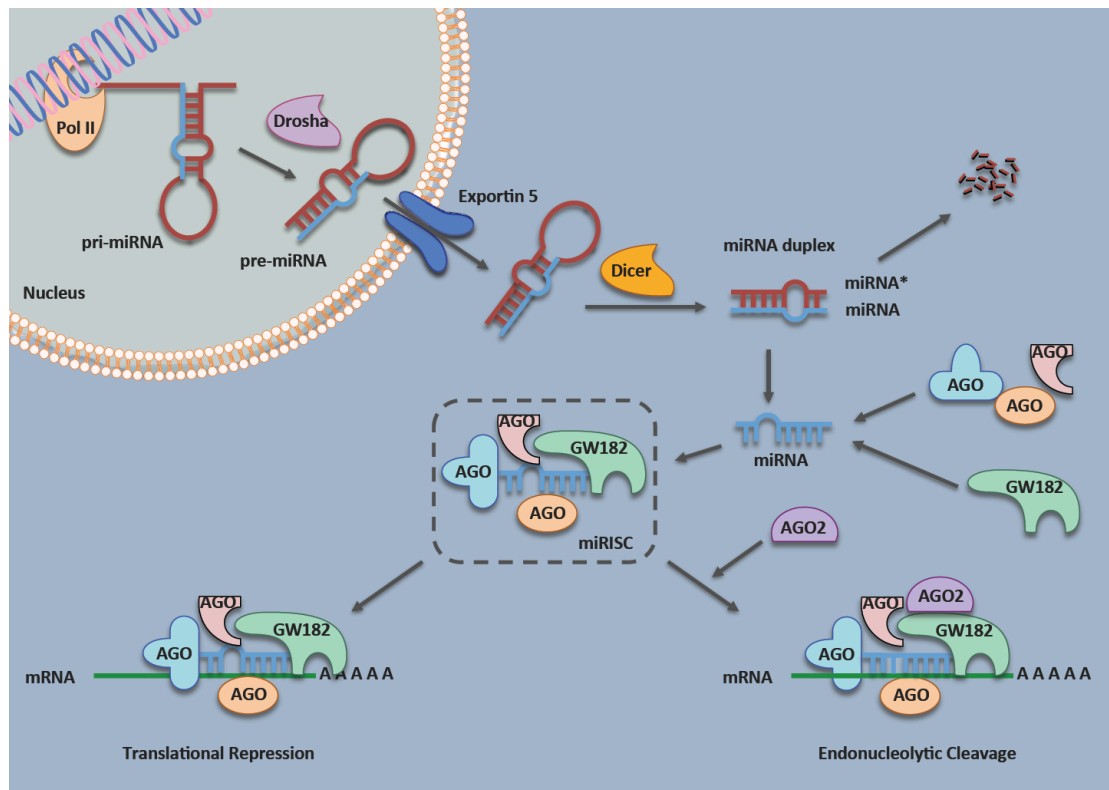


Figure 1.4. miRNA synthesis and mechanism of action. miRNAs are processed from precursor molecules (pri-miRNA). The pri-miRNAs can be produced by two different mechanisms, including the action of RNA polymerase II transcription or the cleavage of introns from protein-coding genes. Then, pri-miRNAs are processed by Drosha in the nucleus into a ~ 70-nucleotide precursor hairpin (pre-miRNA), which is exported to the cytoplasm *via* Exportin 5. In the cytoplasm, pre-miRNA is processed by Dicer into a ~21-nucleotides miRNA/miRNA* duplex. One strand of this duplex is a mature miRNA, which forms a complex with AGO and GW182 (miRISC). The other strand (passenger strand or miRNA*) is released and often degraded. The majority of the miRNAs base-pair imperfectly with sequences in the 3'-UTR of target mRNAs, which inhibits protein synthesis by either repressing translation or promoting mRNA deadenylation and decay. Deadenylation of mRNAs is mediated by GW182 proteins, which interact with AGOs and act downstream. When miRISC-containing AGO2 encounters mRNAs bearing sites nearly perfectly complementary to miRNA, these mRNAs are cleaved endonucleolytically and degraded.

deadenylation. Alternatively, deadenylation may occur independently of the initial translational block, although at a slower rate (Chekulaeva *et al.* 2009; Fabian *et al.* 2010). Although the mechanistic details of miRNA-mediated translational repression are still not well understood, miRNA-mediated mRNA deadenylation is more or less established, being mediated by GW182

proteins, upon interaction with AGOs. While the N-terminal of GW182 interacts with AGO through its GW repeats, the C-terminal region interacts with the poly(A) binding protein and recruits the deadenylases (Eulalio *et al.* 2009; Fabian *et al.* 2010). miRNA-induced mRNA deadenylation may ultimately lead to the decay of target mRNAs through the recruitment of decapping machinery (Bethune *et al.* 2012). Finally, when miRISC-containing AGO2 encounters mRNAs bearing sites nearly perfectly complementary to miRNA, these mRNAs are cleaved endonucleolytically and degraded (Bartel 2009; Voinnet 2009; Fabian *et al.* 2010). Although rare in animals, this is a common mode of miRNA action in plants (Voinnet 2009).

1.1.5.1. Modulation of hepatocellular proliferation and apoptosis

miRNAs are being increasingly described as powerful, novel regulators of hepatocellular proliferation and apoptosis. For instance, MCL-1 is a direct target of miR-20a in hepatocytes (Akgul 2009) and, in hepatocellular carcinoma (HCC), MCL-1 is increased, while miR-20a is found downregulated. Interestingly, miR-20a restoration inhibits HCC cell proliferation and induces apoptosis by directly targeting MCL1 (Fan *et al.* 2013). miR-125b is also found at abnormally low levels in HCC and indeed, the levels of miR-125b are positively associated with apoptosis in the HCC liver. This is most likely the result of miR-125b also targeting MCL1, which increases the MMP and ultimately reduces caspase-3 cleavage (Gong *et al.* 2012). On the contrary, miR-221 is found overexpressed in HCC liver samples, suggesting that it may play a critical role in hepatocarcinogenesis, as an oncogenic miRNA (Gramantieri *et al.* 2007; Pineau *et al.* 2010). In fact, miR-221 expression is significantly higher in stages III and IV, the more severe stages of HCC, when compared with stages I and II, more benign stages (Rong *et al.* 2013). It is possible that miR-221 act as an oncogenic miRNA by targeting phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumor suppressor, thereby inducing TRAIL resistance and enhancing cellular migration through the activation of the AKT pathway (Garofalo *et al.* 2009). miR-372 was very recently suggested to also play a role during HCC progression, and its increased expression correlates with more severe HCC stages. In fact, miR-372 may promote proliferation,

invasion, and migration of HCC cells (Gu *et al.* 2013). Finally, miR-34a, a pro-apoptotic miRNA that induces apoptosis in a p53-dependent manner, is downregulated in human HCC, indicating that it may play a critical role as a tumour suppressor miRNA during oncogenesis and progression of HCC, by targeting multiple pathways (Li *et al.* 2009a; Dang *et al.* 2013).

In addition to HCC, liver regeneration may also be regulated by miRNAs. For instance, miR-19a, -21, and -214 were found to be significantly upregulated after 2/3 liver partial hepatectomy in rats. All these miRNAs are known to target PTEN, releasing the negative regulation on the PI3K/AKT pathway and, thus, likely playing a crucial role in the early regenerative response of the liver after resection (Castro *et al.* 2010). miR-221 overexpression was also shown to accelerate hepatocyte proliferation during liver regeneration (Yuan *et al.* 2013), while miR-127 was downregulated after liver partial hepatectomy, due to a rapid methylation of its promoter, increasing hepatocyte proliferation by relieving miR-127 targets BCL-6 and SET domain-containing protein 8 (Pan *et al.* 2012).

1.1.5.2. The miR-34a/Sirtuin1/p53 pro-apoptotic pathway

In mammals, the *miR-34* family comprises three processed miRNAs encoded by two genes. *miR-34a* is encoded in a single transcript on chromosome 1p36 whereas *miR-34b* and *miR-34c* share a common primary transcript on chromosome 11q23 (Bommer *et al.* 2007). It has already been shown that all three *miR-34* genes can be targeted by p53, for instance after DNA damage. In fact, *miR-34* elements are not activated in p53-null mouse embryonic fibroblasts after DNA damage (He *et al.* 2007). miR-34a, in particular, has been reported to act as an inducer of senescence, cell cycle arrest or apoptosis (Hermeking 2010). Its expression is significantly decreased in multiple types of diseases such as human neuroblastoma (Brodeur 2003; Welch *et al.* 2007), B-lymphoid malignancies (Sotillo *et al.* 2011), colon cancer, prostate cancer, pancreatic cancer (Bagchi *et al.* 2008), atherosclerotic cardiovascular diseases (Zhao *et al.* 2010), among others. Most miR-34a targets regulate cell-cycle progression, cellular proliferation, apoptosis, DNA repair, and angiogenesis. In particular, ectopic overexpression of miR-34a in different tumour cell lines results in

re-activation of the apoptotic pathway, underscoring the role of miR-34a as a potent tumour suppressor gene (Chang *et al.* 2007; Raver-Shapira *et al.* 2007). Induction of apoptosis by miR-34a, which can occur through both p53-dependent and -independent mechanisms, depends on the cellular context and expression levels of miR-34a target proteins.

The best characterized direct target of miR-34a is Sirtuin 1 (SIRT1), a NAD-dependent deacetylase that modulates apoptosis in response to oxidative and genotoxic stress (Yamakuchi *et al.* 2008) (**Fig. 1.5.**). The deacetylation reaction induced by SIRT1 involves an amide cleavage of NAD⁺ with the formation of nicotinamide and a covalent ADP-ribose peptide-imidate intermediate. Then, the intermediate is resolved to form O-acetyl-ADP-ribose and the deacetylated substrate is released. The NAD⁺ is essential to provide the driving force for the SIRT1 deacetylation reaction (Sauve *et al.* 2001; Borra *et al.* 2004). SIRT1 possesses two nuclear localization signals and two nuclear export signals and, as such, can be found either in the nucleus or in the cytosol (North *et al.* 2007; Tanno *et al.* 2007). In addition, SIRT1 has different roles in different mammalian tissues. For instance, SIRT1 activation promotes survival of neurons and protects cardiomyocytes from death. In the liver, SIRT1 promotes fatty acid oxidation and gluconeogenesis during nutrient deprivation *via* liver X receptor, peroxisome proliferator-activated receptor (PPAR) γ co-activator 1 α (PGC-1 α), and PPAR α . In white adipose tissue, SIRT1 decreases fat storage by repressing PPAR γ . SIRT1 also promotes insulin secretion and pancreatic beta cell survival by suppressing uncoupling protein 2 and interacting with FOXO, respectively. In the skeletal muscle, SIRT1 promotes mitochondrial biogenesis through activation of PGC-1 α (Haigis *et al.* 2006). In particular, SIRT1 deacetylates PGC-1 α to promote the transcription of mitochondrial fatty acid oxidation genes and initiate fatty acid oxidation during fasting conditions (Canto *et al.* 2008, 2009).

Importantly, by repressing SIRT1, miR-34a increases p53 acetylation and transcription, leading to induction of pro-apoptotic genes such as PUMA and, finally, apoptosis. Furthermore, this mechanism comprises a positive feedback loop, since miR-34a is itself a direct target of p53 (Chang *et al.* 2007). In fact, p53 activation leads to increased production of miR-34a and suppression of SIRT1. The decrease in SIRT1 expression allows for an

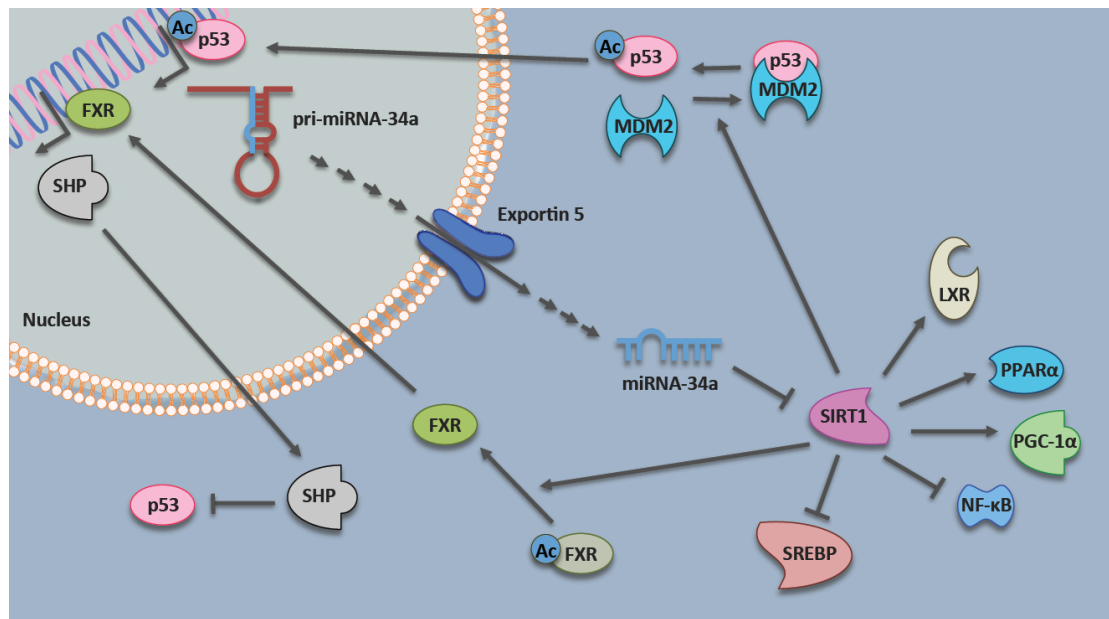


Figure 1.5. The miR-34a/SIRT1/p53 pro-apoptotic pathway. Acetylation of p53 decreases its binding affinity to MDM2. This allows p53 nuclear translocation and transcription of miR-34a. After export to the cytoplasm, miR-34a interacts and inhibits SIRT1. This decreases SIRT1 activity in promoting fatty acid oxidation and gluconeogenesis during nutrient deprivation *via* deacetylation and activation of liver X receptor (LXR), PGC-1 α , and PPAR α . SIRT1 can also deacetylate and inhibit SREBP and NF- κ B repressing lipogenesis and inflammation, respectively. Moreover, SIRT1 can deacetylate and activate FXR that acts as a nuclear receptor that transcribes small heterodimer partner (SHP). SHP in the cytoplasm can inhibit p53. Finally, SIRT1 can also deacetylate p53, which increases MDM2 binding. By repressing SIRT1, miR-34a increases p53 acetylation and transcription activity, leading to induction of pro-apoptotic genes such as PUMA and miR-34a itself, and, finally, apoptosis.

increase in p53 acetylation and p53 activity. Finally, increased p53 further drives miR-34a production, which increases p53 acetylation and p53 activity, completing the positive feedback loop (Yamakuchi *et al.* 2009).

1.1.6. Bile acids

Cholesterol breakdown in the liver gives rise to bile acids as end products (Chiang 2002). Bile acids help in the secretion of endogenous metabolites and xenobiotics from the liver and allow the absorption of lipids and lipophilic nutrients from the intestine. Bile acids also control glucose and lipid metabolism in the enterohepatic system and energy expenditure in peripheral tissues (Nguyen *et al.* 2008; Lefebvre *et al.* 2009).

The bile acid pool is formed by primary and secondary bile acids. In humans, primary bile acids include cholic acid (CA) and chenodeoxycholic acid, while secondary bile acids are deoxycholic acid (DCA), lithocholic acid, and ursodeoxycholic acid (UDCA). Whereas primary bile acids are synthesized directly from cholesterol in the liver, secondary bile acids are derived from primary bile acids in the intestine by the action of bacterial enzymes. For example, 7-dehydroxylation of CA or epimerization of the hydroxyl group in C-7 of chenodeoxycholic acid yields DCA and UDCA, respectively (Ridlon *et al.* 2006). Cholesterol 7 α -hydroxylase (CYP7A1), a microsomal cytochrome P450 enzyme, catalyzes the first and rate-limiting step in the production of primary bile acids from cholesterol (Lefebvre *et al.* 2009). Once produced in the liver, bile acids are transported across the canalicular membrane of hepatocytes into the bile and are stored in the gallbladder. This is important in order to avoid a constant secretion of bile acids to the intestine. In fact, after a meal, the duodenum releases cholecystokinin that stimulates the gallbladder to contract and all bile acids are released into the intestine, reabsorbed in the ileum, and transported back to the liver *via* portal blood for re-excretion into the bile. This process is known as enterohepatic circulation of bile acids, which is important to reduce bile acids synthesis (Chiang 1998; Hofmann 1999).

Bile acid transporters act as the crucial players during enterohepatic circulation. In order to be able to contact with the portal blood, to collect nutrients and bile acids, as well as with the bile, to excrete bile acids, hepatocytes are polarized cells with basolateral or sinusoidal (in contact with portal blood) and apical or canalicular (in contact with bile) membrane domains (Trauner *et al.* 2003). Because the bile is highly concentrated in bile acids, bile acid transporters carry them and other organic compounds against their concentration gradients through the canalicular membrane. At this level, the major transporter of bile acids is the bile salt export pump.

To decrease intrinsic hydrophobicity, bile acids usually give rise to bile salts by conjugation with taurine or glycine in the peroxisomes. However, they cannot cross the hepatocyte membrane and require active transport mechanisms for cellular uptake (Meier 1995). In this case, basolateral bile salts transport into hepatocytes occurs through two transporters: the

Na⁺-dependent taurocholate transporter and the organic anion transporter. Of note, in the intestine, bile salts are deconjugated, and then mostly reabsorbed at the terminal ileum (Trauner *et al.* 2003; Lefebvre *et al.* 2009).

Bile acids can control their own synthesis by activating nuclear receptors, including the farnesoid X receptor (FXR). This represents a crucial feedback mechanism in bile acid synthesis, since activation of FXR protects against the toxic accumulation of bile acids in the liver, limits the overall circulation of bile and reduces hepatic exposure to toxic bile acids. In addition, FXR acts as a nuclear receptor that regulates, directly or through the nuclear receptors small heterodimer partner, a wide variety of target genes involved in the control of not only bile acid synthesis but also lipid and glucose homeostasis (Lefebvre *et al.* 2009).

In addition to their primary role in the liver and intestine, bile acids are also important signalling molecules. In particular, they are key modulators of apoptosis in the liver, as well as in other cell types (Amaral *et al.* 2009). Given the fact that apoptosis has long been described as a key event during hepatobiliary diseases (Patel *et al.* 1995), the signalling properties of bile acids are of significant interest. In a broad, vast sense, it appears that bile acid cytotoxicity is related with chemical structure. Indeed, during cholestasis, accumulation of hydrophobic bile acids within hepatocyte induces cell death, while more hydrophilic bile acids displaying cytoprotective functions (Hofmann *et al.* 1984; Amaral *et al.* 2009).

1.1.6.1. Induction of apoptosis

DCA is a secondary bile acid converted from CA by intestinal anaerobic bacteria during enterohepatic circulation (Ridlon *et al.* 2006). Despite its physiological properties, excessive accumulation of DCA and other hydrophobic bile acids is associated with cytotoxic effects. In fact, previous studies have argued that hydrophobic bile acids, including DCA, may induce apoptosis in hepatocytes upon activation of death receptors (Higuchi *et al.* 2003). In that regard, the Fas receptor appears to act as a key player during DCA-induced apoptosis (**Fig. 1.6.**), as hepatocytes from mice lacking the Fas receptor are unable to undergo apoptosis in response to DCA treatment (Qiao *et al.* 2001). DCA-induced activation of Fas receptor signalling results in JNK

activation and repression of CYP7A1 mRNA levels. In fact, DCA can activate JNK in wild type but not Fas receptor null hepatocytes, confirming the role of Fas signalling in JNK activation by DCA (Gupta *et al.* 2004). Moreover, DCA-induced JNK1 signalling appears to be mostly cytotoxic, whereas JNK2 activation by DCA is, rather, cytoprotective in hepatocytes. In fact, DCA-induced apoptosis is enhanced after loss of JNK2 and diminished after JNK1 loss (Qiao *et al.* 2003). Intriguingly, DCA also increases TRAIL receptor expression, as its promoter possesses a bile acid response element in hepatocytes. This increase in TRAIL receptor sensitizes hepatocytes to apoptosis (Higuchi *et al.* 2004).

In addition to activating the death receptor pathway, DCA also activates the mitochondrial pathway of apoptosis in hepatocytes (Rodrigues *et al.* 1998b). In fact, DCA induces mitochondrial perturbation with decreased $\Delta\Psi_m$ and enhanced generation of ROS (**Fig. 1.6.**). In turn, $\Delta\Psi_m$ and ROS might directly participate in apoptosis or occur concomitantly with additional mitochondrial dysfunctions, such as MMP and cytochrome c and Smac/DIABLO release. Furthermore, rats fed a diet containing 0.4% of DCA display increased p53 levels, as well increased levels of BAX at the mitochondrial membrane (Rodrigues *et al.* 1998b). Interestingly, production of ROS by DCA can also activate the insulin receptor (INSR)/PI3K/AKT pathway in primary rodent hepatocytes. This activation can be inhibited by treatment with antioxidants, confirming the role of ROS in activating the AKT pathway. Therefore, in normal conditions, it is possible that DCA-induced ROS may help in the regulation of hepatic glucose and lipid metabolism, through activation of AKT and glycogen synthase. However, during cholestasis, where there is a prolonged exposure to high concentrations of DCA, it induces oxidative stress capable of inhibiting the PI3K/AKT pathway and inducing apoptosis (Fang *et al.* 2004). In fact, at low concentrations, DCA and insulin cooperate to activate the insulin pathway (Han *et al.* 2004). However, when inhibitors or toxics were used to block the insulin pathway, DCA-induced apoptosis significantly increased (Dent *et al.* 2005). Apart from inducing ROS, DCA was also shown to increase cyclin D1 expression, which in turn increases BAX mitochondrial translocation and cytochrome c release. This mechanism appears to be p53-dependent, as p53 silencing abolished

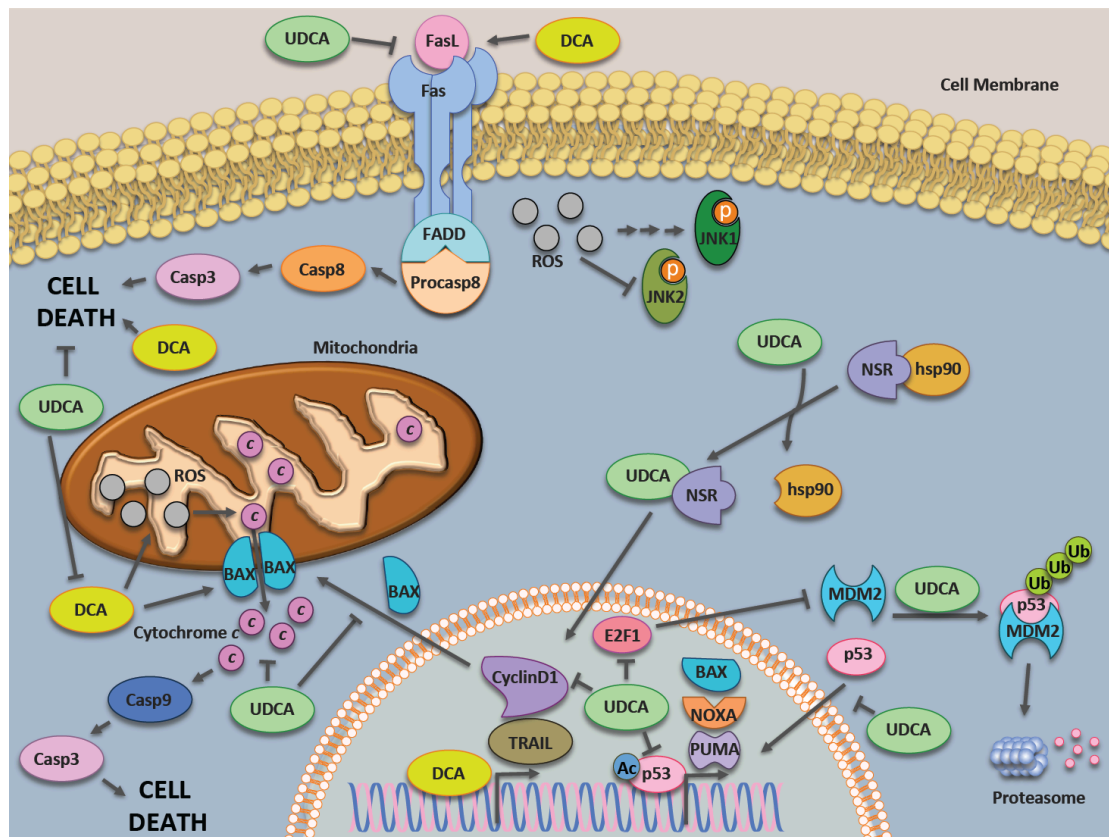


Figure 1.6. Bile acids as inducers or inhibitors of cell death. DCA induces apoptosis in hepatocytes by activating the Fas receptor. Interestingly, DCA-induced activation of the Fas receptor signalling resulted in JNK1 activation that is cytotoxic, whereas DCA-induced JNK2 activation is cytoprotective in hepatocytes. DCA also induces mitochondrial perturbation with decreased $\Delta\Psi_m$ and enhanced ROS generation. In addition, DCA induces an increase in BAX at the mitochondrial membrane, which could result in the formation of BAX homodimers. In addition, DCA increases cyclin D1 expression that increases BAX mitochondrial translocation with cytochrome c release. DCA also increases the expression of TRAIL receptor by acting in its promoter since it has a bile acid response element in hepatocytes. On the other hand, UDCA has a protective effect against MMP and decreased $\Delta\Psi_m$, which reduces ROS production and apoptosis. Interestingly, UDCA completely abolishes mitochondrial changes induced by DCA, such as higher levels of BAX and ROS levels. Importantly, UDCA interacts with nuclear steroid receptors (NSR), leading to NSR/heat shock protein 90 (hsp90) dissociation and nuclear translocation of the UDCA/NSR complex. Once in the nucleus, UDCA inhibits E2F1-mediated apoptosis with decreased MDM2 degradation. MDM2/p53 association increases with decreased BAX, PUMA and NOXA expression and apoptosis. In addition, UDCA modulates p53-induced apoptosis by altering p53 transactivation and DNA binding activity, and preventing its accumulation in the nucleus. Casp3, caspase-3; Casp8, caspase-8; Casp9, caspase-9.

DCA-induced BAX mitochondrial translocation (Castro *et al.* 2007a). p21, an inhibitor of cyclin-dependent kinases and a p53 target could additionally mediate the induction of cyclin D1 by DCA. In fact, p21 potentiates DCA-induced p53-dependent apoptosis in hepatocytes (Qiao *et al.* 2002).

Finally, a very recent study showed that DCA is able to inhibit miR-199a-5p in hepatocytes, which in turn is involved in the regulation of ER stress by repressing inositol-requiring enzyme-1 α and activating transcription factor 6. In normal conditions, ER stress induces JNK with subsequent AP-1 activation, and AP-1 induces miR-199a-5p expression. miR-199a-5p targets and represses the inositol-requiring enzyme-1 α and the activating transcription factor 6, which inhibits ER stress. This feedback loop may protect hepatocytes from sustained ER stress and ultimately shelter the liver from injury and apoptosis. Significantly, inhibitors of miR-199a-5p, including DCA, abolished this protective effect and triggered a more pronounced ER stress response, leading to hepatocyte cell death (Dai *et al.* 2013). Therefore, it is likely that modulation of miRNAs by DCA constitutes a novel mechanism by which this bile acid can induce apoptosis.

1.1.6.2. Inhibition of apoptosis

UDCA is an endogenous secondary bile acid with strong cytoprotective and anti-apoptotic properties in hepatocytes (Amaral *et al.* 2009). In fact, UDCA can protect hepatocytes from the deleterious effects of several cytotoxic agents, including TGF- β 1, anti-Fas antibody, okadaic acid and even DCA (Rodrigues *et al.* 1998a). Specifically, in isolated mitochondria or cultured hepatocytes, UDCA is able to inhibit BAX translocation to the mitochondria and cytochrome c release induced by DCA (**Fig. 1.6.**). In addition, it also impairs MMP and pore formation, further contributing for decreased apoptosis (Rodrigues *et al.* 1999). Importantly, UDCA is also able to completely abolish mitochondrial changes induced by DCA, including BAX translocation to the mitochondria, *in vivo* (Rodrigues *et al.* 1998b).

Additional studies have shown that UDCA also activates the glucocorticoid receptor (GR), a member of the nuclear receptor family that controls metabolism homeostasis, in the absence of a steroid ligand (Kumar *et al.* 1999). This mechanism allows UDCA to block NF- κ B transcription and

the associated inflammatory response (Miura *et al.* 2001). Moreover, by interacting with GR, UDCA itself is translocated to the nucleus, where it may modulate gene expression and, ultimately, apoptosis (Sola *et al.* 2005). Since UDCA is used as a therapeutic drug for patients with cholestatic liver diseases (Beuers *et al.* 1998) and the concentrations of UDCA are elevated in the liver and bile ducts of patients taking UDCA, it is possible that UDCA exerts a strong anti-apoptotic and anti-inflammatory response in the liver of those patients, by interacting with GR (Ewerth *et al.* 1985).

UDCA has been additionally shown to halt TGF- β 1-mediated rat hepatocyte apoptosis, by inhibiting E2F1 activity and preventing MDM2 degradation. By doing so, MDM2/p53 binding is increased, and BAX expression and apoptosis decreased (Sola *et al.* 2003) (**Fig. 1.6.**). Interestingly, GR expression is decreased during TGF- β 1-induced hepatocyte apoptosis and pre-treatment with UDCA increases both GR expression and GR nuclear translocation. Moreover, UDCA-dependent modulation of E2F1/MDM2/p53 pro-apoptotic pathway appears to largely rely on GR, since silencing of GR in hepatocytes leads to a decrease in MDM2 levels and an increase in p53 (Sola *et al.* 2004).

When in the nucleus, UDCA may interact with the chromatin and several transcription factors. In fact, microarray studies revealed that UDCA modulates the expression of at least 96 genes in primary rat hepatocytes, most of them involved in apoptosis and cell cycle regulation (Castro *et al.* 2005). In particular, UDCA was shown to modulate p53-induced apoptosis by altering p53 transactivation and DNA binding activity, while also preventing its accumulation in the nucleus (Amaral *et al.* 2007). In fact, overexpression of p53 in hepatocytes significantly increases apoptosis, which is associated with transactivation of the *bax* gene promoter and, consequently, BAX mitochondrial translocation with cytochrome c release and caspase-3 activation. Of note, pre-incubation of cells with UDCA abrogates all apoptotic changes induced by p53 overexpression as it induces reverse p53 translocation, from the nucleus to the cytosol and, in addition, increases its association with MDM2 (Amaral *et al.* 2007). In a more recent study, it was shown that ubiquitination and functional proteasome degradation are key processes during inhibition of p53-induced apoptosis by UDCA; apart from

increasing MDM2/p53 binding, UDCA also stimulates MDM2-dependent ubiquitination of p53, which inhibits p53 transcriptional activity (Amaral *et al.* 2010) (**Fig. 1.6.**).

Similarly to DCA, very few studies have investigated the cross-talk between UDCA and miRNAs. A study by our group has, however, shown that UDCA modulates miRNA expression during liver regeneration (Castro *et al.* 2010). For instance, UDCA was shown to downregulate miRNA-451 expression, a miRNA that typically inhibits hepatocyte cell proliferation. In addition, UDCA increased the expression of miRNAs belonging to the miR-17–92 cluster, as well as miR-21, which promote cell proliferation while inhibiting apoptosis. Altogether, UDCA may modulate miRNA expression to favour cell proliferation and inhibit apoptosis in the setting of liver regeneration (Castro *et al.* 2010).

The United States Food and Drug Administration has already approved the use UDCA for the treatment of primary biliary cirrhosis. Despite its clinical efficacy in this pathology, the exact mechanisms by which UDCA exerts its cytoprotective actions and, in particular, inhibits hepatocyte apoptosis to improve liver function are still under investigation. Nevertheless, several studies have already demonstrated that UDCA may be clinically relevant for other liver diseases, particularly non-alcoholic fatty liver disease (NAFLD).

1.2. Non-alcoholic fatty liver disease

NAFLD is defined as the accumulation of liver fat exceeding 5% of hepatocytes, in the absence of significant alcohol intake (20 g/day for men and 10 g/day for women), viral infection, or any other specific aetiology of liver disease. In addition, NAFLD encompasses a spectrum from simple steatosis to NASH; whereas simple steatosis is characterized by a relatively favourable clinical course, NASH more frequently progresses to cirrhosis and HCC, leading to liver-related morbidity and mortality (Cohen *et al.* 2011).

To clearly identify and classify NAFLD, it is crucial to perform a histological characterization of a liver biopsy to identify steatosis, liver injury with hepatocyte ballooning, inflammation, and fibrosis. In steatosis, portal inflammation is usually mild or absent and there is almost none ballooning injury. Fibrosis, if present at all, should be limited to mild periportal or

perisinusoidal fibrosis. On the other hand, NASH diagnosis implies the presence of ballooning injury in which cells become enlarged and cytoplasm becomes irregularly clumped with nonvesiculated areas. However, it is possible to identify steatotic vacuoles in ballooned cells, but they should not fill the cytoplasm (Kleiner *et al.* 2012). In addition, the most classic appearing balloon cells will contain Mallory-Denk bodies (Kleiner *et al.* 2012), found near the nucleus, which contain hyperphosphorylated and misfolded cytokeratin 8 and 18 filaments (Omary *et al.* 2009). Early in the disease, fibrosis is mild and inflammation is lobular and associated with steatosis and fibrosis. Latter in the disease, steatosis is more spread all over the liver and inflammation may become more prominent (Chalasani *et al.* 2008). Periportal fibrosis may then occur and extend into the surrounding parenchyma. Eventually, hepatocytes trapped by collagen from fibrosis will undergo apoptosis, and regeneration will create solid nodules of hepatocytes, in such a way that the end stage may resemble cirrhosis. Until now, apoptosis has not been included as a criteria in the classification of NAFLD, but has been shown to be deeply correlated with NAFLD severity (Kleiner *et al.* 2012). Taking all that information into consideration, Kleiner and Brunt proposed a widely used scoring system, named the NAFLD Activity Score (NAS). The score is defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2); thus ranging from 0 to 8. Fibrosis was not included in this classification, as it is less reversible and a result of the disease activity. Briefly, cases with NAS of 0 to 2 are considered not diagnostic of NASH and cases with scores of 5 or higher are diagnosed as NASH. Cases with activity scores of 3 and 4 are considered an early stage of NASH (Kleiner *et al.* 2005).

1.2.1. NAFLD epidemiology

Several epidemiological studies in Europe have shown that the prevalence of NAFLD ranges from 26-40% in patients with chronic liver disease (Bedogni *et al.* 2007; Radu *et al.* 2008; Zois *et al.* 2010). Surprisingly, NAFLD has a high prevalence in obese children; the Raine cohort analyzed 1170 Australian adolescents and 12.8 % had NAFLD (Ayonrinde *et al.* 2011). Nevertheless, the prevalence of NAFLD increases with age, with higher values in males between 40 and 65 years (Sanyal 2002;

Frith *et al.* 2009). The Rotterdam study, which analyzed the prevalence of NAFLD in the elderly (2811 participants from the Netherlands with a mean age 76.4 ± 6.0 years), found that the prevalence of NAFLD was of 35.1%, a value that decreased with advancing age until 24.3%, for participants over 85 years old. This may be explained by the dietary composition or intake, that varies with age (Koehler *et al.* 2012).

The prevalence of NAFLD is increasing worldwide in parallel with the increase in obesity and type 2 diabetes. It is known that NAFLD is particularly prevalent in type 2 diabetic patients (Bellentani *et al.* 2010). This assumption was confirmed by two major European epidemiological studies, which reported prevalence rates of NAFLD of 42.6–69.5% in type 2 diabetic patients (Targher *et al.* 2007; Williamson *et al.* 2011). These studies indicated that approximately 50% of adults in the European Union with type 2 diabetes might eventually develop NAFLD. As for obesity, the Finnish type 2 diabetes survey, which collected information from 2849 patients (45-74 years old), identified that increasing body mass index (BMI) had a greater effect on alanine aminotransferase, aspartate aminotransferase, and NAS (Pajunen *et al.* 2011).

Apart from its significant health issues, NAFLD already represents an important economic cargo for European countries, with patients having up to 26% higher overall health-care costs at a 5-year follow-up (Baumeister *et al.* 2008). As such, NAFLD constitutes a major potential threat to public health both in terms of health and of economical factors, and more suitable forms of treatment are urgently needed.

1.2.2. NAFLD pathogenesis

For a long time, NAFLD pathogenesis was explained on the basis of the “two hits” hypothesis. The first “hit” was the reversible deposit of triacylglycerols in hepatocytes that lead to the development of steatosis and sensitize hepatocytes for the second “hit”. In the second “hit”, free fatty acids (FFAs), mobilized from the adipose tissue and taken up by the liver, are oxidized in the mitochondria of hepatocytes. If the FFAs supply exceeds the liver metabolic capacity, triacylglycerol accumulation occurs leading to oxidative stress- and cytokine- induced liver injury (Day *et al.* 1998). Despite

the “two hits” hypothesis no longer being considered due to its rather simplistic view, when considering more recent data, it is still considered useful in revising the main mechanisms involved in NAFLD pathogenesis.

In fact, other factors have been recently described as playing a key role in NAFLD pathogenesis, including adipokines (adiponectin, leptin, and resistin) and cytokines (such as TNF- α , interleukin-6, and interleukin-1 β), which are secreted by adipocytes or inflammatory cells that infiltrate into the adipose tissue in insulin-resistant states. The adipocytokines exert a cross talk between adipose, skeletal muscle and hepatic tissues (Bugianesi *et al.* 2005b). Interestingly, in NAFLD patients, adiponectin serum levels are decreased, and inversely correlated with hepatic IR and fat content (Bugianesi *et al.* 2005c), as well as the extent of fibrosis in the liver (Musso *et al.* 2005). In addition, high levels of TNF- α and low levels of adiponectin have been proposed as independent predictors of NASH in human patients, since low serum adiponectin is associated with more extensive necroinflammation (Hui *et al.* 2004). On the contrary, resistin serum levels are increased in patients with NASH and a decrease in resistin levels could be positively correlated with improvement of hepatic insulin sensitivity and decreased hepatic fat content (Bajaj *et al.* 2004). Finally, leptin has been positively correlated with hepatic fat content but not with inflammation or fibrosis in NASH patients (Chitturi *et al.* 2002b). Cytokines are involved in the recruitment and activation of Kupffer cells and hepatic stellate cells, which contribute to the progression of NAFLD from steatosis to NASH by increasing inflammation and fibrosis. They can also affect the insulin signalling pathway, thus also playing a role in the development of IR (Ogawa *et al.* 2008).

Under physiological conditions, the liver is not intended to function as a storage unit for fat and, as such, the steady state concentration of hepatic triacylglycerol is low. However, an alteration in the feeding and fasting status comprises a high amount of trafficking of both triacylglycerol and fatty acids into and out of the liver. After a meal, dietary fatty acids are absorbed from the small intestine, assembled into triacylglycerol and incorporated into chylomicrons. These are then secreted into lymphatics and enter the plasma as triacylglycerol-rich chylomicrons. In the plasma, triacylglycerol-rich chylomicrons deliver almost 70% of their fatty acids to the adipose tissue, with

the remaining being taken up by the liver (Donnelly *et al.* 2005). In addition, when carbohydrates are ingested at very high levels, fatty acids are synthesized *de novo* within the liver (Cohen *et al.* 2011). In fact, in overweight non-diabetic subjects, a HFD is able to promote an increase of almost 40% in liver fat content in only 10 days. These fatty acids may be converted into other lipid species, such as glycerolipids, glycerophospholipids and sterols, which can be packaged with apolipoprotein B 100 into very low-density lipoprotein (VLDL) particles and secreted from the liver into the plasma. The reason for this may be that the liver has a limited capacity to store lipids and any excess is either oxidised or released as VLDL (Westerbacka *et al.* 2005). Nevertheless, and importantly, overload of FFA in the liver promotes the expression of pro-inflammatory cytokines, impairs insulin signalling, and stimulates apoptosis induced by death receptors, oxidative stress or ER impairment (Wang *et al.* 2006a; Srivastava *et al.* 2008).

1.2.2.1. Insulin resistance

IR is a common characteristic feature of NAFLD, even when subjects are not obese (Fabbrini *et al.* 2009; Yki-Jarvinen 2010). Several studies have described that insulin resistant subjects with NAFLD have reduced insulin sensitivity not only in the skeletal muscle but also in the liver and adipose tissues (Bugianesi *et al.* 2005a; Gastaldelli *et al.* 2007; Lomonaco *et al.* 2012). It is known that insulin inhibits lipolysis in the adipose tissue; if IR develops, the adipose tissue does not respond to insulin-inhibition of lipolysis increasing the release of FFA to the blood (Arner 2002). In addition, the presence of increased lipolysis and/or increased fat intake, together with increased insulin levels, due to IR, promotes hepatic triacylglycerol synthesis (Gastaldelli *et al.* 2007). Moreover, the increased circulating plasma levels of triacylglycerol and FFAs, due to obesity, contribute to IR in peripheral tissues like the skeletal muscle (Kahn *et al.* 2000). The muscle and the liver take up these FFAs, saturating their oxidative capacity, (Bugianesi *et al.* 2005a) and accumulating it as ectopic fat, mainly as intramyocellular and hepatic lipids, respectively (Hwang *et al.* 2007; Machado *et al.* 2012).

The insulin signalling pathway follows different routes in the different metabolic tissues (**Fig. 1.7., upper panel**). In the adipose and skeletal

muscle tissues, insulin binds to the INSR, thus allowing its tyrosine autophosphorylation and activation. This is followed by the sequential activation IRS-1, PI3K and AKT. This last kinase activates the glucose transporter 4 (GLUT-4), found in cytoplasmic vesicles, and moves to the plasma membrane in order to allow glucose uptake (Saltiel *et al.* 2001). In addition, PI3K activates phosphodiesterases that degrade cAMP and decrease its amounts in the cell. Low levels of cAMP induce PKA activation, which activates lipoprotein lipase (Kitamura *et al.* 1999). In the liver, the engaged INSR activates a different substrate, IRS-2, through which PI3K and AKT phosphorylate and inactivate glycogen synthase kinase-3. Its inactivation then releases glycogen synthase, which increases the synthesis of glycogen (Cross *et al.* 1995; Previs *et al.* 2000).

Both FFA and TNF- α interfere with the insulin signalling pathway and contribute to IR in the adipose and skeletal tissues by inducing IRS-1 Ser312 phosphorylation in humans (or Ser307 in rats), rather than tyrosine phosphorylation (**Fig. 1.7., lower panel**) (Sykietis *et al.* 2001; Gao *et al.* 2002). By doing so, cell glucose uptake is interrupted and glucose is retained in the extracellular space, inducing hyperglycaemia that stimulates the release of insulin from pancreatic β cells (Chitturi *et al.* 2002a). Furthermore, in the adipose tissue, IR also leads to increased cAMP levels, which activate PKA and ultimately lipoprotein lipase, resulting in triacylglycerol degradation and FFAs release into the blood stream (Anthonsen *et al.* 1998). On the other hand, IR in the liver has a different effect. In fact, it decreases glycogen synthesis and increases glycolysis, gluconeogenesis, and the release of glucose into the blood stream. In addition, insulin also stimulates the expression of lipogenic genes, thus determining the synthesis of fatty acids (Lopez *et al.* 1996). Overall, IR leads to a condition in which normal insulin levels fail to achieve a normal metabolic response, upon which higher levels of insulin are needed (Bugianesi *et al.* 2005b).

It appears that TNF- α can also induce IR by modulating JNK activity. JNK activation increases IRS-1 serine phosphorylation and prevents its interaction with INSR. In agreement, disruption of the JNK-binding motif in IRS-1 significantly reduces IRS-1 Ser307 phosphorylation and increases

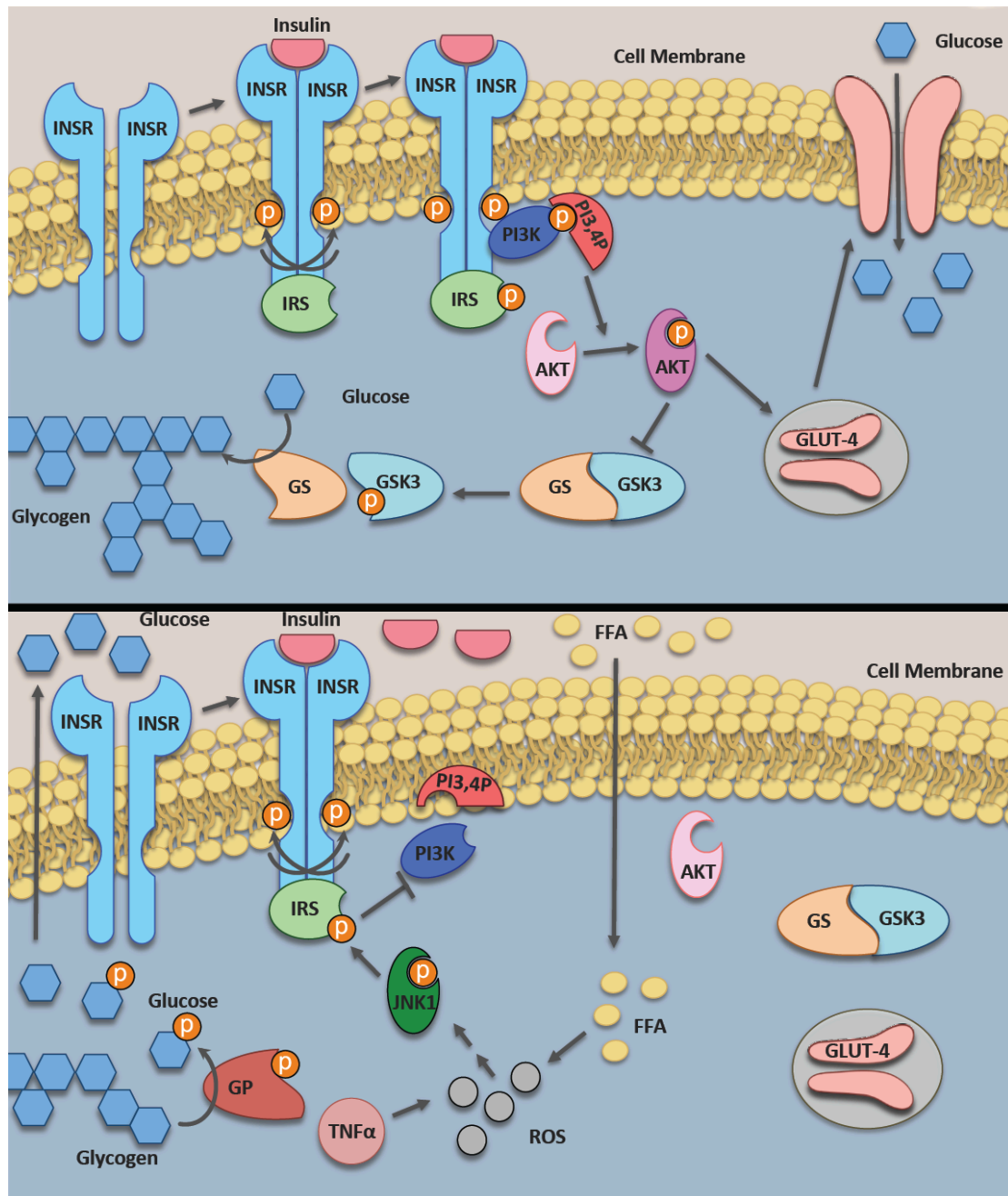


Figure 1.7. The insulin signalling pathway under physiological and insulin resistance conditions. Under physiological conditions (upper panel) insulin binds to INSR, which allows its tyrosine autophosphorylation and activation with latter phosphorylation in tyrosine and activation of IRS. This is followed by the activation of PI3K and AKT. This last kinase activates GLUT-4 that is found in vesicles in the cytoplasm and moves to the plasmatic membrane in order to allow glucose uptake. In addition, AKT phosphorylates and inactivates GSK-3. GSK-3 inactivation then releases glycogen synthase (GS) and allows its activation to increase the glycogen synthesis in the liver. FFA and TNF- α interfere with the insulin signalling pathway and contribute to IR (lower panel). The increase in FFA and TNF- α leads to ROS production that activates JNK1. JNK1 then phosphorylates IRS serine residues rather than tyrosine phosphorylation. This serine phosphorylation is incompatible with

tyrosine phosphorylation. Moreover, IRS serine phosphorylation decreases AKT phosphorylation, which impairs GLUT-4 activation and translocation to the plasma membrane. In addition, this serine phosphorylation decreases glycogen synthesis and increases glycolysis with release of glucose into the blood stream inducing hyperglycaemia that stimulates the release of insulin from pancreatic β cells. GP, glycogen phosphorylase; GSK-3, glycogen synthase kinase-3.

insulin-stimulated tyrosine phosphorylation and AKT activation (Lee *et al.* 2003). Furthermore, acute oxidative stress leads to accumulation of activated JNK in the nucleus, whereas chronic oxidative stress activates JNK in the cytosol (Berdichevsky *et al.* 2010). Due to this differential activation, chronic oxidative stress induces IR and glucose intolerance in muscle and adipose tissues, while acute oxidative stress increases AKT phosphorylation and reverses hyperglycaemia-induced IR, restoring insulin stimulation of glucose uptake (Houstis *et al.* 2006). Apart from JNK, other serine/threonine kinases, such as I κ B Kinase β and protein kinase C- θ , are also capable of phosphorylating IRS-1 Ser307 (Gao *et al.* 2002; Kim *et al.* 2004). In fact, these pro-inflammatory kinases are activated in the skeletal muscle of insulin-resistant subjects, leading to a decrease in AKT phosphorylation and impairing GLUT-4 activation and translocation to the plasma membrane (Bandyopadhyay *et al.* 2005).

1.2.2.2. The metabolic syndrome

Dysfunctional lipid metabolism, obesity and IR contribute to the development of the metabolic syndrome. According to the Third Report of The National Cholesterol Education Program Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults, the metabolic syndrome is characterized by the presence of 3 or more criteria out of 5, namely abdominal obesity (waist circumference in men > 102 cm and in women > 88 cm); serum triacylglycerol $\geq$ 150 mg/dL (1.7 mmol/L); serum high-density lipoprotein cholesterol (HDL) < 40 mg/dL (1 mmol/L) in men and < 50 mg/dL (1.3 mmol/L) in women; hypertension (systolic blood pressure $\geq$ 130 mmHg and/or diastolic blood pressure $\geq$ 85 mmHg); and fasting glucose $\geq$ 110 mg/dL (6.1 mmol/L) (ATPIII 2001). Interestingly, insulin

regulates both triacylglycerol and HDL serum concentrations; following IR, triacylglycerol levels increase, while HDL levels decrease. Despite patients with NAFLD commonly presenting hyperlipidaemia, the co-existence of low HDL levels doubles the risk of NAFLD (Clark *et al.* 2003).

Obesity has been described as being crucial in the development of the metabolic syndrome since, in obese patients, there is an increase in adipose energy storage, which results in lipolysis and increased FFA flux to other tissues, such as liver and skeletal muscle. At the end, triacylglycerol storage in these tissues increases, promoting IR and other adverse effects (Kahn *et al.* 2000). In addition, accumulated visceral adipose tissue in obese patients produces and secretes adipokines, which leads to hypertension (Katagiri *et al.* 2007). Moreover, it has been described that individuals with metabolic syndrome have also higher rate of sodium and water reabsorption at the proximal tubular level, all of that contributing to the development of hypertension (Strazzullo *et al.* 2006).

1.2.2.3. Oxidative stress

Oxidative stress constitutes another important pathogenic factor for NAFLD (**Fig. 1.8.**). Upon an excessive supply of FFAs to the liver, mitochondrial and peroxisomal β -oxidation of FFAs increase. This leads to the formation of ROS, which induce hepatocyte toxicity, inflammation and fibrosis. (Sanyal *et al.* 2001; Bugianesi *et al.* 2004). Moreover, patients with NASH display decreased mitochondrial respiratory chain complexes, leading to inefficient ATP production (Cortez-Pinto *et al.* 1999; Perez-Carreras *et al.* 2003), further stressing the role of oxidative stress and mitochondrial dysfunction in NAFLD. Animal models have also confirmed this theory; in *ob/ob* mice, there is an increase in FFA β -oxidation in the mitochondria and peroxisomes, leading to oxidative stress and, ultimately, to IR (Garcia-Ruiz *et al.* 2006). In fact, lipid peroxidation can inhibit mitochondrial cytochrome c oxidase by forming adducts with this enzyme (Chen *et al.* 2000). In addition, excessive β -oxidation can increase ROS levels, leading to depletion of mitochondrial DNA. This severely affects mitochondrial function, impairing the synthesis of enzymes involved in the mitochondrial respiratory chain, but also

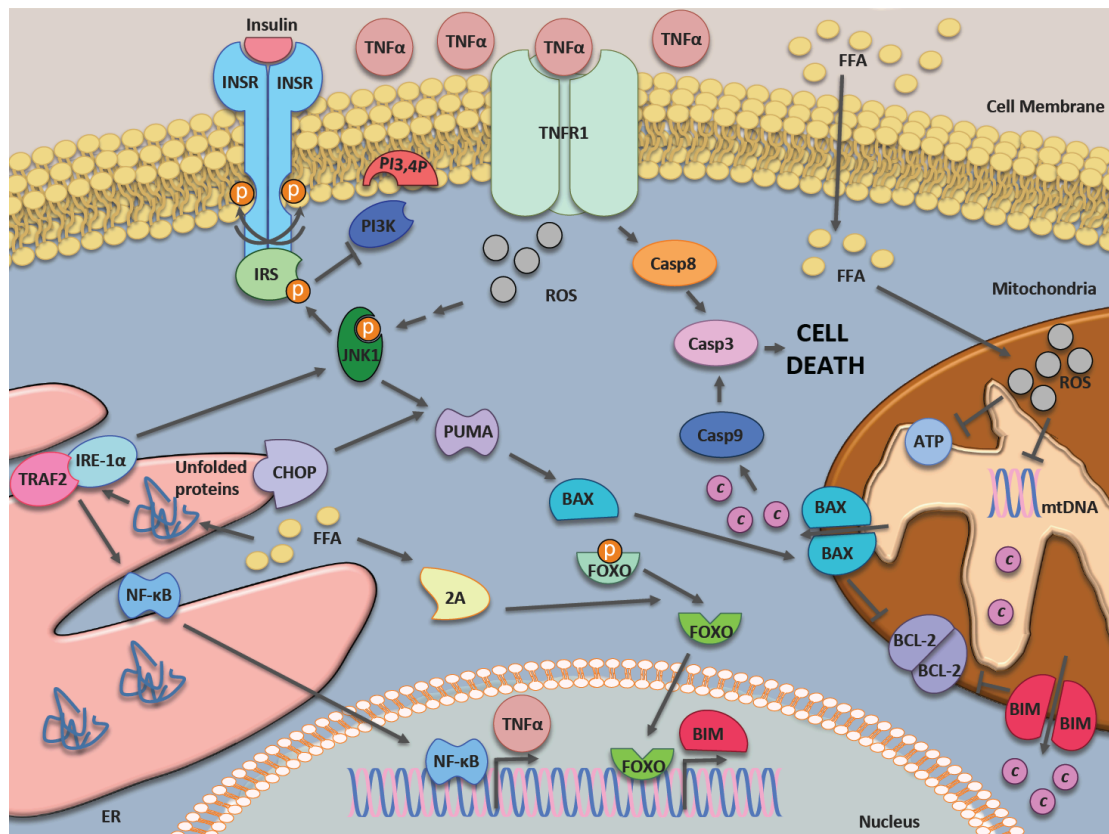


Figure 1.8. Cell death, oxidative stress and endoplasmic reticulum stress interplay.

Excessive supply of FFAs to the liver increases mitochondrial and peroxisomal β -oxidation of FFAs. This increases ROS levels that then decrease mitochondrial respiratory chain complexes leading to inefficient ATP production and depletion in mitochondrial DNA. In addition, patients with NASH have high levels of TNF- α in the blood that also increases ROS levels, which induces JNK1 phosphorylation. Moreover, patients with NAFLD have demonstrated high levels of ER stress due to reduced UPR in the liver. In obesity and NAFLD, there is an inability to resolve ER stress leading to the accumulation of unfolded proteins in the ER lumen, which leads to the suppression of insulin signalling pathway through IRE-1 α /TRAF2-dependent activation of JNK1 and subsequent Ser307 phosphorylation of IRS-1. In addition, JNK1 cooperates with ER stress-induced expression of CHOP to upregulate PUMA. Then, PUMA activates BAX, which translocates to mitochondria, causing mitochondrial dysfunction and caspase-dependent apoptosis. Furthermore, it has been described that toxic saturated FFA, such as palmitic acid, stimulates protein phosphatase 2A activity that promotes FOXO dephosphorylation and activation. One of the transcriptional targets of FOXO3a is BIM that increases FFA-dependent apoptosis. Finally, it has been described that in response to ER stress, IRE1 α can directly interact with TRAF2 to bind to the IKK complex and then activate NF- κ B to induce TNF- α expression leading to the inflammatory response observed in NASH patients. 2A, Phosphatase 2A; CHOP, CAAT/enhancer binding homologous protein; IRE-1 α , inositol-requiring enzyme 1 α ; TRAF2, TNF receptor associated protein 2.

inducing steatosis and liver lesion (Demeilliers *et al.* 2002). In fact, mitochondrial DNA depletion has been described in NASH patients (Rolo *et al.* 2012). Furthermore, FFAs also activate the transcription factor PPAR α , which induces the expression of genes involved in FFA β -oxidation, further increasing ROS production and ultimately inducing IR, lipolysis and FFA uptake by the liver (Kersten *et al.* 1999; Sanyal *et al.* 2001).

In vitro studies have shown that TNF- α is also capable of increasing ROS levels, thus inhibiting enzymes involved in the mitochondrial respiratory chain (Sanchez-Alcazar *et al.* 2003). In fact, a positive correlation between high levels of TNF- α in the blood and a reduced activity of the mitochondrial respiratory chain has been found in NASH patients (Perez-Carreras *et al.* 2003). These high levels of TNF- α are obtained after release from adipose tissue and even from hepatocytes or Kupfer cells (Crespo *et al.* 2001) and, during lipolysis, a result of NF- κ B activation (Cai *et al.* 2005).

In sum, the main consequences of oxidative stress in hepatocytes are lipid peroxidation, cell degeneration, cell death, increased expression of pro-inflammatory cytokines, liver stellate cell activation, and fibrogenesis. As such, oxidative stress is one of the main mechanisms involved in the progression of NAFLD from simple steatosis to NASH and, to more advanced lesions (Chitturi *et al.* 2001; Ferret *et al.* 2001).

1.2.2.4. ER stress

Several studies have demonstrated high levels of ER stress markers and reduced unfolded protein response (UPR) in the liver of NAFLD patients, hinting at a likely role for ER stress during disease pathogenesis (Puri *et al.* 2008) (**Fig. 1.8.**). For instance, in obese humans, ER stress is present in the adipose and liver tissues. Interestingly, after weight loss, ER stress is significantly reduced, as evidenced by the decreased expression of translation initiation factor eIF2 α and JNK phosphorylation, as well as by the UPR activation (Gregor *et al.* 2009). In addition, a rat model with dietary ingestion of FFAs has demonstrated the existence of liver injury, ER stress, and increased caspase-3 activity long before body fat accumulation and circulating TNF- α appear (Wang *et al.* 2006a). In fact, there are well known physical and functional links between the ER and the mitochondria, where

ER-mitochondrial coupling may promote mitochondrial respiration and be influenced by ER stress and UPR activation (Bravo *et al.* 2011). In addition, chronic or severe ER stress may modify cellular metabolism and mitochondrial respiration. Therefore, it is likely that mitochondrial dysfunction in NAFLD also involves ER stress and UPR activation (Wang *et al.* 2011).

When the cell is faced with unfolded proteins, the UPR is crucial to restore ER homeostasis by reducing the protein load entering the ER lumen and increasing the capacity of the ER to fold and degrade proteins (Tsai *et al.* 2010). However, in several diseases including NAFLD, there is an inability to resolve ER stress, leading to activation of the UPR; knockdown of proteins of the UPR pathway results in ER stress and hepatic steatosis as a result of the inability to oxidize FFA (Rutkowski *et al.* 2008). Thus, the UPR appears to have an important role in promoting lipid homeostasis by maintaining ER homeostasis following ER stress. Moreover, the exacerbation of hepatic steatosis in the context of NAFLD might lead to several impairments in the UPR, reducing its ability to resolve ER stress and restore ER homeostasis (Rutkowski *et al.* 2008; Zhang *et al.* 2011). Interestingly, FFAs are assembled into saturated phospholipids that integrate the ER membrane. A high accumulation of saturated phospholipids in the ER membrane, as a result of high FFA levels, decreases the stiffness of the ER membrane, contributing to its loss of functionality and ER stress (Borradaile *et al.* 2006). Also, ER stress ends up activating TNF- α , in a NF- κ B-dependent manner, leading to the inflammatory response observed in NASH patients (Hu *et al.* 2006). In addition, mice deficient in X-box-binding protein-1, a transcription factor that modulates the ER stress response, develop ER stress, hyperactivation of JNK, reduced insulin signalling and systemic IR, as well as type 2 diabetes (Ozcan *et al.* 2004). In fact, activated JNK cooperates with ER stress-induced CAAT/enhancer binding homologous protein to up-regulate PUMA. PUMA then activates BAX, which translocates to mitochondria, causing mitochondrial dysfunction and caspase-dependent apoptosis (Kakisaka *et al.* 2012).

1.2.2.5. Apoptosis

Hepatocyte apoptosis is a crucial event in several liver diseases, including NAFLD (**Fig. 1.8.**). NASH patients display a significant increase in

hepatocyte levels of caspase-3 and -7, as well as apoptosis (Feldstein *et al.* 2003). Interestingly, apoptosis and NF- κ B activity were increased in NASH patients, correlating with inflammation and fibrosis, but not with steatosis (Ribeiro *et al.* 2004). Moreover, the increased expression of death receptors in NAFLD, namely Fas (Feldstein *et al.* 2003), TNF-R1 (Crespo *et al.* 2001; Ribeiro *et al.* 2004), and TRAIL (Farrell *et al.* 2009), all correlate with increased hepatocyte apoptosis. In fact, TNF-R1 knockout mice on a high-carbohydrate diet display less steatosis and liver injury in comparison with wild-type controls (Feldstein *et al.* 2004). Interestingly, TNF-R1, Fas and TRAIL have all been described to activate JNK1 in response to bile acids or FFAs (Higuchi *et al.* 2004; Malhi *et al.* 2006).

Apart from the activation of the death receptor pathway of apoptosis, several NAFLD human and animal studies have demonstrated that hepatocytes also display both structural and functional abnormalities in mitochondria and, as a consequence, mitochondrial-dependent apoptosis. In particular, in the setting of NAFLD, mitochondria become enlarged and develop crystalline inclusions that change its structure, in parallel with enhanced production of ROS, accumulation of lipid peroxides and release of cytochrome *c* into the cytoplasm (Caldwell *et al.* 2004). Regarding proteins of the BCL-2 family, it was found that both BAX and BCL-2 expression is increased in NASH patients. Of note, BCL-2 is not expressed under physiological conditions in hepatocytes, suggesting that its activation during NASH may represent an adaptive phenomenon to resist to apoptosis in response to obesity-related stress. Still, in these same patients, apoptosis was still evident, suggesting that the increased expression of BCL-2 is either insufficient to antagonize apoptosis or prevents a worse scenario, where the levels of apoptosis in liver tissue could further compromise its functions (Ramalho *et al.* 2006).

FFAs and free cholesterol, derived from lipotoxicity, appear to be the main inducers of the mitochondrial pathway of apoptosis in NAFLD (Li *et al.* 2009c). In addition, high levels of free cholesterol also increase hepatocyte susceptibility to TNF and Fas, in nutritional and genetic models of hepatic steatosis. In this context, increased triacylglycerol levels also increase hepatic inflammation and TNF expression by activating NF- κ B (Mari *et al.*

2006). One mechanism by which FFAs induce apoptosis involves stimulation of protein phosphatase 2A that promotes FOXO3a dephosphorylation and activation. One of the transcriptional targets of FOXO3a is the BCL-2-interacting mediator of cell death, which amplifies FFA-dependent apoptosis (Barreiro *et al.* 2007). FFAs also appear to activate JNK1 and phosphorylate c-Jun, leading to increased PUMA transcription and apoptosis during lipogenic hepatocyte injury (Cazanave *et al.* 2009). Moreover, JNK was also described as being activated in a HFD animal model, resulting in BAX activation without changes in BCL-2 or BCL-x_L. This imbalance of pro- and anti-apoptotic proteins of the BCL-2 family further contributes to an increase in hepatocyte apoptosis during NAFLD (Wang *et al.* 2008).

1.2.2.6. miRNAs

Because of their crucial role in lipid metabolism, cell growth and differentiation, apoptosis and inflammation, miRNAs are now being regarded as important regulators of NAFLD pathogenesis (Sayed *et al.* 2011). In fact, miRNAs are differentially expressed in human NASH (Cheung *et al.* 2008), and in genetic (Li *et al.* 2009b) and diet-induced (Pogribny *et al.* 2010) mouse models of NASH. For instance, in human NASH livers, 46 miRNAs were found to be under- or overexpressed, when compared with control samples (Cheung *et al.* 2008) and in *ob/ob* mice, 11 miRNAs were found to be deregulated in NAFLD animals, in comparison with control mice. Among those, eight miRNAs (miR-34a, -31, -103, -107, -194, -335-5p, -221, and -200a) were upregulated and three miRNAs (miR-29c, -451, and -21) were downregulated (Li *et al.* 2009b). These findings support the participation of miRNAs in the pathophysiological processes of NAFLD.

miR-122 is a liver specific miRNA, expressed only in hepatocytes. Due to this specific localization, miR-122 plays a key regulatory role in lipid and fatty acid metabolism, as well as cholesterol accumulation (Hu *et al.* 2012). Recent studies showed that miR-122 null mice accumulate triacylglycerol in the liver resulting from the upregulation of enzymes responsible for triacylglycerol synthesis and storage, regulated by miR-122. In addition, miR-122 knock out mice also display hepatic inflammation, progressive fibrosis and, ultimately, HCC (Hsu *et al.* 2012). miR-122 also regulates

fibrogenic factors, including Kruppel-like factor 6 that targets TGF- β 1. As such, when inhibited, miR-122 leads to activation of hepatic stellate cells and fibrogenic processes. In fact, miR-122 null mice show evidences of steatosis and abnormal levels of VLDL and HDL (Tsai *et al.* 2012). Finally, miR-122, -34a and -16 serum levels in NAFLD patients were found to be significantly higher than in control subjects, with miR-122 and -34a positively correlating with disease severity, liver enzymes levels, fibrosis and inflammation. In addition, miR-122 levels were also positively correlated with serum lipids (Cermelli *et al.* 2011).

miR-33a plays a key role in bile acid synthesis, fatty acid oxidation and cholesterol homeostasis (Gerin *et al.* 2010; Najafi-Shoushtari *et al.* 2010; Allen *et al.* 2012). In particular, when cellular cholesterol levels decrease, miR-33a expression is co-induced with sterol regulatory element-binding protein 2 (SREBP2) mRNA. Interestingly, miR-33a silencing promoted regression of atherosclerosis in mice, which suggests that miR-33a acts in synergy with SREBP2 to regulate cholesterol homeostasis (Rayner *et al.* 2011). A recent study further showed that cholesterol might repress miR-33a levels to increase CYP7A1 expression as well as cholesterol efflux transporters. On the contrary, SREBP2 and miR-33a activation down-regulates cholesterol efflux transporters and bile acid synthesis, which results in increased intrahepatic cholesterol. This mechanism integrates bile acids and cholesterol metabolism to control lipid homeostasis; as such any imbalance in this regulatory circuit will increase hepatocyte lipid content and ultimately induce NAFLD (Li *et al.* 2013).

miR-296-5p was identified as a direct negative regulator of PUMA expression during hepatocyte lipoapoptosis. Interestingly, in NASH patients, hepatic miR-296-5p levels are reduced and associated with increased PUMA expression, confirming an inverse correlation between both in NAFLD patients. In agreement, palmitic acid (PA) also reduces miR-296-5p levels, which further contributes to its inherent lipotoxicity, in part, through overexpression of PUMA (Cazanave *et al.* 2011).

Finally, miR-21 is another miRNA intrinsically related with NAFLD, as it represents a crucial factor affecting PTEN expression during the metabolic syndrome, both in the human liver and in primary human hepatocytes.

Indeed, excessive circulating unsaturated FFAs, such as oleic acid and PA, increase miR-21 expression resulting in PTEN down-regulation and development of steatosis (Vinciguerra *et al.* 2009). Interestingly, liver-specific PTEN knockout mice develop hepatic steatosis, inflammation, and fibrosis, all constituting biochemical and histological evidences of NASH (Watanabe *et al.* 2005).

The exacerbation of inflammation observed at more severe stages of NAFLD increases p53 expression levels (Panasiuk *et al.* 2006) that mediate mitochondrial pathways of apoptosis in several models of NASH (Farrell *et al.* 2009). Interestingly, p53 is a key modulator of steatosis and its regulatory control appears to fail in NAFLD because activated p53 upregulates pro-apoptotic miR-34a (Lee *et al.* 2010). In fact, miR-34a is upregulated in the livers of mice fed with a HFD, as well as in patients with metabolic syndrome and NASH (Cheung *et al.* 2008). Moreover, the main target of miR-34a, SIRT1, has a central role in regulating hepatic fatty acid metabolism; it abolishes ectopic fat accumulation by inducing fatty acid β -oxidation and decreases *de novo* fatty acid synthesis. Accordingly, hepatocyte-specific SIRT1 knockout mice fed with a HFD display significant levels of hepatic steatosis and inflammation (Purushotham *et al.* 2009).

Activation and stabilization of p53 during NAFLD, results in a feed-forward regulatory mechanism to activate downstream genes involved in apoptosis, oxidative stress and IR (Stambolic *et al.* 2001; Derdak *et al.* 2011). miR-34a could be one of such targets. In agreement, pharmacologic inhibition of p53 attenuates hepatic steatosis and liver injury in mice fed a HFD (Derdak *et al.* 2012).

In the context of NAFLD, SIRT1 appears to be the most interesting miR-34a-target, as it may attenuate steatosis by several distinct mechanisms. It can deacetylate and inactivate SREBP1c, a transcriptional regulator of *de novo* fatty acid synthesis (Ponugoti *et al.* 2010). In addition, SIRT1 can also deacetylate and activate PGC1 α (Rodgers *et al.* 2005). Activation of PGC1 α increases the expression of fatty acid oxidizing enzymes and malonyl-CoA decarboxylase, *via* PPAR α (Lee *et al.* 2004; Purushotham *et al.* 2009). Additionally, the interaction between SIRT1 and PPAR α is necessary for efficient PGC1 α activation (Purushotham *et al.* 2009). Finally, SIRT1 can

deacetylate and promote cytosolic translocation of liver kinase B1 (Lan *et al.* 2008) that decreases intrahepatic malonyl-CoA by engaging the liver kinase B1/5'-adenosine monophosphate-activated protein kinase/acetyl-CoA carboxylase signalling cascade (Hou *et al.* 2008). In turn, malonyl-CoA regulates the mitochondrial uptake of long-chain fatty-acyl-CoA molecules for oxidation (Viollet *et al.* 2006), decreasing excessive lipid accumulation (Purushotham *et al.* 2009). SIRT1 also plays a major role in modulating the insulin signalling pathway. First, SIRT1 is able to repress transcription of a negative regulator of the insulin pathway, the protein-tyrosine phosphatase 1B (PTPN1) (Sun *et al.* 2007). PTPN1 acts by dephosphorylating both INSR and IRS (Seely *et al.* 1996; Goldstein *et al.* 2000). In addition, SIRT1 deacetylates IRS-2, thus allowing its phosphorylation and activation (Zhang 2007). Altogether, SIRT1 inhibition will interfere with the insulin signalling pathway at both the protein and mRNA levels. Furthermore, SIRT1 silencing in adipocytes inhibits insulin-stimulated glucose uptake and GLUT4 translocation, tyrosine phosphorylation of IRS-1, and phosphorylation of AKT and ERKs, accompanied by increased phosphorylation of JNK and serine phosphorylation of IRS-1. By contrast, SIRT1 activation increases glucose uptake and insulin signalling and decreases serine phosphorylation of IRS-1 (Yoshizaki *et al.* 2009).

Given these observations, it may be hypothesized that deregulation of p53 in the liver during obesity may favour excess accumulation of lipids by activating miRNA-34a and decreasing SIRT1 expression.

1.2.3. Current therapeutic options for patients with NAFLD

The main recommendation for treating NAFLD patients consists in treating both the liver disease and the associated metabolic co-morbidities, including obesity, hyperlipidemia, IR and type 2 diabetes mellitus. Because patients with steatosis have a good prognosis with lifestyle changes, from a liver standpoint, treatments aimed at improving liver disease should be limited to those with NASH (Chalasani *et al.* 2012). Still, a growing number of studies show that several different agents may be important in managing and treating patients with NAFLD.

The first therapeutic option for obese patients with NAFLD refers to

lifestyle changes. Because NAFLD is strongly correlated with obesity, either due to the lack of physical exercise and/or a deficient diet, lifestyle changes have been proposed as a strategy to manage NAFLD (Chalasani *et al.* 2012). In fact, it was shown that weight loss by dietary intervention and/or exercise is able to decrease liver enzymes (St George *et al.* 2009), reduce liver fat (Haufe *et al.* 2011), and improve liver histology (Promrat *et al.* 2010). When lifestyle changes prove not to be sufficient, bariatric surgery is seen as a viable hypothesis for obese patients. Bariatric surgery, aiming to stimulate weight loss, may be performed by using a gastric band (reducing the size of the stomach), sleeve gastrectomy (removal of a portion of the stomach) or gastric bypass surgery (resecting and re-routing the small intestines to a small stomach pouch) (Green 2012). Several studies have already shown that bariatric surgery is able to improve liver steatosis and IR. However, the lack of randomised clinical trials and quasi-randomised clinical studies does not allow for a definitive assessment on the benefits and harms of bariatric surgery, as a therapeutic approach for patients with NASH (Chavez-Tapia *et al.* 2010). Due to this controversy, it is still premature to establish the bariatric surgery as the first line of treatment for NAFLD. However it is not contraindicated in otherwise eligible obese individuals with NAFLD or NASH, without established cirrhosis (Chalasani *et al.* 2012).

Because NAFLD is associated with IR, the role of metformin, an antidiabetic drug used in overweight and obese patients with normal kidney function, to improve aminotransferases and liver histology in patients with NASH has been explored. Although some studies demonstrated a reduction in IR and aminotransferase levels, they failed to show a significant improvement in liver histology (Nair *et al.* 2004). As such, metformin is not recommended as a specific treatment for liver disease in adults with NASH (Chalasani *et al.* 2012). Other antidiabetic drugs, namely thiazolidinediones and PPAR γ agonists pioglitazone and rosiglitazone, have also been tested regarding their effects on aminotransferases and liver histology in adults with NASH. Although positive results were obtained for rosiglitazone, the results also shown that it also significantly increased the risk of myocardial infarction and the risk of death from cardiovascular causes (Nissen *et al.* 2007). In fact, rosiglitazone is no longer marketed in Europe and its use is highly restricted in

the United States (Chalasani *et al.* 2012). On the other hand, a study using pioglitazone in NAFLD patients resulted in steatosis, inflammation, and hepatocellular ballooning reductions, as well as improvements in IR and aminotransferases, although it did not improve the histological features of NASH (Sanyal *et al.* 2010). Therefore, in contrast with rosiglitazone, pioglitazone can be used to treat NASH; however, it should be noted that majority of the patients who participated in this clinical trial were non-diabetic and that the long-term safety and efficacy of pioglitazone in patients with NASH is still not established (Chalasani *et al.* 2012).

Because NAFLD is associated with dyslipidaemia, statins have also been evaluated as a possible new therapeutic option. Long-term statin treatment of NAFLD patients was shown to slightly reduce aminotransferases activity, theoretically contributing to a decrease in cardiovascular-related and liver-related morbidity and mortality (Athyros *et al.* 2010). However, and on the contrary, a more recent study showed that the use of statins increased aminotransferases in NAFLD patients (Chalasani *et al.* 2012). Given the lack of evidence to show that patients with NAFLD are at increased risk for serious drug-induced liver injury from statins, statins may be used to treat dyslipidemia in patients with NAFLD but should not be used to specifically treat NASH (Chalasani *et al.* 2012).

The use of antioxidants to treat NAFLD, in particular, vitamin E, has also been explored. A study analysed the effect of treating NAFLD patients with vitamin E for 96 weeks. Vitamin E treatment improved histological features of NASH, in particular, steatosis, hepatocellular ballooning, lobular inflammation and NAS, although it showed no improvement in IR (Sanyal *et al.* 2010). In another study, analysing vitamin E treatment for 24 weeks, patients with biopsy-proven NASH improved fasting insulin values and decreased aminotransferases and IR levels. However, serum cholesterol, triacylglycerol, fasting blood glucose levels and BMI remained unchanged. In addition, steatosis decreased without changes in necroinflammation and fibrosis (Yakaryilmaz *et al.* 2007). A multi-approach study analysed the co-treatment of vitamin E and vitamin C for 6 months in patients with NASH together with weight-loss counselling and encouragement to follow a low-fat diet. Indeed, fibrosis decreased after the treatment; however, there was no

improvement in necroinflammation and transaminases levels (Harrison *et al.* 2003). In addition, dietary supplementation of vitamin E has been shown to significantly increase the risk of prostate cancer among healthy men (Klein *et al.* 2011). Even so, vitamin E may be considered as a pharmacotherapy in non-diabetic adult biopsy-proven NASH patients, although until further data supporting its effectiveness becomes available, it should not be recommended to treat NASH in diabetic patients, NAFLD without liver biopsy, NASH cirrhosis, or cryptogenic cirrhosis (Chalasani *et al.* 2012).

Altogether, it is clearly urgent to find novel, more effective and with less side effects therapies for treating NAFLD.

Several preclinical studies have demonstrated that UDCA can act as an hepatoprotector, immunomodulator and anti-apoptotic agent, that could interfere with the progression NAFLD (Ratziu 2012). Still, the therapeutic usefulness of UDCA has already been explored in a small number of NAFLD clinical trials, and much remains uncertain. As such, UDCA is still not used in the clinical practice, as a NAFLD therapeutic agent (Liechti *et al.* 2012). The first clinical trial included 166 NASH patients treated with UDCA (13-15 mg/kg/day) for 2 years. The UDCA-treated group did not show any signs of histological improvement (steatosis, fibrosis, necroinflammation, Mallory-Denk bodies, and hepatocellular ballooning) when compared with the placebo-treated group (Lindor *et al.* 2004). In another clinical trial, 48 patients with NASH were subjected to treatment with UDCA (12-15 mg/Kg/day) in combination with 800 IU vitamin E for 2 years. Although UDCA alone showed no evidences of improvement, co-treatment of UDCA and vitamin E ameliorated liver transaminase levels and steatosis (Dufour *et al.* 2006). A more recent clinical trial using high doses of UDCA (23-28 mg/Kg/day) for 18 months also failed to provide evidences that UDCA could improve transaminase levels and histological parameters in NASH patients, compared with placebo-treated patients. However, UDCA did show some improvement in liver lobular inflammation (Leuschner *et al.* 2010). In fact, the most recent clinical trial available showed that higher doses of UDCA (28-35 mg/Kg/day for 12 months) were able to improve transaminase levels in NASH patients, as well as several metabolic parameters including glycaemia and serum insulin. Further, UDCA also improved IR and serum fibrosis markers.

Unfortunately, in this clinical trial, no histological evaluations were performed (Ratziu *et al.* 2011). In addition, some argue that, in all these studies, the number of patients was too small, the treatment time was too short, or a real control group was missing (at least in some cases) (Ratziu 2012). Therefore, the controversy about the effective role of UDCA as a therapeutic agent for NASH and NAFLD remains.

Interestingly, the taurine-conjugated form of UDCA, tauroursodeoxycholic acid (TUDCA), has been described as an efficient chaperone that reduces ER stress. Because ER stress is linked to IR, steatosis and the overall metabolic syndrome, studies were performed where TUDCA was administered to obese and diabetic mice, resulting in normalization of hyperglycemia, systemic insulin sensitivity, steatosis, and enhancement of insulin actions in hepatic, skeletal muscle, and adipose tissues (Ozcan *et al.* 2006). In addition, TUDCA administration *per se* in *ob/ob* mice down-regulates the expression of genes involved in *de novo* lipogenesis, thus reducing hepatic fat content. Still, it failed to improve glucose homeostasis (Yang *et al.* 2010).

In order to improve the efficacy of UDCA for diseases like NAFLD, a side chain-modified derivative of UDCA has been developed, to originate 24-norursodeoxycholic acid (norUDCA), with distinct pharmacological and physiological properties, as compared with UDCA. It is unable to conjugate with taurine and glycine, has more extensive cholehepatic shunting, does not accumulate in the enterohepatic circulation and does not cause hepatotoxicity (Cohen *et al.* 1986). In an animal model of liver inflammation and cholestasis, norUDCA reversed liver injury, fibrosis and decreased serum lipids. In addition, norUDCA reduces triacylglycerol catabolism and FFA utilization, which favours the FFA incorporation into triacylglycerol (Moustafa *et al.* 2012). Interestingly, in a mouse model of steatosis, norUDCA appeared to improve liver damage by down-regulating fatty acid synthesis, increasing cholesterol efflux from the liver to the blood, as well as bile acid synthesis. Importantly, bile acid destoxification and flow might decrease cell death, inflammation and ameliorate liver fibrosis (Beraza *et al.* 2011). Still, more studies are needed in order to ascertain the potential therapeutic of UDCA and its derivatives in NAFLD pathogenesis. The derivatives seem to be very promising therapeutic

tools; however, clinical trials are essential to confirm their therapeutic relevance.

OBJECTIVES

The studies presented in this thesis were driven by the ambition to better understand the role of apoptosis and miRNAs in NAFLD pathogenesis, and contribute to the knowledge on novel targets for therapeutic intervention in NAFLD. To accomplish this aim, we designed three major objectives. Because it was recently established that insulin resistance and apoptosis are two key events during NAFLD pathogenesis, our first goal was to determine the differences in insulin and apoptosis signalling pathways, in insulin sensitive organs, in patients with different degrees of NAFLD. In addition, taking into account that the miR-34a/SIRT1/p53 is a known pro-apoptotic pathway and that SIRT1 is a critical regulator of metabolism and insulin signalling pathways, in our second goal we sought to understand whether the miR-34a/SIRT1/p53 pathway could play a role in NAFLD pathogenesis and whether it could be modulated by UDCA. Finally, because levels of cytotoxic DCA are augmented in NASH patients and UDCA inhibits DCA-induced hepatocyte cell death, we further explored the role of DCA in modulating the miR-34a/SIRT1/p53 pro-apoptotic pathway *in vitro* and *in vivo*.

The specific questions addressed in this thesis are:

1. How do insulin signalling and apoptosis correlate with NAFLD severity? Is JNK a mechanistic link?
2. Does the miR-34a/SIRT1/p53 pro-apoptotic pathway contribute to hepatocyte apoptosis and NAFLD severity? Are UDCA and DCA key endogenous modulators?
3. Do p53 and JNK act as upstream regulators of the miR-34a/SIRT1/p53 pro-apoptotic pathway? If so, can they be targeted by endogenous bile acids?

Taken together, the results presented herein provide a significant contribution to our understanding on the molecular mechanisms governing NAFLD progression, as well as on the effects of bile acids and miRNAs on

Objectives

cell function and injury during NAFLD pathogenesis, thus putting in perspective potential therapeutic options.

Apoptosis and Insulin Resistance in Liver and Peripheral Tissues of Morbid Obese Patients is Associated with Different Stages of Non-alcoholic Fatty Liver Disease

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2.1. Abstract

Background and Aims: Non-alcoholic fatty liver disease (NAFLD) is associated with insulin resistance (IR) and characterized by different degrees of hepatic lesion. Its pathogenesis and correlation with apoptosis and IR in insulin-target tissues remains incompletely understood. Our aim was to investigate how insulin signalling, caspase activation and apoptosis, correlate with different NAFLD stages in the liver and/or in the muscle and visceral adipose tissues.

Methods: Liver, muscle and adipose tissue biopsies from 26 morbid obese patients undergoing bariatric surgery were grouped according to the Kleiner/Brunt scoring system in simple steatosis, less severe and more severe non-alcoholic steatohepatitis (NASH). Apoptosis was assessed by DNA fragmentation, and caspase-2 and -3 activation. Insulin signalling and c-Jun NH₂-terminal kinase (JNK) proteins were evaluated by Western blot.

Results: Caspase-3 and -2 activation, and DNA fragmentation were markedly increased in the liver of patients with severe NASH *versus* simple steatosis ($p < 0.01$). The muscle tissue, and to a less extent the liver, showed decreased tyrosine phosphorylated insulin receptor and insulin receptor substrate in severe NASH, comparing with simple steatosis ($p < 0.01$, muscle; $p < 0.05$, liver). Concomitantly, AKT phosphorylation decreased in muscle, liver and visceral adipose tissues in severe NASH (at least $p < 0.05$). Finally, JNK phosphorylation was significantly increased in NASH, compared with simple steatosis, both in muscle ($p < 0.01$) and liver ($p < 0.05$).

Conclusions: Our results demonstrate a link between apoptosis, IR and different NAFLD stages, where both JNK and caspase-2 may play a key regulatory role.

Keywords

Cell death; caspase-2; Insulin resistance; JNK; non-alcoholic steatohepatitis; obesity; steatosis

2.2. Introduction

Non-alcoholic fatty liver disease (NAFLD) comprises a range of liver lesions from simple steatosis to inflammation, steatohepatitis and cirrhosis. While simple steatosis is usually benign, non-alcoholic steatohepatitis (NASH) is characterized by chronic hepatocyte injury and inflammation and/or fibrosis, which can lead to advanced fibrosis, cirrhosis, hepatocellular carcinoma, and liver-related death (Vanni *et al.* 2010). Increased fat accumulation appears to be the primary factor leading to insulin resistance (IR) and imbalanced lipid synthesis and oxidation, culminating in hepatic steatosis. In addition, recent data suggest that mechanisms driving disease progression can also induce steatosis, which should therefore be considered part of the liver's early "adaptive" response to stress (Marchesini *et al.* 2001). Further stress signals, including oxidative stress and lipid peroxidation, lead to hepatocyte injury and inflammation, likely playing an important role in the transition of simple steatosis to NASH (Feldstein *et al.* 2004). Interestingly, cell death by apoptosis may also constitute an important component of disease progression (Kusminski *et al.* 2009). In fact, we and others have already demonstrated that hepatocyte apoptosis is a prominent pathological feature in patients with NASH and NAFLD (Ribeiro *et al.* 2004; Puri *et al.* 2008). Nevertheless, exact mechanisms of hepatocyte apoptosis in NASH and underlying factors of NAFLD progression and pathogenesis remain incompletely understood.

Individuals with NAFLD typically display IR at the level of the muscle (reduced glucose uptake), liver (impaired suppression of hepatic glucose production) and adipose tissue (high lipolytic rates and increased circulating non-esterified fatty acids (NEFAs)) (Gastaldelli *et al.* 2010). In particular, by increasing visceral adiposity, obesity leads to increased plasma concentration of NEFAs, thus being strongly associated with both hepatic and muscular IR and fat deposition (Gastaldelli *et al.* 2007). The pathways by which increased visceral adiposity leads to IR are not fully understood. Some authors suggest that lipolysis induced by tumour necrosis factor alpha (TNF- α) and interleukin 6, resulting in inhibition of insulin receptor substrate (IRS) and increased plasma NEFAs, may represent a major mechanism (Hotamisligil *et al.* 1996; Bruce *et al.* 2004). Still, the exact contribution of TNF- α in IR remains scattered, as other studies suggest that *in vivo* TNF- α neutralization has no

effects on insulin sensitivity (Ofei *et al.* 1996). Therefore, the absence of TNF- α might only partially protect against obesity-induced insulin resistance (Ventre *et al.* 1997).

In turn, it is well established that these and other pro-inflammatory cytokines are produced by M1 macrophages that have infiltrated in the adipose tissue (Schenk *et al.* 2008). These results were then expanded to show that c-Jun NH₂-terminal kinase 1 (JNK1) activation in adipocytes is also responsible for inducing interleukin 6, either directly or by activating macrophages (Sabio *et al.* 2008). In fact, it is now generally established that JNK1 is central in obesity-induced IR, although JNK2 might also play a contributing role (Tuncman *et al.* 2006).

Interestingly, JNK1 has opposite roles in the pathogenesis of hepatic steatosis, in a tissue-specific manner (Sabio *et al.* 2009). Studies in type II diabetes patients suggest that intramyocellular lipid accumulation and muscle IR precede the development of hepatic IR and type 2 diabetes (Belfort *et al.* 2005). Other studies have shown that hepatic steatosis may induce hepatic IR, or that the initial IR site is located in the periphery, probably in the skeletal muscle, followed by the liver, which further increases the degree of IR (Bugianesi *et al.* 2005b).

The purpose of this study was to evaluate and identify particular mechanisms of liver cell apoptosis at different stages of NAFLD, and how they might correlate with insulin signalling cascade activation, using three major insulin-target tissues, namely the liver, muscle and adipose tissues. Finally, we also sought to investigate whether JNK activation might represent an additional mechanism linking apoptosis and IR, at different NAFLD stages.

2.3. Materials and Methods

2.3.1. Patients

This study included consecutive patients undergoing bariatric surgery for morbid obesity, defined either as body mass index (BMI) superior to 40 Kg/m², or BMI superior to 35 Kg/m² with major associated complications. All fulfilled the inclusion criteria and accepted to participate in the study, with written informed consent. Exclusion criteria were the presence of other

causes for liver disease, including alcohol ingestion superior to 20 g/day, chronic viral infection B and/or C, α -1 anti-trypsin deficiency, primary biliary cirrhosis, hemochromatosis, autoimmune hepatitis, and Wilson's disease as well as the use of anti-obesity, anti-diabetic, and/or lipid lowering pharmacological treatments. The study protocol conformed to the Ethical Guidelines of the 1975 Declaration of Helsinki, revised in 2000, as reflected in *a priori* approval by the Hospital de Santa Maria Human Ethics Committee.

2.3.2. Clinical data, laboratory assays and histology

Evaluations were performed in the morning before the surgical procedure. Demographic data were obtained via structured interviews and questionnaires, evaluating age, gender, personal and family history, associated pathology, arterial systolic and diastolic pressure, alcohol habits referring to current and past consumption, cigarette consumption, and pharmacological treatments. Anthropometric data included weight, height, abdominal circumference, waist-to-hip ratio, and BMI.

Laboratory assays included total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triacylglycerol, aspartate aminotransferase, alanine aminotransferase, γ -glutamyltranspeptidase, fasting serum glucose, fasting insulin, and serum glucose 2 hours after 75 g glucose (OGTT). For evaluation of insulin sensitivity, the homeostasis model assessment of insulin resistance (HOMA-IR) test was performed.

Biopsies from liver, muscle and visceral adipose tissues were obtained during bariatric surgery. Tissue samples were immediately flash-frozen in liquid nitrogen and kept at -80°C . Liver biopsies were processed conventionally for diagnostic purposes, and for histological grading and staging. Paraffin-embedded sections were stained with haematoxylin and eosin. Sweet and Gordon's methods were used for reticulin, chromotrope aniline blue for connective tissue and Perl's Prussian blue for iron. Liver histology was scored according to NAFLD histology scoring system. The severity of steatosis was graded from 0 to 3, inflammation from 0 to 3, hepatocellular ballooning from 0 to 2, and fibrosis was staged from 0 to 4 (**Table 2.1.**). Each liver specimen was assessed for the presence or absence

of NASH by pattern recognition and for the NAFLD activity score (NAS), which is the sum of steatosis, inflammation and hepatocyte ballooning (Neuschwander-Tetri *et al.* 2003; Kleiner *et al.* 2005). Eleven patients were classified as NASH and 15 patients as simple steatosis. NASH patients were further divided in patients with NASH score ≥ 3 and < 5 (group 1 or less severe NASH; $n = 5$), and patients with NASH score ≥ 5 (group 2 or more severe NASH; $n = 6$).

Table 2.1. Histological data of the patient population.

Variable	Steatosis ($n=15$)	NASH 1 ($n=5$)	NASH 2 ($n=6$)
Steatosis grade			
0 (none)	1 (7)	0 (0)	0 (0)
1 (mild)	6 (40)	1 (20)	0 (0)
2 (moderate)	5 (33)	4 (80)	1 (17)
3 (severe)	3 (20)	0 (0)	5 (83)
Lobular inflammation			
0 (none)	14 (93)	0 (0)	0 (0)
1 (<2) ^a	1 (7)	0 (0)	1 (17)
2 (2–4) ^a	0 (0)	5 (100)	5 (83)
3 (>4) ^a	0 (0)	0 (0)	0 (0)
Portal inflammation score			
None/mild	5 (33)	2 (40)	0 (0)
More than mild	10 (67)	3 (60)	6 (100)
Hepatocellular ballooning			
None	15 (100)	5 (100)	3 (50)
Few/many	0 (0)	0 (0)	3 (50)
Fibrosis stage			
0 (none)	3 (20)	0 (0)	0 (0)
1 (mild)	8 (53)	1 (20)	3 (49)
2 (moderate)	4 (27)	4 (80)	1 (17)
3 (bridging)	0 (0)	0 (0)	1 (17)
4 (cirrhosis)	0 (0)	0 (0)	1 (17)
NAFLD activity score			
Score <3	15 (100)	0 (0)	0 (0)
Score ≥ 3 and <5	0 (0)	5 (100)	0 (0)
Score ≥ 5	0 (0)	0 (0)	6 (100)

Data are presented as n (%). ^a Under x200 magnification.

2.3.3. Immunoblotting

Total protein extracts were subjected to SDS-PAGE electrophoresis (Ramalho *et al.* 2006). Blots were incubated with primary rabbit polyclonal antibodies against INSR, pINSR Tyr^{1162/1163}, IRS-1, pIRS-1 Tyr⁶³², IRS-2, caspase-3, and caspase-2 or primary mouse monoclonal antibodies reactive to AKT, pAKT Ser⁴⁷³, JNK, and pJNK Thr¹⁸³/Tyr¹⁸⁵ (Santa Cruz Biotechnology, Santa Cruz, CA), and finally with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase and β -actin were used as loading control for muscle tissue and for liver and adipose tissues, respectively. Membranes were processed for protein detection using Super Signal substrate (Pierce, Rockford, IL).

2.3.4. Immunoprecipitation

One mg total liver protein samples were incubated with 1 μ g of IRS-2 antibody (Santa Cruz Biotechnology), overnight at 4°C. Samples with antibody were added to Ezview Red Protein G Affinity Gel (Sigma-Aldrich Corp., St. Louis, MO), and incubated overnight at 4°C. Finally, this mixture was denaturated for 10 min at 95°C and phosphorylation of IRS-2 determined by Western blot analysis. The blots were incubated overnight at 4°C with a mouse monoclonal antiphosphotyrosine antibody coupled with horseradish peroxidase (Millipore, Temecula, CA). IRS-2 expression was determined in the same membrane after stripping off the immune complex for the detection of phosphotyrosine.

2.3.5. Caspase activity

General caspase-3-, -6-, -8-, and -9-like activities were evaluated by enzymatic cleavage of the chromophore p-nitroanilide from the substrate N-Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Caspase-3), N-Acetyl-Val-Glu-Ile-Asp-p-nitroanilide (Caspase-6), N-Acetyl-Ile-Glu-Pro-Asp-p-nitroanilide (Caspase-8), and N-Acetyl-Leu-Glu-His-Asp-p-nitroanilide (Caspase-9) (Sigma-Aldrich Corp.). The proteolytic reaction was carried out using 50 μ g total protein and 50 μ M substrate. The reaction mixtures were incubated at

37°C for 1 h, and the formation of p-nitroanilide was measured at 405 nm, using a 96-well plate reader.

2.3.6. Measurement of apoptosis

Transferase mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining was performed according to the manufacturer's instructions (Serologicals Corp., Norcross, GA). Specimens were examined using a bright-field microscope Leica DM2500 (Leica Microsystems, Portugal) and data expressed as the number of TUNEL-positive cells/high-power field (x400).

2.3.7. Densitometry and statistical analysis

The relative intensities of protein bands were analysed using the Quantity One Version 4.6 densitometric analysis program (Bio-Rad Laboratories). Statistical analysis was performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

2.4. Results

2.4.1. Clinical, anthropometric, and biochemical data

Among the patients fulfilling the inclusion criteria and accepting to participate in this study, 26 were selected after undisputed classification of their liver biopsies as either steatosis or NASH. This representative group was then divided into simple steatosis and NASH (groups 1 and 2), as described in Materials and Methods (**Table 2.1.**). The clinical data and characteristics of patients are summarized in **Table 2.2.** It is important to acknowledge that simple steatosis and NASH exhibit age and gender differences in both prevalence and severity. In this regard, patients presented a similar age and BMI across the whole NAFLD spectrum, although the female gender was not equally represented. Patients with NASH presented much higher TC, LDL and triacylglycerol levels, compared with those with

Table 2.2. Clinical, anthropometric and biological data of the patient population.

Variable	Steatosis (n=15)	NASH 1 (n=5)	NASH 2 (n=6)
Age (years)	44±3	47±4	45±8
Men (n)	5	0	2
Women (n)	10	5	4
BMI (kg/m ²)	45±8	47±7	46±8
WHR, women	0.959±0.223	1.010±0.024	0.928±0.307
WHR, men	1.021±0.123	—	1.011±0.027
Systolic BP (mmHg)	133.5±5.1	140.8±4.8	133.5±3.9
Diastolic BP (mmHg)	81.6±2.9	73.0±8.4	74.8±5.7
Total cholesterol (mmol/l)	4.7±0.3	4.9±0.3	5.5±0.3 ^a
HDL-cholesterol (mmol/l)	1.4±0.1	1.1±0.1 ^a	1.1±0.1 ^a
LDL-cholesterol (mmol/l)	2.7±0.2	3.2±0.2	3.4±0.4 ^a
Triacylglycerol (mmol/l)	1.1±0.1	1.4±0.1 ^a	1.9±0.2 ^a
Fasting glucose (mmol/l)	5.0±0.8	5.2±1.0	6.2±1.5
Fasting insulin (pmol/l)	79±14	184±34 ^a	131±29
2-h OGTT glucose (mmol/l)	6.4±0.5	6.9±0.5	8.5±0.9 ^{a,c}
HOMA-IR	1.5±0.2	2.6±0.5 ^b	3.3±0.5 ^b
Total bilirubin (mmol/l)	14.2±7.5	11.1±2.6	15.3±10.4
AST, women (U/l)	19±5	30±15	28±9
AST, men (U/l)	22±8	—	41±1 ^a
ALT, women (U/l)	20±4	23±5	42±12 ^{a,c}
ALT, men (U/l)	35±16	—	56±11
γ-GT, women (U/l)	14±5	30±8	19±3
γ-GT, men (U/l)	37±24	—	60±30

Data are present as mean ± SD. ^a $p < 0.05$ and ^b $p < 0.01$ for difference from steatosis; ^c $p < 0.05$ for difference from NASH 1. ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyltranspeptidase.

simple steatosis ($p < 0.05$). Patients with less severe NASH had TC, LDL and triacylglycerol values between those of simple steatosis and more severe NASH. HDL levels were significantly lower in patients with NASH, when compared with those with simple steatosis ($p < 0.05$). Fasting glucose and 2-hour OGTT glucose levels were significantly increased in patients with more severe NASH compared with those with simple steatosis and less severe NASH ($p < 0.05$), which may indicate a lower glucose tolerance and higher IR in these patients. In fact, this was also supported by the HOMA-IR test that showed a progressive increase from simple steatosis, to less severe and to more severe NASH ($p < 0.01$), thus suggesting that higher IR might correlate with more severe NAFLD stages.

2.4.2. Caspase-2, -3 and apoptosis increases in the liver of patients with NASH

Hepatocyte apoptosis is a key feature of NASH and it is possible that it may correlate with NAFLD severity (Feldstein *et al.* 2003; Malhi *et al.* 2008). In the present study, we showed that active caspase-2 was 2.9- and 3.5-fold increased in more severe NASH, compared with less severe NASH and steatosis, respectively ($p < 0.01$) (**Fig. 2.1.a**). Similarly, executioner active caspase-3 was increased by 3.4-fold in more severe NASH ($p < 0.01$) (**Fig. 2.1.b**). Caspase-3-like activity assays further demonstrated increasing activity from simple steatosis to more severe NASH ($p < 0.05$) (**Fig. 2.1.c**). Other caspases, such as caspase-6, -8, and -9 were not significantly altered between groups (data not shown), further underlying the likely significance of elevated active caspase-3 and, particularly, active caspase-2 during NAFLD. Finally, end-stage apoptosis was evaluated using the TUNEL assay in liver tissue sections. According with the previous results, the number of TUNEL-positive cells increased from 0.5 ± 0.2 positive cells/HPF in simple steatosis to 0.7 ± 0.3 in less severe NASH, to 1.6 ± 0.3 in more severe NASH ($p < 0.01$) (**Fig.2.1.d**). These results underline the significance of apoptosis in NASH and further suggest the participation of apoptosis in advancing NASH to more severe stages. In particular, caspase-2 activation might function as an important component of NAFLD.

2.4.3. INSR and IRS phosphorylation are strongly impaired in the muscle and liver of patients with NASH

It is well established that the insulin signalling pathway is impaired in obese patients as a result of decreased insulin receptor INSR and IRS phosphorylation (Goodyear *et al.* 1995). However, the degree of IR in insulin-target tissues in different NAFLD stages is largely unknown. In the muscle, total INSR expression was decreased in patients with NASH. More importantly, INSR phosphorylation, decreased by 2.8-fold in more severe NASH, compared with simple steatosis ($p < 0.01$) (**Fig. 2.2.a**). In the liver, total INSR expression was similarly reduced, while INSR phosphorylation was ~ 1.4- and 2-fold lower in less severe and more severe NASH, respectively,

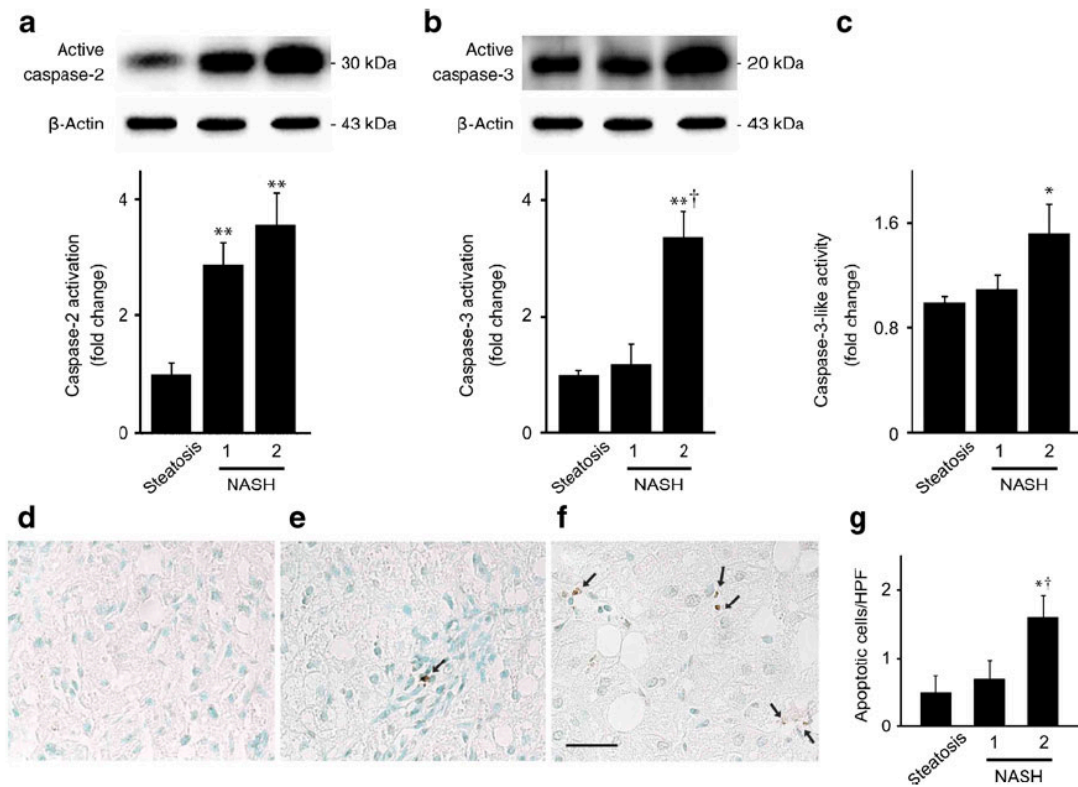


Figure 2.1. Caspase-2 and -3 activation and TUNEL-positive cells are increased in the liver of patients with NASH. Total proteins were extracted for immunoblot analysis as described in the Methods. Representative immunoblots for active caspase-2 (**a**) and active caspase-3 (**b**), with corresponding histograms comparing simple steatosis with less severe and more severe NASH (NASH 1 and 2). Protein blots were normalized to endogenous β -actin. (**c**) Caspase-3-like activity was analyzed by enzymatic cleavage of the chromophore p-nitroanilide from a substrate. Results are expressed as mean $\pm$ SEM (fold change). TUNEL staining of representative paraffin-embedded liver tissue sections of patients with steatosis (**d**), less severe NASH (**e**) and more severe NASH (**f**), with (**g**) quantification of apoptotic cells. Arrows indicate apoptotic cells; scale bar 10 μ m; magnification x400. Results (**g**) are expressed as mean $\pm$ SEM of TUNEL-positive cells/high-power field (HPF). * p < 0.05 and ** p < 0.01 for difference from steatosis; † p < 0.05 for difference from less severe NASH (NASH 1).

compared with simple steatosis (p < 0.05 for more severe NASH) (**Fig. 2.2.b**). In adipose tissue, INSR expression was reduced in NASH, but INSR tyrosine

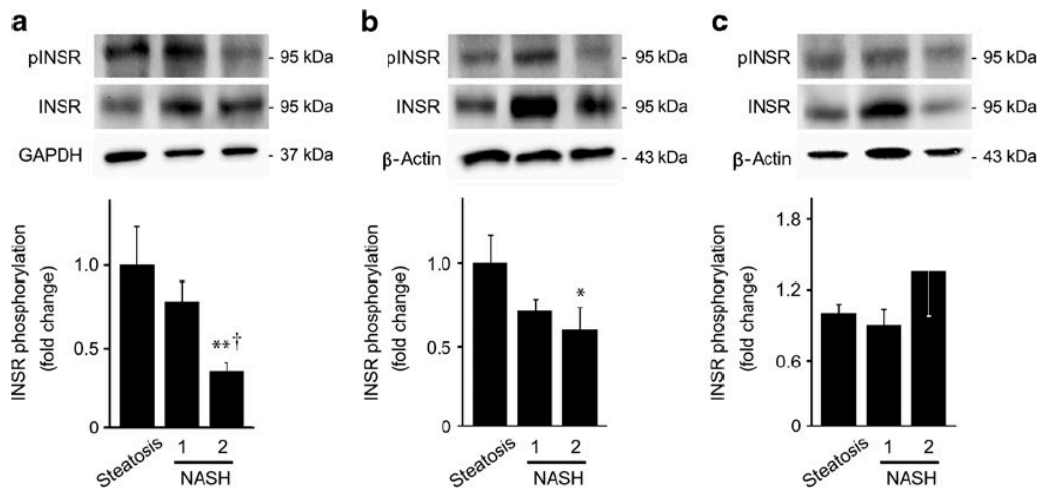


Figure 2.2. INSR production and tyrosine phosphorylation are decreased in muscle and liver tissue of patients with NASH. Total proteins were extracted for immunoblot analysis as described. Representative immunoblots for INSR and phosphorylated INSR (pINSR), with corresponding pINSR/INSR histogram for conditions as labelled in (a) muscle, (b) liver and (c) adipose tissue. Liver and adipose tissue protein blots were normalized to endogenous β -actin, while muscle tissue protein blots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are expressed as mean $\pm$ SEM (fold change); * $p < 0.05$ and ** $p < 0.01$ for difference from steatosis; $^{\dagger}p < 0.01$ for difference from less severe NASH (NASH 1).

phosphorylation did not significantly change between NAFLD disease stages (**Fig. 2.2.c**). As for IRS-1 tyrosine phosphorylation, it was found to be decreased in less severe NASH by almost 2-fold ($p < 0.05$) and in more severe NASH by almost 15-fold ($p < 0.01$), compared with simple steatosis, in the muscle (**Fig. 2.3.a**). While IRS-1 is the major substrate leading to stimulation of glucose transport in muscle and adipose tissues, IRS-2 is the main mediator of insulin signalling in the liver. In these samples, IRS-2 tyrosine phosphorylation, as determined by immunoprecipitation, was similarly decreased in patients with severe NASH ($p < 0.05$), although to a lesser extent than IRS-1 phosphorylation in the muscle (**Fig. 2.3.b**). In adipose tissue, IRS-1 tyrosine phosphorylation remained relatively unchanged (**Fig. 2.3.c**).

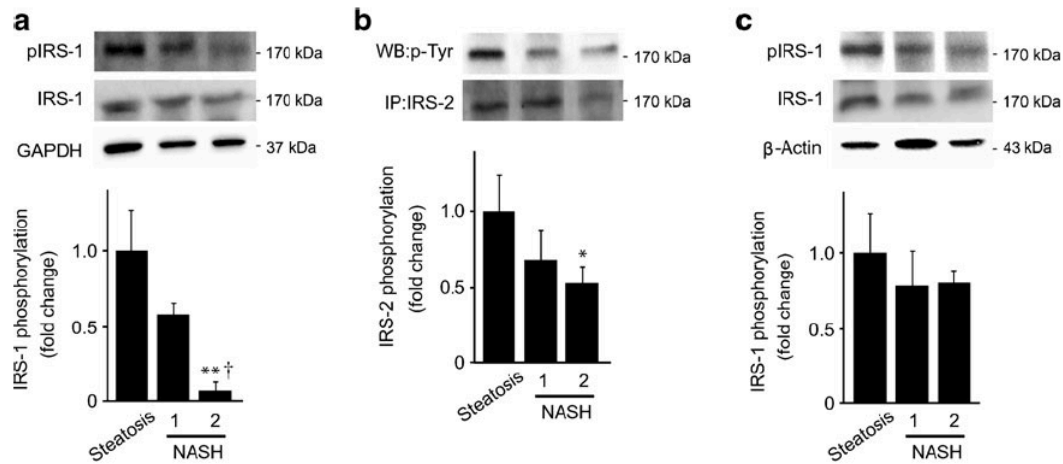


Figure 2.3. Tyrosine phosphorylation of IRS is decreased in both muscle and liver tissue of patients with NASH. Total proteins were extracted for either western blot (WB) analysis or immunoprecipitation (IP) as described. Representative immunoblots for phosphorylated IRS-1 (pIRS-1) and IRS-1, with corresponding pIRS-1/IRS-1 histogram as conditions as labelled for **(a)** muscle and **(c)** adipose tissues. Adipose tissue protein blots were normalized to endogenous β -actin, while muscle tissue protein blots were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **b** Representative immunoblot for IRS-2 and phosphorylated IRS-(p-Tyr), with corresponding phosphorylated IRS-2/IRS-2 histogram in liver tissue. Results are expressed as mean $\pm$ SEM (fold change); * $p < 0.05$ and ** $p < 0.01$ for difference from steatosis; † $p < 0.01$ for difference from less severe NASH (NASH 1).

All together, these results indicate that IR becomes more pronounced in muscle than in liver of morbid obese patients, and less evident in adipose tissue, in more severe stages of the disease, thus suggesting a correlation between IR and different stages of NAFLD.

2.4.4. AKT phosphorylation decreases in muscle, liver and adipose tissues of patients with NASH

We next investigated whether the apparent increased IR, from simple steatosis to more severe NASH, was also evident at the AKT level. In the muscle, AKT phosphorylation decreased in NASH by almost 2-fold, compared with simple steatosis ($p < 0.01$) (**Fig. 2.4.a**). Similarly, total AKT was

significantly decreased among groups. The same trend in AKT phosphorylation was observed in the liver, although in a less pronounced manner ($p < 0.05$ for more severe NASH) (**Fig. 2.4.b**), corroborating previous results (Piro *et al.* 2008; Dongiovanni *et al.* 2010). Surprisingly, in adipose tissue, AKT phosphorylation was also significantly impaired by ~ 3 -fold in NASH, compared with simple steatosis ($p < 0.01$) (**Fig. 2.4.c**). While some authors have shown that insulin-stimulated AKT phosphorylation is reduced in different tissues of obese patients, including in skeletal muscle, others have failed to detect alterations in insulin-induced AKT activation in skeletal muscle of overweight type 2 diabetic patients, or even in obese subjects with or without type 2 diabetes (Sesti 2006). Our results corroborate the first suggestion, showing that in morbidly obese patients with NAFLD, AKT phosphorylation decreases in muscle, liver and adipose tissues in more severe stages of the disease.

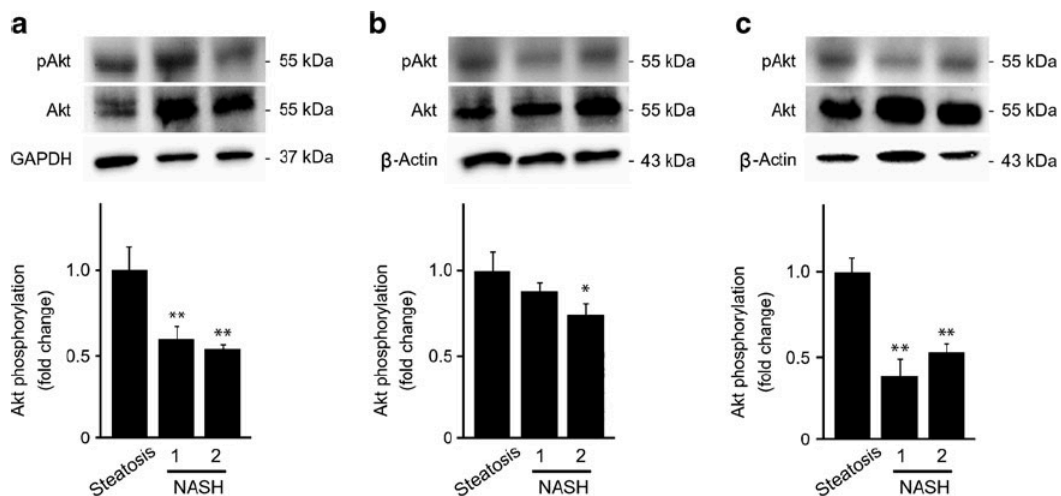


Figure 2.4. AKT phosphorylation is decreased in muscle, liver and adipose tissue of patients with NASH. Total proteins were extracted for immunoblot analysis as described. Representative immunoblots for AKT and phosphorylated AKT (pAKT), with corresponding histogram of pAkt/Akt in conditions as labelled are shown for (a) muscle, (b) liver and (c) adipose tissues. Liver and adipose tissue protein blots were normalized to endogenous β -actin, while muscle tissue protein blots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are expressed as mean $\pm$ SEM (fold change); * $p < 0.05$ and ** $p < 0.01$ for difference from steatosis.

2.4.5. JNK phosphorylation is associated with IR and apoptosis in patients with NASH

JNK1 may directly induce IR (Tanti *et al.* 2009). In addition, some studies have demonstrated a link between JNK activation, caspase-2 and/or -3 activation and apoptosis (Troy *et al.* 2001; Wang *et al.* 2008), while NEFAs have been shown to activate hepatocyte apoptosis in a JNK-dependent manner (Malhi *et al.* 2006).

Therefore, we tested the hypothesis that phosphorylated total JNK is a crucial mediator between metabolic stress and IR and apoptosis, in insulin-target tissues of NAFLD patients, at different disease stages. Total JNK decreased from steatosis to NASH, in muscle and adipose tissues, but not in the liver (**Fig. 2.5.**). More importantly, JNK phosphorylation was

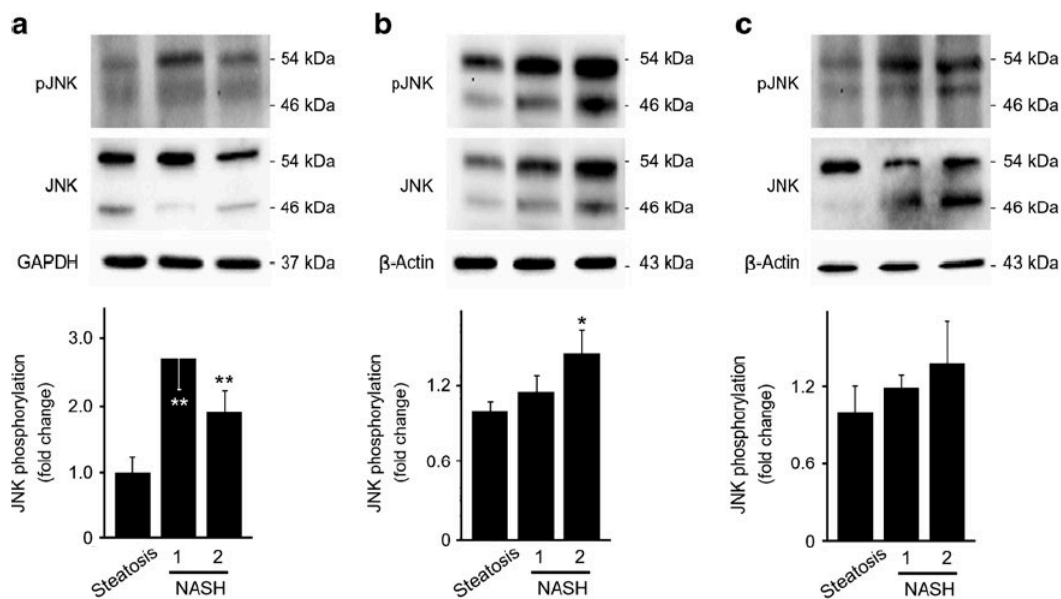


Figure 2.5. JNK expression and phosphorylation are increased in muscle and liver tissue of patients with NASH. Total proteins were extracted for immunoblot analysis as described. Representative immunoblots for total JNK and phosphorylated JNK (pJNK), with corresponding histogram of pJNK/JNK in conditions as labelled are shown for (a) muscle, (b) liver and (c) adipose tissue. Liver and adipose tissue protein blots were normalized to endogenous β -actin, while muscle tissue protein blots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are expressed as mean $\pm$ SEM (fold change); * $p < 0.05$ and ** $p < 0.01$ for difference from steatosis.

significantly increased in NASH, compared with simple steatosis, both in the muscle ($p < 0.01$) and in the liver ($p < 0.05$) (**Fig. 2.5.a** and **2.5.b**). Although not significant, a similar trend was found in adipose tissue (**Fig. 2.5.c**). Thus, increased JNK phosphorylation may explain increased IR and apoptosis in NASH, compared with simple steatosis.

2.5. Discussion

The complex mechanisms leading to development of steatosis, and its progression to different degrees of NASH are largely unknown. In addition, human studies exploring changes in liver and peripheral tissues in signalling pathways involved in cell death and IR remain scant. In this study, we investigated apoptosis in liver tissue, and insulin signalling in insulin-target tissues of patients with different NAFLD stages. Our results show that IR is differentially sensed in muscle, liver and adipose tissues and that apoptosis and IR increase with more severe NAFLD stages in morbid obese patients.

This study includes severely obese patients undergoing bariatric surgery, who typically have very mild liver involvement, with either isolated steatosis or mild NASH. Thus, the results may not be generalized to the typical obese NASH patients with moderate to severe disease. Another limitation of this study is the lack of a real control group, with normal liver, muscle and visceral fat histology, mostly due to ethical issues. Although important, this control group is not crucial when comparing apoptosis and insulin signalling proteins among patients with different stages of NAFLD, which was a major goal of our study.

Both NAFLD and IR have been associated with occurrence of hepatocyte apoptosis (Feldstein *et al.* 2005; Kusminski *et al.* 2009; Schattenberg *et al.* 2009). In particular, we have previously shown that caspase-3 activation and apoptosis are present in the liver of non-obese NASH patients (Ribeiro *et al.* 2004; Ramalho *et al.* 2006). Our current results confirm the same in morbid obese patients. In contrast, little is known about caspase-2 physiological functions, which may include regulation of apoptosis, cell cycle and tumour formation (Vakifahmetoglu-Norberg *et al.* 2010), and virtually no information is available on the role of caspase-2 in the liver. Here, we show that caspase-2 activation increases in the liver from simple steatosis

to NASH. Interestingly, caspase-2 deficient mice display reduced body fat content, when compared with age-matched wild-type mice (Zhang *et al.* 2007), while diet-induced obese rats on dietary L-arginine supplementation gain much less white-fat, concomitant with reduced mRNA levels of caspase-2 (Jobgen *et al.* 2009). In turn, TNF- α was shown to activate caspase-2, but not caspases-3 and -8, and induce apoptosis in HepG2 cells (Tsagarakis *et al.* 2011), while human pancreatic β -cell death induced by saturated fatty acids also relates with increased caspase-2 activity and not caspase-3 (Furstova *et al.* 2008). It is likely that caspase-2 activation affects both the metabolic syndrome and apoptosis during NAFLD. Whether caspase-2 activation results from increased accumulation of liver fat, and/or represents an active apoptosis mechanism during NAFLD or is rather a secondary event remains to be established.

In this study, we report a maximum decrease in tyrosine phosphorylation of INSR and IRS-1 at the muscle level, correlating with more severe NAFLD stages. While this is consistent with the notion that IR in the skeletal muscle manifests long before hyperglycaemia becomes evident (DeFronzo 2009), it also supports the idea that fat deposition in the muscle might be a conditioning factor for the appearance and progression of NAFLD and, particularly, NASH. Importantly, a blockage in the insulin signalling cascade at the IRS-1 level is thought to be the primary defect leading to IR in the muscle. This appears to be also the case for NAFLD progression, as IRS-1 tyrosine phosphorylation was almost 15-fold downregulated in more severe NASH, compared with simple steatosis. The mechanism by which obesity induces IR in skeletal muscle is likely related with the accumulation of intramyocellular fat and fatty acid metabolites (Belfort *et al.* 2005). Still, the cause for deposit of intramyocellular fat and its metabolites has yet to be defined.

The less evident decrease of INSR and IRS-2 activation in the liver, compared with the muscle during NAFLD progression, may indicate that IR in the liver is on a lag phase. Activation of AKT appears to corroborate these results in muscle and liver tissues.

Interestingly, both INSR and IRS-1 activation in adipose tissue remained unchanged with NAFLD progression. Although adipose tissue is an important site of IR in NAFLD (Bugianesi *et al.* 2005a), and visceral fat is strongly associated with both hepatic and muscular IR (Patel *et al.* 2008), it appears that NAFLD progression is not dependent on, or a consequence of, adipose tissue IR. Still, AKT activation was also significantly impaired in visceral fat in more advanced phases of NAFLD, namely in NASH, likely independently from a blockage at the IRS-1 level. Alternatively, phosphorylation of IRS-1 at different residues may still be occurring, thus impairing insulin signalling (Boura-Halfon *et al.* 2009).

JNK activation has been previously shown in human NASH (Puri *et al.* 2008). In addition, JNK appears to play a crucial role in inducing IR and/or steatohepatitis in rodents (Wang *et al.* 2008; Singh *et al.* 2009). However, its phosphorylation state in other insulin-target tissues or in different stages of NAFLD has never been evaluated before. This is particularly relevant, since obesity is known to cause broad chronic low-grade inflammatory responses that lead to activation of stress pathways, in particular JNK. Our findings show that, in muscle tissue, total JNK phosphorylation is significantly increased in NASH, comparing with simple steatosis. In the liver, this increase was more moderate and in visceral fat was absent. These results suggest that in human subjects with morbid obesity, JNK phosphorylation may be responsible for aggravating IR as NAFLD advances to more severe forms. Interestingly, JNK activation may also induce apoptosis, thus providing a link between IR and cell death in different NAFLD stages (Schattenberg *et al.* 2006). In fact, a connection between liver JNK, caspase-2 and apoptosis is an attractive hypothesis, as it was recently shown that caspase-2 and JNK-mediated signalling is one of the mechanisms involved in age-related muscle cell apoptosis (Braga *et al.* 2008). Curiously, we have very recently demonstrated that caspase-2 is a specific key downstream target of JNK in amyloid β -induced apoptosis (Viana *et al.* 2010). However, the role of different JNK isoforms in different human insulin-target tissues remains to be explored. In fact, this might constitute a roadblock to a possible therapeutic approach involving JNK inhibition, as hepatic JNK1 in mice appears to inhibit liver steatosis in opposite to adipose tissue JNK1 (Sabio *et al.* 2009). In

addition, JNK2 appears to inhibit hepatocyte cell death by blocking the mitochondrial pathway of apoptosis in high fat diet-fed mice (Singh *et al.* 2009).

In conclusion, this study clarifies the involvement of apoptosis, caspase-2, and IR in NAFLD stage and in different insulin-target tissues. In particular, more severe cases of NAFLD are associated with increased caspase-2, and -3 activation as well as apoptosis in the liver. In addition, IR may target primarily the muscle tissue as NAFLD advances to more severe cases, although the liver tissue is also affected. Finally, JNK is suggested as a mechanistic link between IR and apoptosis during NAFLD progression, and may represent an attractive pharmacological target for the development of drugs for the treatment of IR-associated NAFLD.

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miR-34a/SIRT1/p53 is Suppressed by Ursodeoxycholic Acid in Rat Liver and Activated by Disease Severity in Human Non-alcoholic Fatty Liver Disease

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3.1. Abstract

Background and Aims: Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of stages from simple steatosis to nonalcoholic steatohepatitis (NASH). However, disease pathogenesis remains largely unknown. microRNA (miRNA or miR) expression has recently been reported altered in human NASH, and modulated by ursodeoxycholic acid (UDCA) in rat liver. Here, we aimed to evaluate the miR-34a/Sirtuin 1(SIRT1)/p53 pro-apoptotic pathway in human NAFLD, and to elucidate its function and modulation by UDCA in rat liver and primary rat hepatocytes.

Methods: Liver biopsies were obtained from NAFLD morbid obese patients undergoing bariatric surgery. Rat livers were collected from animals fed 0.4% UDCA diets. Primary rat hepatocytes were incubated with bile acids or free fatty acids (FFAs) and transfected with a specific miRNA-34a precursor and/or with a p53 overexpression plasmid. P53 transcriptional activity was assessed by ELISA and target reporter constructs.

Results: miR-34a, apoptosis and acetylated p53 increased with disease severity, while SIRT1 diminished in NAFLD liver. UDCA inhibited the miR-34a/SIRT1/p53 pathway in the rat liver *in vivo* and in primary rat hepatocytes. miR-34a overexpression confirmed its targeting by UDCA, which prevented miR-34a-dependent repression of SIRT1, p53 acetylation and apoptosis. Augmented apoptosis by FFAs in miR-34a overexpressing cells was also inhibited by UDCA. Finally, p53 overexpression activated miR-34a/SIRT1/p53, which in turn was inhibited by UDCA, *via* decreased p53 transcriptional activity.

Conclusions: Our results support a link between liver cell apoptosis and miR-34a/SIRT1/p53 signalling, specifically modulated by UDCA, and NAFLD severity. Potential endogenous modulators of NAFLD pathogenesis may ultimately provide new tools for therapeutic intervention.

Keywords: Apoptosis; miRNAs; NAFLD; p53; SIRT1; UDCA

3.2. Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a disease spectrum ranging from simple steatosis to steatohepatitis (NASH), fibrosis and cirrhosis. NAFLD has the potential to progress to hepatocellular carcinoma or liver failure, ultimately leading to early death (Cheung *et al.* 2009). Nevertheless, the biological mechanisms of disease progression are not entirely understood. Consolidated data, including our own, suggest that apoptosis may play a determinant role in the pathogenesis of NAFLD (Canbay *et al.* 2004; Ferreira *et al.* 2011). In addition, microRNAs (miRNAs or miRs) were recently suggested to play an important role in NAFLD (Sayed *et al.* 2011). In fact, miRNAs are differentially expressed in human NASH (Cheung *et al.* 2008), and in genetic (Li *et al.* 2009b) and diet-induced (Pogribny *et al.* 2010) mouse models of NASH.

The role of miRNAs in modulating apoptosis during human NAFLD pathogenesis has not been fully addressed. miR-34a is a prime putative player that induces senescence, cell cycle arrest and apoptosis (Hermeking 2010). The induction of apoptosis by miR-34a presumably depends on cellular context and expression levels of miR-34a target proteins involved in regulating cell death.

Sirtuin 1 (SIRT1), a NAD-dependent deacetylase that modulates apoptosis in response to oxidative and genotoxic stress, represents the best-characterized direct target of miR-34a (Yamakuchi *et al.* 2008). By repressing SIRT1, miR-34a increases p53 acetylation and transcription, leading to the induction of pro-apoptotic genes such as PUMA and, finally, apoptosis. Furthermore, this mechanism represents a positive feedback loop, as miR-34 family members are themselves direct p53 transcriptional targets (Chang *et al.* 2007). Interestingly, the exacerbation of inflammation in human NAFLD increases p53 expression (Panasiuk *et al.* 2006), shown to be biologically active in mediating mitochondrial pathways of apoptosis in an animal model of NASH (Farrell *et al.* 2009). Thus, we hypothesize that miR-34a may play a key role during hepatocyte apoptosis and NAFLD pathogenesis, and that strategies aimed at inhibiting miR-34a and p53 activity, or restoring SIRT1 function, may be beneficial in NAFLD.

We have shown that ursodeoxycholic acid (UDCA) is an important

signalling molecule that modulates liver cell apoptosis (Rodrigues *et al.* 1998a), and appears to do so by inhibiting p53-dependent apoptotic pathways (Sola *et al.* 2003; Castro *et al.* 2005; Amaral *et al.* 2007). Importantly, we have also demonstrated that UDCA is a potent modulator of gene transcription, including several p53-related transcripts (Castro *et al.* 2005), and miRNA expression (Castro *et al.* 2010). Therefore, we aimed to explore the function of the miR-34a/SIRT1/p53 pathway and its modulation by UDCA in primary rat hepatocytes and in rat liver *in vivo*. In addition, we seek to establish whether miR-34a/SIRT1/p53 signalling is modulated in human liver and how this correlates with apoptosis and NAFLD severity.

3.3. Materials and Methods

3.3.1. Patients

This study included consecutive NAFLD patients undergoing bariatric surgery for morbid obesity, as described in Supplementary Materials and Methods. All comprised patients fulfilled the inclusion criteria (Ferreira *et al.* 2011) and accepted to participate in the study, giving written informed consent. The study protocol conformed to the Ethical Guidelines of the 1975 Declaration of Helsinki, revised in 2000, as reflected in a priori approval by the Hospital de Santa Maria Human Ethics Committee. Grading of liver biopsies and definition of experimental groups are described in Supplementary Materials and Methods.

3.3.2. Animals and diets

Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) were fed a diet of standard laboratory chow supplemented with 0.4% (wt/wt) UDCA (n = 12) or no bile acid addition (control; n = 12) for 2 weeks as previously described (Castro *et al.* 2010). Animal protocols were approved and performed as described in Supplementary Materials and Methods.

3.3.3. Cell culture and treatments

Primary rat hepatocytes were isolated from male rats by collagenase perfusion as previously described (Castro *et al.* 2010). Cell culture treatments

and incubations were performed as described in Supplementary Materials and Methods.

3.3.4. Quantitative RT-PCR (qRT-PCR) and immunoblotting

RNA extraction and qPCR analysis of miR-34 family members, miR-122, -143 and -451 was performed as previously described (Castro *et al.* 2010). Immunoblot analysis was performed as previously described (Ferreira *et al.* 2011); the membranes were blotted with antibodies for SIRT1, β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 and acetylated p53 (acetyl-p53) Lys 379 (human Lys 382) (Cell Signaling Technology, Danvers, MA, USA).

3.3.5. Measurement of lipid droplets and cell death

Intracellular neutral lipids were stained with Nile Red, whereas general cell death and apoptosis were assessed by LDH, TUNEL and Hoechst, as described in Supplementary Materials and Methods.

3.3.6. Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the Quantity One Version 4.6 densitometric analysis program (Bio-Rad Laboratories). Statistical analysis was performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

3.4. Results

3.4.1. The miR-34a/SIRT1/p53 pro-apoptotic pathway is modulated by disease severity in human NAFLD

There is currently no proven pharmacological treatment of NAFLD, in part due to the poor understanding of the underlying mechanisms of pathological progression from simple steatosis to NASH and cirrhosis. Here, we show for the first time that miRNAs belonging to the miR-34 family progressively increase with disease severity in the liver of human NAFLD

patients (**Fig. 3.1.A**). In particular, miR-34a expression increased by ~ 2-fold in less severe NASH ($p < 0.05$), and > 3-fold in more severe NASH ($p < 0.01$),

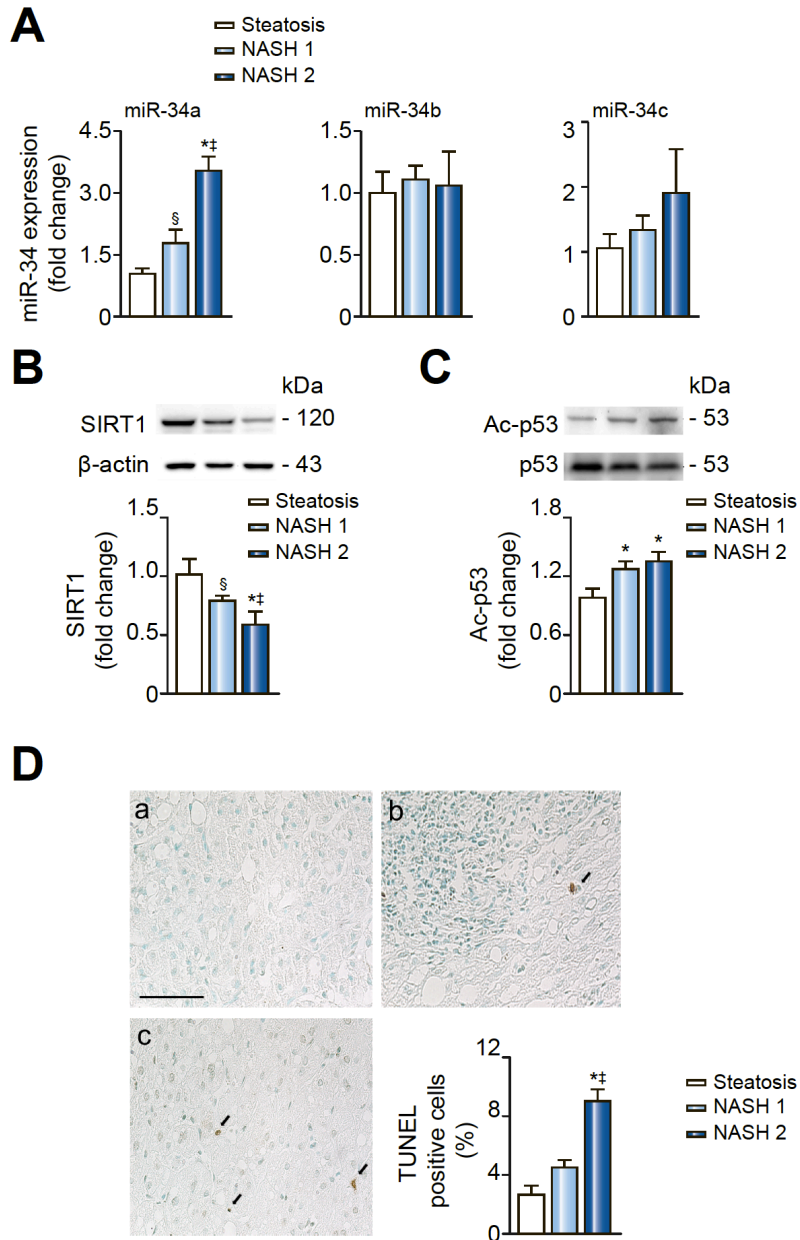


Figure 3.1. The miR-34a/SIRT1/p53 pathway is activated in the liver of NAFLD patients and correlates with disease severity in patients with steatosis ($n = 15$), less severe NASH (NASH 1; $n = 5$), and more severe NASH (NASH 2; $n = 8$). (A) qRT-PCR analysis of miR-34 family. (B and C) Immunoblotting and densitometry of SIRT1 and acetyl-p53 (Ac-p53). (D) TUNEL staining of paraffin-embedded liver tissue sections and percentage of apoptotic cells. Arrows indicate apoptotic cells. Scale bar, 5 μ m. Results are expressed as mean $\pm$ SEM. $^{\S}p < 0.05$ and $^*p < 0.01$ from steatosis; $^{\ddagger}p < 0.01$ from NASH 1.

compared with steatosis. The robustness and significance of our analysis was confirmed by the steady decrease of miR-122, -143, and -451 from human steatosis to more severe NASH ($p < 0.01$) (**Suppl. Fig 3.1.**), previously demonstrated in animal models of NAFLD (Murakami *et al.* 2006; Alisi *et al.* 2011).

Hepatic SIRT1 expression is reduced in different animal models of NAFLD (Colak *et al.* 2011), suggesting that its pharmacological activation may constitute a potential therapeutic strategy. Because a p53/miR-34a/SIRT1 axis has been described in the context of chronic lymphocytic leukemia (Audrito *et al.* 2011), we then determined whether SIRT1 and p53 acetylation could be targeted by miR-34a in human NAFLD. While SIRT1 expression decreased from steatosis to less ($p < 0.05$) and more severe ($p < 0.01$) NASH (**Fig. 3.1.B**), p53 acetylation was increased ($p < 0.01$) (**Fig. 3.1.C**). Finally, the number of TUNEL-positive cells increased from steatosis to less severe NASH (~ 50%), and more severe NASH (~3-fold; $p < 0.01$) (**Fig. 3.1.D**), further suggesting that activation of the miR34-a/SIRT1/p53 pro-apoptotic pathway contributes for NAFLD severity.

3.4.2. UDCA targets the miR-34a/SIRT1/p53 pathway in rat liver and primary rat hepatocytes

We have recently demonstrated that UDCA modulates miRNA expression in the rat liver, during liver regeneration (Castro *et al.* 2010). We now confirmed that UDCA feeding reduces rat liver miR-34a expression by almost 40%, compared with control diet-fed animals ($p < 0.01$) (**Fig. 3.2.A**). As miR-34a up-regulation during rat liver regeneration is associated with suppression of hepatocyte proliferation (Chen *et al.* 2011), these results support our previous suggestion that UDCA promotes liver regeneration by modulating miRNA expression (Castro *et al.* 2010). In addition, UDCA-fed rats showed a ~ 2-fold increase in SIRT1 protein ($p < 0.01$) and a concomitant decrease on acetylated p53 levels ($p < 0.01$), without altering total p53 expression in the rat liver (**Fig. 3.2.B**).

We next tested whether the miR-34a/SIRT1/p53 pathway was specifically modulated by UDCA in cultured primary rat hepatocytes. While

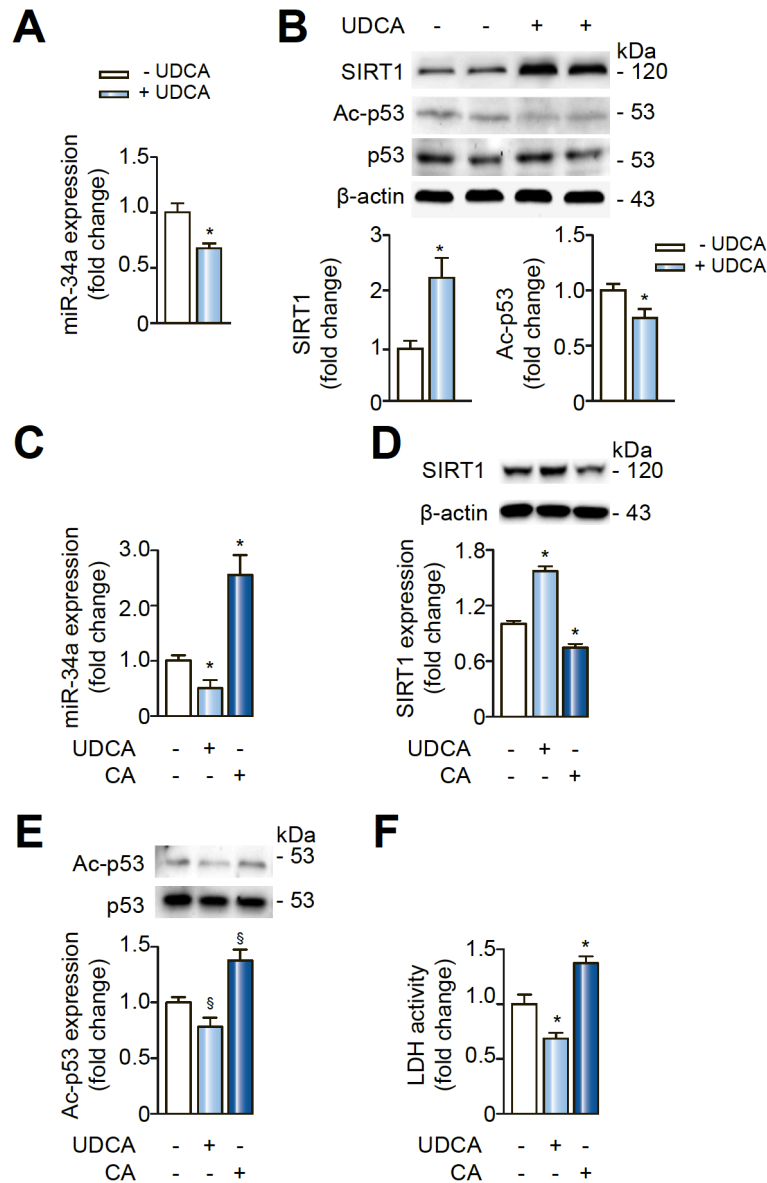


Figure 3.2. UDCA inhibits the miR-34a/SIRT1/p53 pathway in rat liver and in cultured primary rat hepatocytes. (A) qRT-PCR analysis of miR-34a in rat liver from control ($n = 12$) and UDCA-fed ($n = 12$) animals. (B) Immunoblotting and densitometry of SIRT1, acetyl-p53 (Ac-p53), and p53 in rat liver. (C) qRT-PCR analysis of miR-34a expression in cells treated with UDCA, CA or no addition (control) from 4 different experiments. (D and E) Immunoblotting and densitometry of SIRT1 and Ac-p53 in cells. (F) LDH activity assay in cells. Results are expressed as mean $\pm$ SEM fold change. $^{\$}p < 0.05$ and $^*p < 0.01$ from control diet-fed rats or control cells.

UDCA decreased miR-34a expression by ~ 50% ($p < 0.01$), cholic acid (CA) was a strong inducer of miR-34a ($p < 0.01$) (**Fig. 3.2.C**). In addition, in agreement with *in vivo* data, UDCA increased SIRT1 by > 50% ($p < 0.01$) (**Fig. 3.2.D**), while significantly inhibiting acetylated p53 ($p < 0.05$) (**Fig. 3.2.E**). CA inhibited SIRT1 ($p < 0.01$) and increased p53 acetylation ($p < 0.05$) by ~ 35%. Finally, UDCA decreased LDH release from cultured hepatocytes ($p < 0.01$), while CA had an opposite effect ($p < 0.01$) (**Fig. 3.2.F**). Interestingly, tauroursodeoxycholic and taurocholic acids had very similar effects to their unconjugated counterparts, both on the miR-34a/SIRT1/p53 pathway and on cellular toxicity (data not shown). This suggests that UDCA specifically inhibits miR-34a/SIRT1/p53 signalling, an effect that was sustained, until 40 h of incubation ($p < 0.05$) (**Suppl. Fig. 3.2.A - C**), and dose-dependent (**Suppl. Fig. 3.2.D - F**). Incubation of hepatocytes with 400 μ M UDCA was already cytotoxic ($p < 0.05$) and unable to significantly inhibit the miR-34a/SIRT1/p53 pathway.

3.4.3. UDCA modulates apoptosis in a miR-34a/SIRT1/p53-dependent manner

To characterize the cellular effects of UDCA in modulating miR-34a expression, we transfected primary rat hepatocytes with a miR-34a precursor, in the presence or absence of UDCA. miR-34a was markedly increased compared with pre-miR control ($p < 0.01$) and UDCA significantly counteracted miR-34 overexpression ($p < 0.05$) (**Fig. 3.3.A**). In addition, miR-34a overexpression led to a 2-fold decrease in SIRT1 expression ($p < 0.05$) (**Fig. 3.3.B**, left panel). Notably, UDCA reverted the loss of SIRT1 induced by miR-34a overexpression ($p < 0.05$), whereas silencing of SIRT1 attenuated the cytoprotective functions of UDCA (data not shown). To further validate modulation of SIRT1 by UDCA *via* miR-34a, we co-transfected cells with a luciferase reporter construct containing the wild-type miR-34a binding site within SIRT1 3'UTR (Luc-SIRT1 Wt 3'UTR), or a mutated sequence (Luc-SIRT1 Mut 3'UTR), together with pre-miR-34a. While miR-34a overexpression led to a decrease in luciferase activity ($p < 0.05$), UDCA resulted in increased firefly activity in both control and miR-34a

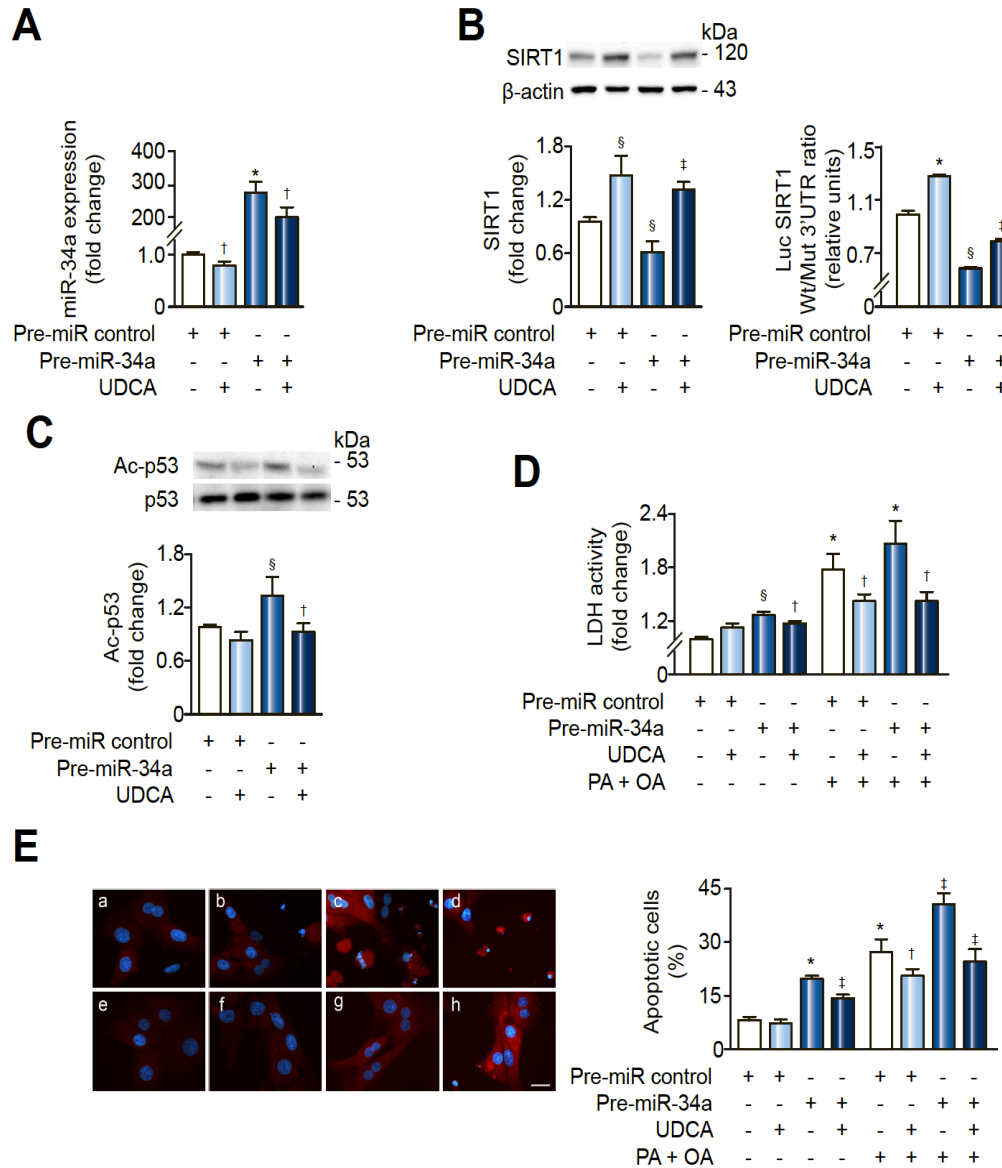


Figure 3.3. miR-34a dependent modulation of apoptosis by UDCA targets SIRT1 and p53 in cultured primary rat hepatocytes. (A) qRT-PCR analysis of miR-34a overexpression. (B) Immunoblotting and densitometry of SIRT1 (left panel) and ratio of luciferase activity between SIRT1 3'UTR constructs containing wild-type (Wt) and mutant (Mut) miR-34a binding sites (right panel). (C) Immunoblotting and densitometry of acetyl-p53 (Ac-p53). (D) LDH activity. (E) Nuclear morphology, intracellular lipid accumulation and percentage of apoptosis in control (a), miR-34a overexpression (b), palmitic acid (PA) + oleic acid (OA) (c), miR-34a overexpression with PA + OA (d), UDCA (e), miR-34a overexpression with UDCA (f), PA + OA with UDCA (g), and miR-34a overexpression with PA + OA and UDCA (h). Scale bar, 5 μ m. Results are expressed as mean $\pm$ SEM fold change from 3

different experiments. [§] $p < 0.05$ and ^{*} $p < 0.01$ from Pre-miR-Control; [†] $p < 0.05$ and [‡] $p < 0.01$ from respective Pre-miR-34a.

overexpressing cells ($p < 0.01$) (**Fig. 3.3.B**, right panel). Neither pre-miR-34a nor UDCA had any major effect in cells transfected with Luc-SIRT1 Mut 3'UTR. Importantly, UDCA also had little effect in cells transfected with a luciferase reporter plasmid harboring the SIRT1 promoter (data not shown), indicating that SIRT1 is mostly regulated by UDCA at the posttranscriptional level, at least in part, through miR-34a. Interestingly, tauroursodeoxycholic acid is a potent inhibitor of c-Jun N-terminal kinase 1 (JNK1), which in turn has been shown to promote SIRT1 degradation (Gao *et al.* 2011). Nevertheless, UDCA alone did not significantly modulate total or phosphorylated JNK1 in primary rat hepatocytes (data not shown), although it may still be engaging distinct additional mechanisms of SIRT1 post-transcriptional regulation.

We have previously shown that p53 is a key molecular target of UDCA in regulating apoptosis (Amaral *et al.* 2007; Castro *et al.* 2007a); however, its role in modulating p53 acetylation is uncertain. Following miR-34a overexpression, p53 acetylation was increased ($p < 0.05$), an effect abrogated by UDCA ($p < 0.05$) (**Fig. 3.3.C**). Concomitant results were obtained when specifically inhibiting miR-34a *in vitro*, where the negative regulation of the miR34-a/SIRT1/p53 pathway by anti-miR34a was potentiated in the presence of UDCA (**Suppl. Fig. 3.3.**).

Finally, we evaluated whether modulation miR-34a/SIRT1/p53 by UDCA had a significant impact on cell death. In addition, we also examined whether increased miR-34a expression could sensitize hepatocytes to the deleterious effects of free fatty acids (FFAs), and whether UDCA could also be protective. Cells were double-stained with Hoechst and Nile red to determine apoptosis and neutral lipid accumulation, respectively. In the absence of FFAs, miR-34a significantly increased cytotoxicity ($p < 0.05$) (**Fig. 3.3.D**) and apoptosis ($p < 0.01$) (**Fig. 3.3.E**). In agreement with its effects on miR-34a expression, UDCA proportionally inhibited miR-34a-induced cell toxicity ($p < 0.05$). When cells were incubated with

800 μ M palmitic and oleic acids (1:2), LDH increased by 80% ($p < 0.01$) and apoptosis by > 3 -fold ($p < 0.01$). This was consistent with a strong activation of the miR-34a-dependent pathway by FFAs (data not shown). More importantly, miR-34a overexpression significantly increased FFAs-induced apoptosis ($p < 0.05$), as well as number and size of lipid droplets. Of note, in FFA-treated cells, UDCA reduced cellular toxicity and apoptosis, independently of miR-34a overexpression ($p < 0.05$). In addition, UDCA reduced intracellular lipid droplet aggregation and size. These results strongly suggest that UDCA specifically targets miR-34a/SIRT1/p53 signalling in inhibiting apoptosis and FFA-induced cytotoxicity in primary rat hepatocytes.

3.4.4. UDCA inhibits p53-dependent induction of the miR-34a apoptotic pathway by reducing p53 transcriptional activity

p53 may directly activate the expression of miR-34a, which is then sufficient to induce apoptosis through both p53-dependent and -independent mechanisms (Chang *et al.* 2007). Therefore, we next investigated whether p53 could induce miR-34a expression in primary rat hepatocytes, and whether this increase was modulated by UDCA. Primary rat hepatocytes were transfected with a construct overexpressing p53, in the presence or absence of UDCA. p53 overexpression led to a 2-fold increase in miR-34a expression, compared to empty vector transfected cells ($p < 0.05$) (**Fig. 3.4.A**). Importantly, UDCA abrogated the induction of miR-34a by p53 ($p < 0.05$). In agreement, p53 overexpression also decreased SIRT1 protein levels ($p < 0.05$), an effect reverted by UDCA ($p < 0.05$) (**Fig. 3.4.B**). Interestingly, whereas p53 overexpression resulted in an ~ 16 -fold increase in total p53, ($p < 0.01$) (**Fig. 3.4.C**), UDCA did not significantly alter p53 protein levels either alone or after p53 overexpression. These results suggest that UDCA modulates p53 at the post-transcriptional level, likely p53 transcriptional activity, as *per* our previous findings (Amaral *et al.* 2007). To ascertain this, we assessed p53 transactivity using nuclear extracts from cells overexpressing p53, in the presence or absence of UDCA (**Fig. 3.4.D**). While p53 overexpression increased p53 activity by ~ 20 -fold ($p < 0.01$), UDCA significantly inhibited p53 activity ($p < 0.05$). We also analyzed the ability of

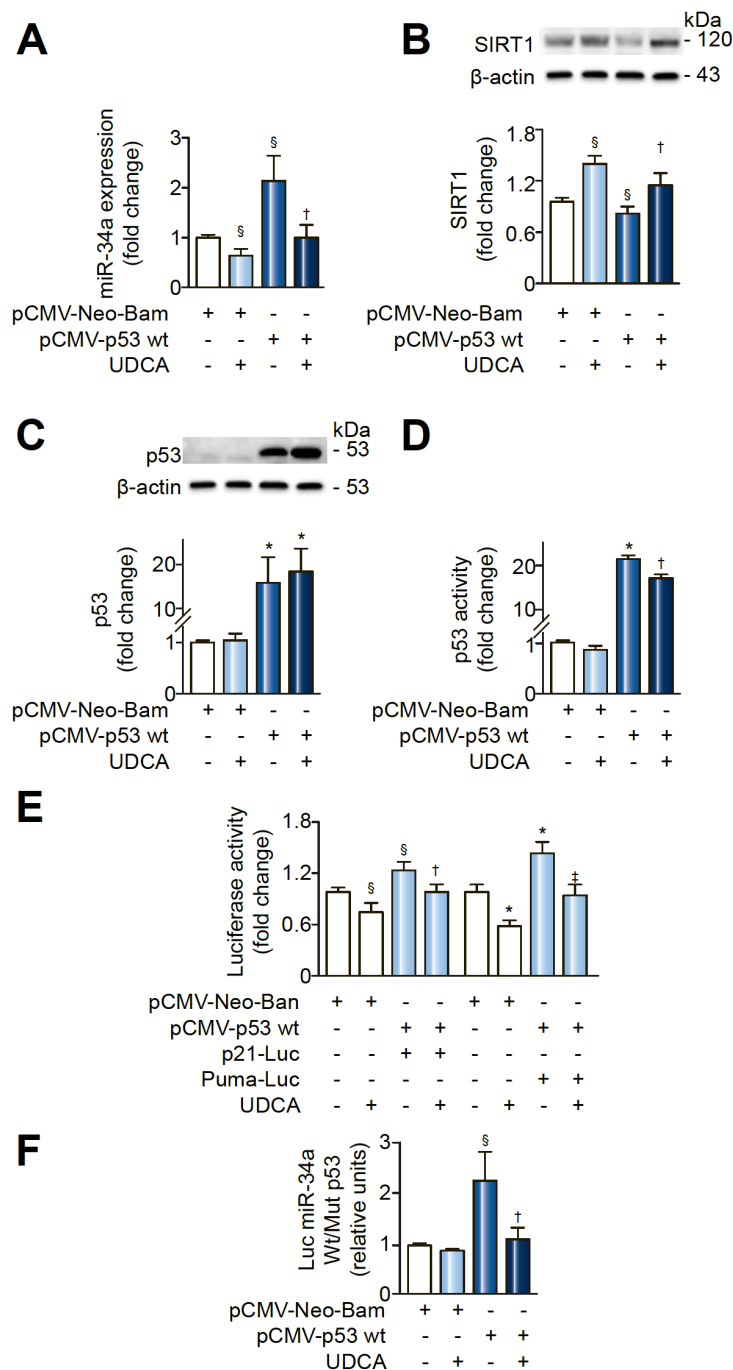


Figure 3.4. UDCA reduces p53 transactivity, inhibiting p53-dependent induction of the miR-34a/SIRT1/p53 pathway in cultured primary rat hepatocytes. (A) qRT-PCR analysis of miR-34a in cells overexpressing p53 (pCMV-p53 Wt), or an empty control vector (pCMV-Neo-Bam). (B and C) Immunoblotting and densitometry of SIRT1 and p53. (D) Nuclear p53 transactivation. (E) p53 transactivation of PUMA (PUMA-Luc) or p21 (p21-Luc) promoter constructs, containing consensus p53 binding sites. (F) p53 transactivation of miR-34a. Results are expressed as mean $\pm$ SEM fold change from 4

different experiments. [§] $p < 0.05$ and ^{*} $p < 0.01$ from pCMV-Neo-Bam; [†] $p < 0.05$ and [‡] $p < 0.01$ from pCMV-p53 Wt.

UDCA to modulate transcriptional activation of p53 targets PUMA and p21, as a measure of p53 activation. p53 overexpression increased the promoter activity of both targets, as compared with cells transfected with the pCMV-Neo-Bam control plasmid (**Fig. 3.4.E**). Notably, UDCA significantly reduced p53-dependent activation of p21 ($p < 0.05$) and PUMA ($p < 0.01$) in both control- and p53-overexpressing cells. Finally, to clearly establish whether modulation of miR-34a by UDCA resulted from diminished p53 activity, cells were co-transfected with luciferase reporter constructs under the transcriptional control of human miR-34a promoter elements containing either wild-type or mutant p53 binding sites. p53 overexpression increased wild-type miR-34a promoter activity by > 2 -fold ($p < 0.05$) (**Fig. 3.4.F**). Importantly, UDCA reduced wild-type miR-34a promoter activity after p53 overexpression ($p < 0.05$). In addition, UDCA also efficiently inhibited apoptosis induced by p53 overexpression (data not shown). These results indicate that UDCA decreases the transcriptional activity of p53 in primary rat hepatocytes and, particularly, p53-mediated activation of miR-34a, providing a functional mechanism for its down-regulation of the miR-34a/SIRT1/p53 pro-apoptotic pathway in the liver.

3.5. Discussion

miRNAs have recently been suggested to play a role in several animal models of NASH (Li *et al.* 2009b; Pogribny *et al.* 2010) and NAFLD (Sayed *et al.* 2011). In particular, we hypothesize that they may constitute modulators of NAFLD progression, as deregulated miRNAs were reported in the transition from hepatic steatosis to steatohepatitis in a rat model (Jin *et al.* 2011). In this regard, our results are the first to show that miR-34a expression in the human liver significantly increases with NAFLD severity. Interestingly, increased miR-34a has been described in circulating serum of NAFLD patients (Cermelli *et al.* 2011). Therefore, miR-34a may represent not only a key therapeutic target in preventing NAFLD progression, but also a novel and noninvasive disease biomarker. We further show that miR-34a expression in the liver of

NAFLD patients is correlated with SIRT1 and acetylated p53 protein levels, as well as with apoptosis. In fact, we and others have previously demonstrated that hepatocyte apoptosis is a key feature of NASH and may correlate with NAFLD severity (Canbay *et al.* 2004; Ferreira *et al.* 2011). In this regard, our current results suggest that the miR-34a/SIRT1/p53 pathway contributes to apoptosis in severe NAFLD. This appears to be a functionally relevant pathway in the liver, as miR-34a expression has been described to increase in the rat liver during aging, with a concomitant decrease in SIRT1 expression (Li *et al.* 2011). Interestingly, activation of miR-34a/SIRT1 in human NAFLD may also contribute to disease severity by differentially deregulating cholesterol metabolism. In a recent study, miR-34a was shown to dephosphorylate HMG CoA reductase (HMGCR), an event correlated with free cholesterol accumulation and histologic severity in NAFLD (Min *et al.* 2012). Altogether, these results underscore the functional relevance of the miR-34a/SIRT1/p53 pathway in NAFLD and further hint at the beneficial effects of targeting miR-34a, an upstream regulator of both SIRT1 and p53.

UDCA is well-established and potent inhibitor of apoptosis in both rat (Rodrigues *et al.* 1998a; Amaral *et al.* 2007) and human hepatocytes (Benz *et al.* 2000). In addition, UDCA modulates several mRNA transcripts and miRNAs involved in apoptosis, cell cycle control, proliferation and cell growth in the rat liver *in vivo* (Castro *et al.* 2005; Castro *et al.* 2010). In the present study, we show that UDCA hampers miR-34a expression, induces SIRT1 expression and inhibits p53 acetylation, in the rat liver both *in vivo* and *in vitro*. This represents a specific effect of UDCA, as primary rat hepatocytes incubated with CA displayed a significant activation of miR-34a/SIRT1/p53 and associated cytotoxicity. In addition, miR-34a overexpression results in cell death and apoptosis in primary rat hepatocytes, *via* SIRT1 and p53, and UDCA strongly inhibits this pathway. Excessive levels of circulating FFAs contribute to hepatocyte lipoapoptosis and NAFLD pathogenesis (Malhi *et al.* 2008). Noteworthy, FFA deleterious effects were sensitized by miR-34a overexpression, and UDCA was still strongly protective, further underscoring the relevance of miR-34a-dependent pathways in NAFLD.

SIRT1, in particular, constitutes an important target regulated by UDCA. This regulation appears to be mostly post-transcriptional, as UDCA

had no significant effect in cells transfected with a reporter plasmid harboring the SIRT1 promoter. Interestingly, hepatic steatosis and inflammation are increased in SIRT1 heterozygous knockout (SIRT1(+/-)) mice (Xu *et al.* 2010), and in mice with hepatocyte-specific deletion of SIRT1 (Purushotham *et al.* 2009). In addition, SIRT1 protein is degraded in response to JNK1 activation, thus contributing to hepatic steatosis in obese mice (Gao *et al.* 2011), and we have shown that both insulin resistance and JNK activation are important factors governing the progression of liver steatosis to more advanced stages of NAFLD (Ferreira *et al.* 2011). Still, we failed to find evidence supporting modulation of SIRT1 by UDCA through JNK1 activation, although additional mechanisms, other than miR-34a-dependent post-transcriptional regulation, are likely to occur.

We also show that miR-34a-dependent modulation of apoptosis by UDCA inhibits p53 acetylation, which is usually indispensable for p53 transcriptional activity (Brooks *et al.* 2011). Interestingly, we have previously shown that UDCA reduces p53 DNA binding activity by stabilizing the p53/MDM2 association (Amaral *et al.* 2007) and enhancing MDM2-dependent ubiquitination of p53 (Amaral *et al.* 2010). Our current results also indicate that p53-dependent apoptosis in primary rat hepatocytes occurs, at least in part, through activation of the miR-34a/SIRT1/p53 pathway, where p53 may function as a transcription factor for miR-34a, in a positive feedback loop (Chang *et al.* 2007). In this regard, we show that inhibition of miR-34a by UDCA results, at least in part, from its ability to inhibit p53 transactivity. Alternatively, UDCA may still directly target miR-34a, as we have already demonstrated that UDCA is a potent and broad modulator of both gene (Castro *et al.* 2005) and miRNA (Castro *et al.* 2010) expression. In addition, it would be interesting to investigate whether downregulation of miR-34a by UDCA is also affecting cholesterol metabolism and, in particular, HMGCR phosphorylation (Min *et al.* 2012). Altogether, the pleiotropic effects of UDCA in the miR-34a/SIRT1/p53 pathway converge in a potent inhibition of p53-mediated apoptosis in liver cells and could prove useful in preventing NAFLD progression.

The role of UDCA in ameliorating NASH and NAFLD has been controversial. While some clinical trials have shown that UDCA in

monotherapy has no positive effect in NASH at 13-15 mg/kg/day dosage (Lindor *et al.* 2004), doses of 28-35 mg/kg/day were somehow effective at improving aminotransferases, serum fibrosis markers, and selected metabolic parameters (Ratziu *et al.* 2011). Furthermore, animal models of NAFLD have shown that UDCA ameliorates insulin sensitivity, and liver steatosis and inflammation (Buko *et al.* 2011; Tsuchida *et al.* 2011). However, whether treatment of NASH with high-dose UDCA will translate into well-established beneficial endpoints is still unclear. Studies in animal models of NAFLD are warranted to establish whether inhibition of apoptosis by UDCA, in particular through the miR-34a/SIRT1/p53, is a relevant event contributing for its beneficial effects.

In conclusion, the hepatic expression of miRNAs is modulated with human NAFLD severity. In particular, miR-34a expression increases from steatosis to less- and more-severe NASH. These changes can potentially affect lipid metabolism, cellular responses to stress and apoptosis. Targeting of the miR-34a/SIRT1/p53 pro-apoptotic pathway by UDCA in primary rat hepatocytes resulted in increased SIRT1 expression, decreased acetylation of p53 and reduced apoptosis, even after miR-34a overexpression, a condition that mimics increased miR-34a expression in severe stage NAFLD. The mechanism by which UDCA inhibits miR-34a expression results, at least in part, by its inhibition of p53 transactivation. Finally, modulation of SIRT1 by UDCA depends almost exclusively on miR-34a, underscoring the relevance of this duality. The miR-34a/SIRT1/p53 pro-apoptotic pathway may represent an attractive pharmacological target for the development of new drugs to arrest NAFLD progression.

Acknowledgments

The authors thank to Dr. Wayne for SIRT1-luciferase reporters, Dr. Lowenstein for SIRT1-3'UTR-luciferase reporters, Dr. Hannon for miR-34a-luciferase reporters, and Dr. Vogelstein for p53 expression and PUMA and p21 luciferase vectors. We specially thank Dr. Steer for providing all the conditions to perform the animal feeding protocol at the University of Minnesota, Minneapolis, MN, USA. The authors also thank all members of the laboratory for insightful discussions.

3.6. Supplementary materials and methods

3.6.1. Patients

Liver biopsies were obtained from patients during bariatric surgery; liver biopsies from normal individuals were not collected due to ethical issues. Biopsies were processed conventionally for diagnostic purposes, histological grading and staging as described previously (Ferreira *et al.* 2011). In particular, all liver specimens were evaluated by an experienced pathologist, blinded to clinical data, according to the NAFLD histology scoring system. The severity of steatosis was graded from 0 to 3, inflammation from 0 to 3, hepatocellular ballooning from none to many, and fibrosis was staged from 0 to 4. Each liver specimen was assessed for presence or absence of NASH, by pattern recognition, and for NAFLD activity score, defined as the sum of steatosis, inflammation and hepatocyte ballooning (Neuschwander-Tetri *et al.* 2003; Kleiner *et al.* 2005). Thirteen patients were classified as having NASH and 15 as having simple steatosis. NASH patients were further divided into those with a NASH score ≥ 3 and < 5 (group 1 or less severe NASH; $n = 5$) and those with a NASH score ≥ 5 (group 2 or more severe NASH; $n = 8$). Clinical, anthropometric, and biological data of patients has been previously reported (Ferreira *et al.* 2011).

3.6.2. Animals and diets

Feeding regimens were conducted according to protocols submitted to and approved by the Institutional Animal Care and Use Committee at the University of Minnesota. In addition, all animals received humane care in compliance with the Institute's guidelines, and as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

3.6.3. Cell culture and treatments

Cells were incubated in Complete William's E medium (Sigma-Aldrich Corp.). When indicated, cells were treated with 100 μM UDCA, 100 μM CA (Sigma-Aldrich Corp.), or no addition (control) for 28 h before processing for

total protein and RNA extraction, and for cell viability assays. In parallel, cells were incubated with 100 μ M UDCA for 16, 28, 40, 52, and 64 h, or 25, 50, 100, 200, and 400 μ M UDCA. For functional analyses, primary rat hepatocytes were transfected at the moment of plating with 100 pM of a specific miR-34a precursor (pre-miR-34a; Applied Biosystems, Foster City, CA, USA), or with a pre-miR negative control using LipofectamineTM 2000 (Invitrogen Corp.). Alternatively, miR-34a inhibition was performed by transfecting primary rat hepatocytes with 100 pM of a miR-34a-specific inhibitor (anti-miRNA-34a; Applied Biosystems), or with an anti-miRNA negative control. After 6 h, cells were incubated with 100 μ M UDCA, or no addition (control). Hepatocytes were harvested at 40 h post-transfection and processed for total RNA and protein extraction, and for cell viability assays.

To induce lipotoxicity, primary rat hepatocytes were incubated with 800 μ M palmitic acid (PA) and oleic acid (OA) (Sigma-Aldrich Corp.) in a molecular ratio of 1:2, or no addition (control), 6 h after pre-miR-34a transfections. PA and OA were dissolved in isopropyl alcohol at a stock concentration of 80 mM. FFAs were added to Complete William's E medium containing 1% bovine serum albumin to ensure a physiologic ratio between bound and unbound FFAs in the media, approximating the molar ratio present in the plasma (Richieri *et al.* 1995). The concentration of isopropyl alcohol was 1% in final incubations. Four hours after FFAs incubation, cells were treated with 100 μ M UDCA, or no addition (control). Hepatocytes were harvested at 24 h post-transfection and processed for total RNA and protein extraction, cell viability assays and Nile Red/Hoechst staining.

To assess the direct effect of miR-34a on SIRT1 expression, we used reporter plasmids consisting of a pMIR-REPORT backbone harboring either wild-type (Luc-SIRT1 Wt 3'UTR) or mutated (Luc-SIRT1 Mut 3'UTR) miR-34a binding sequences within the 3' UTR of SIRT1 (plasmids 20379 and 20380; Addgene, Cambridge, MA, USA) (Yamakuchi *et al.* 2008). After plating, primary rat hepatocytes were co-transfected with 500 ng of either construct, together with 100 pM of pre-miR-34a or anti-miR-34a (Applied Biosystems), or respective controls, using LipofectamineTM 2000 (Invitrogen Corp.).

To assess if UDCA transcriptionally activates SIRT1, we used a pGL3 luciferase reporter plasmid harboring the SIRT1 promoter (a gift of Dr. Alexander Wayne) (Xiong *et al.* 2011). At the time of plating, primary rat hepatocytes were co-transfected with 500 ng of this construct, or with a matching pGL3 reporter control plasmid, using Lipofectamine™ 2000 (Invitrogen Corp.). When indicated, 6 h after transfections, cells were incubated with 100 μ M UDCA, or no addition (control). Reporter assays were performed 48 h post-transfection using the Dual-Luciferase® Reporter Assay System (Promega Corp.) according to the manufacturer's instructions. In all experiments, cells were also co-transfected with pRL-SV40 (Promega Corp.). Renilla luciferase activity was used as a transfection normalization control.

3.6.4. Assessment of p53 transcriptional activity

The TransAM™ p53 transcription factor assay kit (Active Motif, Carlsbad, CA) was used according to the manufacturer's protocol and as previously described (Amaral *et al.* 2007). p53 activation was additionally assessed based on natural reporter genes. We used pBV-Luc vectors harboring the PUMA (PUMA Frag1-Luc or PUMA-Luc) or the p21 (WWP-Luc or p21-Luc) promoters containing p53 responsive elements (16451 and 16591, respectively; Addgene). The pBV-Luc construct was used as a control (plasmid 16539; Addgene). At the time of plating, primary rat hepatocytes were co-transfected with 500 ng of the luciferase reporter constructs and with 2 μ g of the p53 expression vector, using Lipofectamine™ 2000 (Invitrogen Corp.).

To specifically analyze whether UDCA modulates the miR-34a promoter *via* p53, we used pGL4 vectors harboring the putative promoter regions of human mir-34a, containing either wild-type (Luc miR-34a Wt p53) or mutant (Luc miR-34a Mut p53) p53 binding sites (a gift of Dr. Hannon) (He *et al.* 2007). In addition, p53 was overexpressed by transfecting pCMV-Neo-Bam vector encoding wild-type human p53 (pCMV-p53 wt), using pCMV-Neo-Bam empty vector as control (plasmids 16434 and 16440, respectively; Addgene) (Baker *et al.* 1990). When indicated, cells were incubated with 100 μ M UDCA, or no addition (control), 6 h after transfections. Reporter assays were performed 48 h post-transfection using the

Dual-Luciferase® Reporter Assay System (Promega Corp.) according with the manufacturer's instructions. In all experiments, cells were also co-transfected with pRL-SV40 (Promega Corp.). Renilla luciferase activity was used as a transfection normalization control.

3.6.5. LDH assay

LDH, a stable cytosolic enzyme, is released to cell culture media following cell lysis, and can be used as a marker of cell death. Briefly, to assess LDH release, supernatants resulting from a soft centrifugation of the cell culture media at 250 g, were combined in microplates with lactate (substrate), tetrazolium salt (coloring solution), and NAD (co-factor), previously mixed in equal proportions, following the manufacturer's instructions (Sigma-Aldrich Corp.). Multiwell plates were protect from light and incubated for 10 min at room temperature. Finally, absorbance was measured at 490 nm, with 690 nm as reference, using a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories).

3.6.6. TUNEL assay

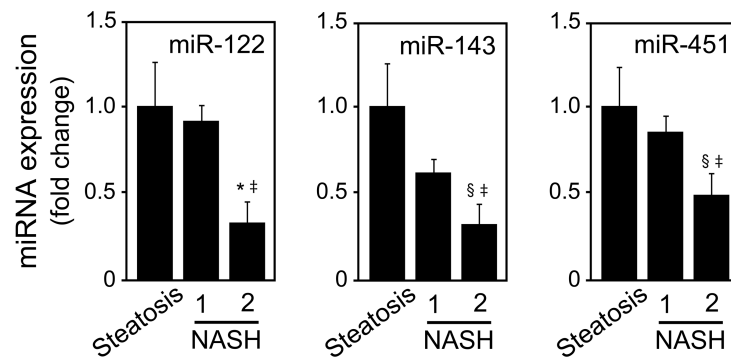
Transferase mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining was performed according to the manufacturer's instructions (Serologicals Corp., Norcross, GA, USA). Liver tissue specimens were examined using a Axioskop bright-field microscope (Carl Zeiss GmbH, Gena, Germany) and data expressed as the number of TUNEL-positive cells/high-power field (x400).

3.6.7. Nile Red/Hoechst double staining

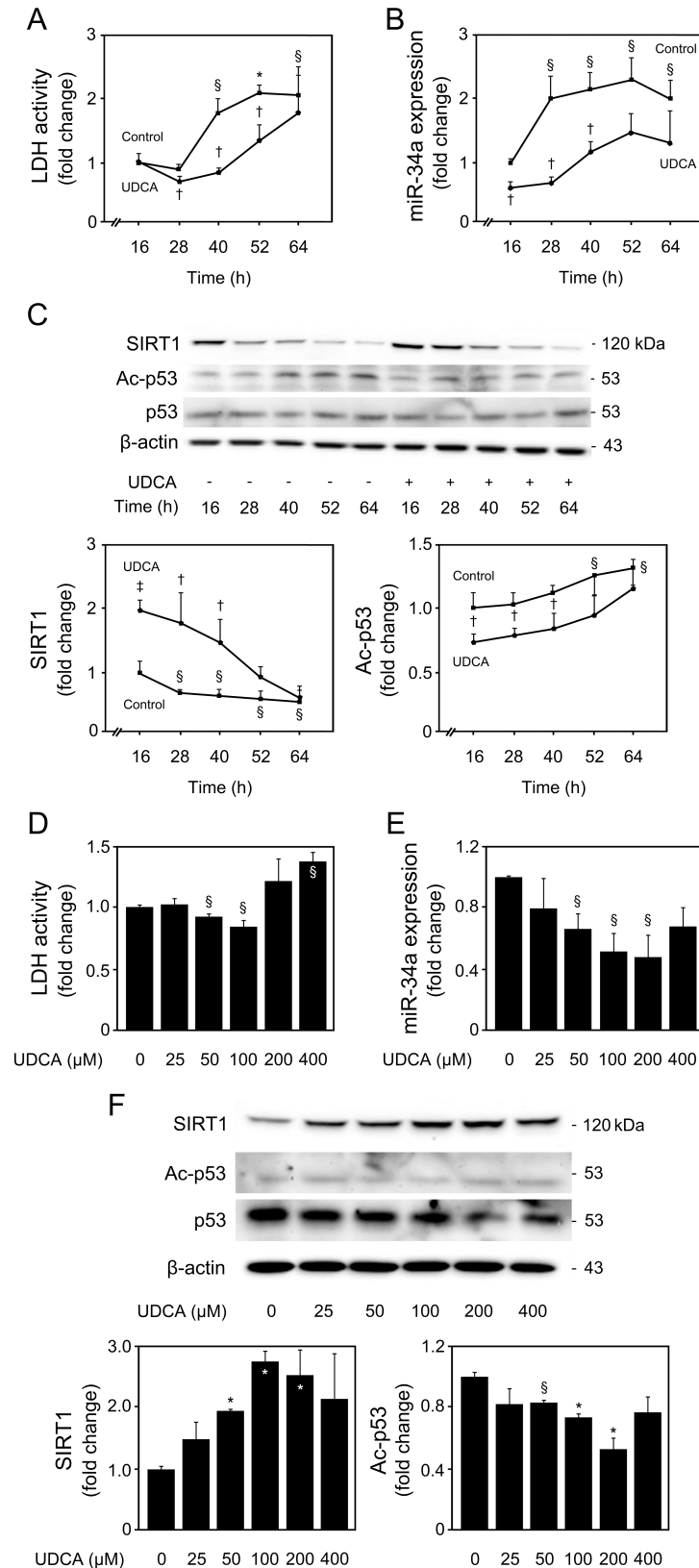
Nile red is a lipophilic dye that stains intracellular lipid droplets, and is routinely used to detect lipid accumulation. Hoechst labeling of attached cells stains chromatin and can be used to detect apoptotic nuclei by morphological analysis. Briefly, after cell treatments, culture medium was gently removed to prevent detachment of cells. Attached primary rat hepatocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at room temperature, washed with PBS, incubated with Hoechst dye 33258 (Sigma-Aldrich Corp.) at 5 µg/mL in PBS for 10 min, washed with PBS,

incubated with Nile Red (Sigma-Aldrich Corp.) at 2.5 µg/mL in PBS for 10 min, washed with PBS, and mounted using Fluoromount-G™ (SouthernBiotech, Birmingham, AL, USA). Fluorescence was visualized using an Axioskop fluorescence microscope (Carl Zeiss GmbH). Blue-fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin disperse over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as by nuclear fragmentation of condensed chromatin. Five random microscopic fields per sample containing approximately 150 nuclei were counted, and mean values expressed as the percentage of apoptotic nuclei. Lipid droplets analysis and evaluation of fluorescence intensity was determined using Image J 1.29x software (N.I.H., USA).

3.8. Supplementary figures

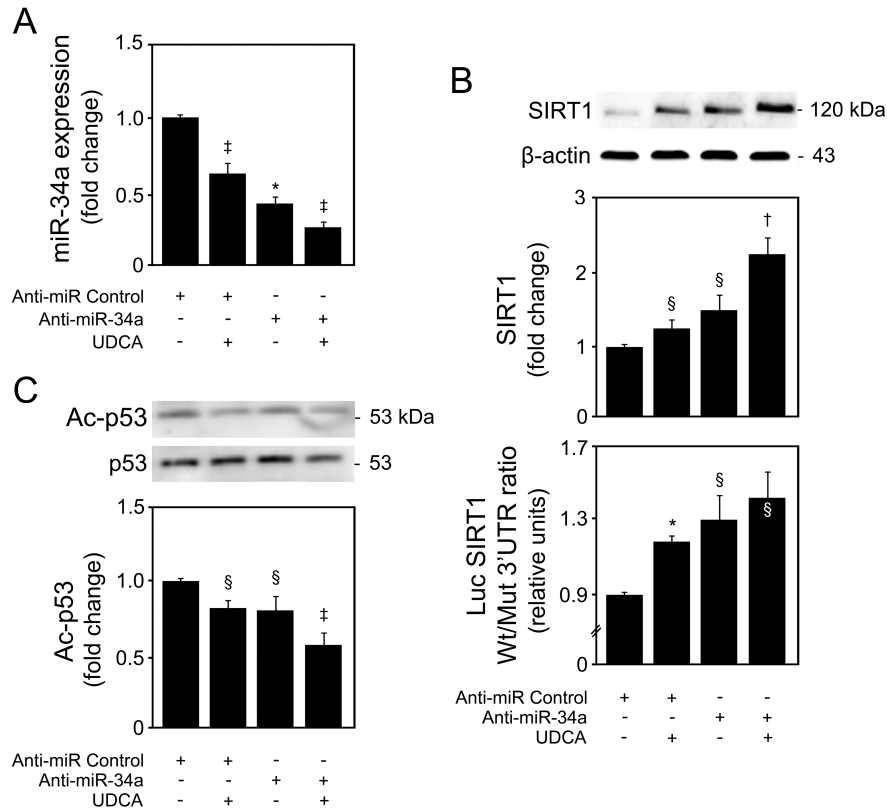


Supplementary Figure 3.1. miR-122, -143, and -451 steadily decrease in the liver of NAFLD patients from steatosis to more severe NASH. miR-122, -143, and -451 expression was assessed by qRT-PCR comparing steatosis ($n = 15$) with less severe (NASH 1; $n = 5$) and more severe NASH (NASH 2; $n = 8$). § $p < 0.05$ and * $p < 0.01$ from steatosis; † $p < 0.01$ from NASH 1.



Supplementary Figure 3.2. Inhibition of the miR-34a/SIRT1/p53 pathway by UDCA in cultured primary rat hepatocytes is dose- and time-dependent. (A) LDH activity assay in primary rat hepatocytes treated with 100 μM UDCA or no addition (control) for 16, 28, 40, 52,

and 64 h from 7 different experiments. **(B)** qRT-PCR analysis of miR-34a in cells. **(C)** Immunoblotting and densitometry of SIRT1 and Ac-p53 in cells. **(D)** LDH activity assay in primary rat hepatocytes treated with 25, 50, 100, 200, and 400 μ M UDCA or no addition (control) for 28 h from 5 different experiments. **(E)** qRT-PCR analysis of miR-34a in cells. **(F)** Immunoblotting and densitometry of SIRT1 and Ac-p53 in cells. Results are expressed as mean $\pm$ SEM fold change. $^{\S}p < 0.05$ and $^*p < 0.01$ from control cells; $^{\dagger}p < 0.05$ from respective time-point controls.



Supplementary Figure 3.3. Modulation of apoptosis by UDCA is dependent on miR-34a expression. (A) Real-time RT-PCR analysis of miR-34a inhibition. (B) Immunoblotting of SIRT1 in cells transfected with miR-34a inhibitor (upper panel). Representative immunoblots are shown. Ratios between luciferase activity from SIRT1 3'UTR constructs containing wild-type (Wt) and mutant (Mut) miR-34a binding sites (lower panel). (C) Immunoblotting of acetyl-p53 (Ac-p53). Representative immunoblots are shown. Results are expressed as mean $\pm$ SEM fold change from 6 different experiments. $^{\S}p < 0.05$ and $^{*}p < 0.01$ from Anti-miR-Control; $^{\ddagger}p < 0.05$ and $^{\ddagger}p < 0.01$ from Anti-miR-34a.

JNK1-activation of the p53/miRNA-34a/Sirtuin1 Pathway Contributes to Apoptosis Induced by DCA in Primary Rat Hepatocytes

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4.1. Abstract

Background and Aims: microRNAs (miRs) are increasingly associated with metabolic liver diseases. We have shown that ursodeoxycholic acid, an endogenous hydrophilic bile acid, counteracts the miR-34a/Sirtuin1(SIRT1)/p53 pathway, activated in the liver of non-alcoholic steatohepatitis (NASH) patients. In contrast, hydrophobic bile acids, particularly deoxycholic acid (DCA), activate apoptosis and are increased in NASH. We evaluated whether DCA-induced apoptosis of primary rat hepatocytes occurs *via* miR-34a-dependent pathways and whether they connect with c-Jun NH₂-Terminal Kinase (JNK) induction.

Methods: Primary rat hepatocytes were incubated with 100 microM DCA, and transfected with a specific miRNA-34a inhibitor or with a p53 overexpression plasmid. p53 transcriptional activity was assessed in nuclear extracts and by using target reporter constructs. Treating cells with Resveratrol overexpressed SIRT1. JNK function was evaluated by silencing experiments. Viability, caspase-3 activity and apoptosis were determined using the ApoTox-Glo™ Triplex Assay.

Results: Our results show that DCA enhances the miR-34a/SIRT1/p53 pro-apoptotic signalling in hepatocytes, in a dose- and time-dependent manner. In turn, miR-34a inhibition and SIRT1 overexpression significantly rescued targeting of the miR-34a pathway and apoptosis by DCA. In addition, p53 overexpression activated the miR-34a/SIRT1/p53 pathway, further induced by DCA. DCA increased p53 expression, as well as p53 transcriptional activation of PUMA and miR-34a itself, providing a functional mechanism for miR-34a activation. Finally, JNK1, but not JNK2, was shown to be a major target of DCA, upstream of p53, in engaging the miR-34a pathway and apoptosis.

Conclusions: These results suggest that the JNK1/p53/miR-34a/SIRT1 pathway may represent an attractive pharmacological target for the development of new drugs to arrest metabolic- and apoptosis-related liver pathologies.

Keywords: Apoptosis; DCA; JNK1; miRNA-34a; p53; SIRT1

4.2. Introduction

Intrahepatic accumulation of hydrophobic bile acids contributes to liver injury, which is associated with development of non-alcoholic steatohepatitis, cholestatic diseases, cholangiocarcinoma, and liver failure (Schmucker *et al.* 1990). Bile acid-induced apoptosis involves activation of the pro-apoptotic stress activated kinase, c-Jun NH₂-terminal kinase (JNK), leading to increased plasma membrane expression of the Fas and TRAIL death receptors and subsequent ligand independent activation (Higuchi *et al.* 2003; Higuchi *et al.* 2004). Caspase-8 is then activated, ultimately leading to apoptosis (Higuchi *et al.* 2003). Conversely, DCA may engage JNK *via* activation of death receptors, namely Fas and TGR5 (Gupta *et al.* 2004; Yang *et al.* 2007). DCA-induced apoptosis may also result from disruption of the mitochondrial transmembrane potential, through increased reactive oxygen species production, leading to translocation of pro-apoptotic BAX protein to the mitochondria and release of cytochrome *c* (Rodrigues *et al.* 2003). Finally, we have shown that DCA-induced apoptosis is also mediated by a cyclin D1/p53-dependent pathway (Castro *et al.* 2007a). Still, the mechanisms by which DCA induces apoptosis in the liver remain scattered, as for the network of targets signalling its actions.

microRNAs (miRNAs or miRs) are known to modulate the expression of numerous genes. In particular, upregulation of members of the miR-34 family induce apoptosis and cell cycle arrest. One of the main targets of miR-34a is Sirtuin1 (SIRT1), a NAD-dependent deacetylase that regulates apoptosis in response to oxidative and genotoxic stress (Yamakuchi *et al.* 2008). Furthermore, SIRT1 is capable of deacetylating all major p53 acetylation sites. SIRT1-mediated deacetylation antagonizes p53-dependent transcriptional activation, inhibiting p53-dependent apoptosis (Brooks *et al.* 2009). Still, the interplay of p53 with miR-34a remains a complex; not only does miR-34a regulates p53 activity through SIRT1, but miR-34a-induced apoptosis and cell cycle arrest are also, at least in part, dependent on the presence of p53 (Hermeking 2010). In fact, activation of p53 has been shown to increase miR-34a transcription, in a positive-feedback loop. Nevertheless, induction of miR-34a expression can also occur independently of p53 (Ichimura *et al.* 2010).

We have recently shown that ursodeoxycholic acid, a strong inhibitor of DCA-induced cell death (Rodrigues *et al.* 1998b; Rodrigues *et al.* 1999; Castro *et al.* 2007a), down-regulates the miR-34a/SIRT1/p53 pro-apoptotic pathway in primary rat hepatocytes. In turn, this pathway was also associated with non-alcoholic fatty liver disease severity (Castro *et al.* 2013). Therefore, in this study, we aimed to evaluate whether DCA modulates miR-34a-dependent pathways, with concomitant outcomes in viability and apoptosis of primary rat hepatocytes, and whether JNK acts as a novel regulator of this pathway. Our results support a link between liver cell apoptosis, miR-34a/SIRT1/p53, and JNK1 signalling, where JNK1-mediated activation of p53 is key to induction of miR-34a by DCA.

4.3. Materials and Methods

4.3.1. Cell culture and treatments

Primary rat hepatocytes were isolated from male rats (100-150 g) by collagenase perfusion (Mariash *et al.* 1986). All experiments involving animals were performed by an Investigator accredited for directing animal experiments (FELASA level C), in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals. Experiments received prior approval from the Portuguese National Authority for Animal Health (DGAV).

Primary rat hepatocytes were isolated from male rats by collagenase perfusion as previously described (Castro *et al.* 2010). After isolation, hepatocytes were resuspended in Complete William's E medium (Sigma-Aldrich Co., St Louis, MO, USA) (Castro *et al.* 2013) and plated on PrimariaTM tissue culture dishes (BD Biosciences, San Jose, CA, USA) at 5×10^4 cells/ cm². Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ for 6 hours, to allow attachment. Plates were then washed with medium to remove dead cells and incubated in Complete William's E medium treated with 10-400 µM DCA (Sigma-Aldrich Co.), or no addition (control) for 24 h before processing for total protein, RNA extraction, and cell viability. Alternatively, primary rat hepatocytes were treated with 100 µM DCA or no

addition (control) for 16, 28, 40, 52, and 64 h before processing for total protein and RNA extractions, cell viability and caspase activity assays, and Hoechst staining. When indicated, cells were co-incubated with 50 μ M of pan-caspase-inhibitor Z-VAD-fmk (Sigma-Aldrich Co.) for 30 min before DCA incubation.

To assess transfection efficiency of primary rat hepatocytes, cells were transfected with the pRNAT-H1.1/neo plasmid, expressing a green fluorescent protein (GFP) (GeneScript, Piscataway, NJ, USA) or with a Dy547-labeled miRIDIAN microRNA Mimic Transfection Control (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using LipofectamineTM (Life Technologies Corp.) (Park *et al.* 2011). Transfection efficiencies for plasmid DNA were typically around 50% while for miRNAs, values varied between 80 to 90%.

For functional analyses, primary rat hepatocytes were transfected at the moment of plating with 100 pM of a specific miR-34a precursor (Pre-miR-34a; Life Technologies Corp.), or with a Pre-miR negative control using LipofectamineTM (Life Technologies Corp.). Alternatively, miR-34a silencing was performed by transfecting primary rat hepatocytes with 100 pM of a miR-34a-specific inhibitor (Anti-miRNA-34a; Life Technologies Corp.), or with an anti-miRNA negative control. After 6 h, cells were incubated with 100 μ M DCA, or no addition (control). Hepatocytes were harvested at 40 h post-transfection and processed for RNA and total protein extraction, cell viability and caspase activity assays, and Hoechst staining.

To assess miR-34a-dependent SIRT1 inhibition, a reporter plasmid driven by the pMIR-REPORT and harbouring either a wild-type (Luc-SIRT1 Wt 3'UTR) or mutated (Luc-SIRT1 Mut 3'UTR) miR-34a target sequence within the 3' UTR of SIRT1 were used (Addgene plasmids 20379 and 20380; Cambridge, MA, USA) (Yamakuchi *et al.* 2008). Upon plating, primary rat hepatocytes were co-transfected with 500 ng of either construct, together with 100 pM of Pre-miR-34a or Anti-miR-34a, or respective controls, using LipofectamineTM.

To assess if DCA interacts with the SIRT1 promoter, a pGL3 reporter plasmid harbouring the SIRT1 promoter luciferase reporter gene was used (Xiong *et al.* 2011). At the time of plating, primary rat hepatocytes were

co-transfected with 500 ng of the luciferase reporter construct, or with a control reporter using LipofectamineTM.

To analyse whether DCA interacts with the miR-34a promoter *via* p53, pGL4 vectors harbouring the putative promoter regions of human miR-34a containing either wild-type (Luc miR-34a Wt p53) or mutant (Luc miR-34a Mut p53) p53 binding sites were used (He *et al.* 2007). In addition, p53 was overexpressed using a pCMV-Neo-Bam vector encoding wild-type human p53 (pCMV p53 Wt), or an empty vector (pCMV-Neo-Bam) (Addgene plasmids 16440 and 16434) (Baker *et al.* 1990).

At the time of plating, primary rat hepatocytes were co-transfected with 500 ng of the luciferase reporter constructs and 2 µg of the p53 expression vector, using LipofectamineTM. Cells were incubated with 100 µM DCA, or no addition (control), 6h after transfections. Reporter assays were performed 48 h post-transfection using the Dual-Luciferase® Reporter Assay System (Promega Corp., Madison, WI, USA) according with the manufacturer's instructions. Cells were also co-transfected with pRL-SV40 (Promega Corp.). Renilla luciferase activity was used as a transfection normalization control for all experiments involving luciferase reporter constructs.

To confirm JNK interaction with the miR-34a/SIRT1/p53 pro-apoptotic pathway, primary rat hepatocytes were transfected with 2 specific short interference RNA (siRNA) nucleotides (siJNK1 and siJNK2) designed to knock down *jnk* gene expression of each isoform of JNK (JNK1 and JNK2) in rats, purchased from Dharmacon (Waltham, MA, USA) (Wang *et al.* 2007). A control siRNA containing a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA was used as control. Primary rat hepatocytes were transfected with siRNA at the final concentration of 125 pM using LipofectamineTM. Cells were incubated with 100 µM DCA, or no addition (control), 6 h after transfections. Hepatocytes were harvested at 24 h posttransfection and processed for RNA and total protein extraction, cell viability and caspase activity assays. To confirm whether DCA interacts with JNK to regulate miR-34a/SIRT1/p53 pro-apoptotic pathway, the p53 binding site miR-34a promoter Luc miR-34a Wt p53 or Luc miR-34a Mut p53 were used (He *et al.* 2007). Moreover, in parallel experiments, miR-34a target sequence within the 3' UTR of SIRT1 were used (Luc-SIRT1 Wt 3'UTR or

Luc-SIRT1 Mut 3'UTR) (Addgene plasmids 20379 and 20380, Cambridge, MA, USA) (Yamakuchi *et al.* 2008). Upon plating, primary rat hepatocytes were co-transfected with 500 ng of either construct, together with 125 pM of JNK's siRNAs purchased from Dharmacon (Waltham, MA, USA), or respective controls, using Lipofectamine™.

To confirm the effects of JNK silencing in the miR-34a/SIRT1/p53 pathway primary rat hepatocytes were transfected with either the binding domain of the JNK interacting protein-1 (JIP-1) (pCMV-Flag-JBD (JIP-1)) (Dickens *et al.* 1997), or dominant negative DN-c-Jun FlagD169 plasmids (Ham *et al.* 1995), using Lipofectamine™. Cells were incubated with 100 μM DCA, or no addition (control), 6h after transfections. Hepatocytes were harvested at 24 h posttransfection and processed for RNA, cell viability and caspase activity assays. p53 activation was assessed based on natural reporter genes. We used pBV-Luc vector harboring the PUMA (PUMA Frag1-Luc or PUMA-Luc) promoter containing p53 responsive elements (16451, Addgene). The pBV-Luc construct was used as a control (plasmid 16539; Addgene). At the time of plating, primary rat hepatocytes were co-transfected with 500 ng of the luciferase reporter constructs, using Lipofectamine™.

4.3.2. Quantitative RT-PCR

RNA was extracted from cell samples using the TRIZOL Reagent following the manufacturer's instructions (Life Technologies Corp., Carlsbad, CA, USA). Real-time RT-PCR was performed in an Applied Biosystems 7300 System (Life Technologies Corp.), to quantitate the expression of miR-34a, miR-195, miR-200a and *sirt1*. U87 snRNA was used as the normalization control for miRNAs. The relative amounts of miRNAs were determined by the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{\text{target}} - Ct_{\text{U87}})_{\text{sample}} - (Ct_{\text{target}} - Ct_{\text{U87}})_{\text{calibrator}}$. To assess SIRT1 and β -actin mRNA levels, the following primer sequences were used: for SIRT1 gene 5' AGG GAA CCT CTG CCT CAT CTA C 3' (forward) and 5' GGC ATA CTC GCC ACC TAA CCT 3' (reverse), and for β -actin gene 5' AGG CCC CTC TGA ACC CTA AG 3' (forward) and 5' GGA GCG CGT AAC CCT CAT AG 3' (reverse). Three independent reactions for each primer sets were assessed in a total volume of 25 μL

containing 2X Power SYBR Green PCR master mix and 0.5 μ M of each primer. The relative amounts of SIRT1 mRNA were determined by the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{SIRT1mRNA} - Ct_{\beta-actin})_{sample} - (Ct_{SIRT1mRNA} - Ct_{\beta-actin})_{calibrator}$.

4.3.3. Immunoblotting

75 μ g of total protein extracts were separated on an 8% sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoretic transfer onto nitrocellulose membranes and blocking with 5% milk solution, blots were incubated overnight at 4°C with primary rabbit polyclonal antibodies against Ac-p53, Ac-H3 histone and H3 histone (Cell Signalling Technology, Danvers, MA, USA) or primary mouse monoclonal antibodies reactive to SIRT1 (Abcam PLC, Cambridge, UK), JNK, pJNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p53 (Cell Signalling Technology) and finally with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 3h at room temperature. Membranes were processed for protein detection using Super Signal substrate (Pierce, Rockford, IL, USA). β -actin was used as a loading control. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories) according to the manufacturer's specifications.

4.3.4. Immunocytochemistry

For SIRT1 localization in primary rat hepatocytes with overexpression of miR-34a incubated with 100 μ M DCA, or no addition (control). Cells were washed twice, fixed with paraformaldehyde (4%, w/v) in PBS and then blocked for 1 h at room temperature in PBS, containing 0.1% Triton-X-100, 1% FBS, and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc.). Cells were incubated with primary mouse monoclonal antibody reactive to SIRT1 (Abcam PLC) at a dilution of 0,5 μ g/mL overnight at 4°C. After washed twice, secondary DyLight 568 conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:200 and added to cells for 2 h at room temperature. Primary rat hepatocytes nuclei were stained with Hoechst 33258 (Sigma-Aldrich Co.) at 50 μ g/mL in PBS, for

6 min at room temperature. Samples were mounted using Fluoromount-G™ (Beckman Coulter, Inc., Brea, CA). Detection of SIRT1 puncta in cells was visualized using an Axioskop fluorescence microscope (Carl Zeiss GmbH, Jena, Germany) with magnification x 630.

4.3.5. Cell viability, cytotoxicity, and caspase activity

The ApoTox-Glo™ Triplex Assay (Promega Corp.) was used according to the manufacturer's protocol. Alternatively, cells were incubated with the Caspase-Glo®-8 or -9 reagents. LDH activity and MTS metabolism were also assessed as measures of cell death and viability, respectively. Briefly, to assess LDH release, supernatants resulting from a soft centrifugation of the cell culture media at 250 g, were combined in microplates with lactate (substrate), tetrazolium salt (coloring solution), and NAD (co-factor), previously mixed in equal proportions, following the manufacturer's instructions (Sigma-Aldrich Corp.). Multiwell plates were protect from light and incubated for 10 min at room temperature. Finally, absorbance was measured at 490 nm, with 690 nm as reference, using a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories). Cell viability was evaluated with CellTiter96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega), using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), according to the manufacturer's instructions. Absorbance was measured at 490 nm, with 620 nm as reference, using a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories). Finally, Hoechst labelling of attached cells was used to detect apoptotic nuclei by morphological analysis, as previously described (Castro *et al.* 2013).

4.3.6. p53 activity

For assaying p53 activity, the TransAM™ p53 transcription factor assay kit (Active Motif, Carlsbad, CA, USA) and the p53/MDM2 ImmunoSet™ Assay (Enzo Life Sciences, Farmingdale, NY) were used according to the manufacturer's protocols.

4.3.7. Densitometry and statistical analysis

The relative intensities of protein bands were analysed using the Quantity One Version 4.6 densitometric analysis program (Bio-Rad Laboratories). Statistical analysis was performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

4.4. Results

4.4.1. DCA induces the miR-34a/SIRT1/p53 pro-apoptotic pathway in primary rat hepatocytes

DCA is a well-known inducer of apoptosis *via* both death receptor and mitochondrial pathways of apoptosis (Higuchi *et al.* 2003; Castro *et al.* 2007a; Castro *et al.* 2007b). Still, its pleiotropic mechanisms of action remain scattered. In contrast, ursodeoxycholic acid is a strong inhibitor of apoptosis, including DCA-induced cell death (Rodrigues *et al.* 1998b; Rodrigues *et al.* 1999; Castro *et al.* 2007a). In addition, we have recently demonstrated that its cytoprotective mechanisms appear to involve inhibition of the miR-34a/SIRT1/p53 pro-apoptotic pathway (Castro *et al.* 2013). Therefore, we analysed whether DCA could induce apoptosis through the miR-34a signalling pathway in primary rat hepatocytes. As a proof-of-principle, we started by evaluating the dose-dependent effects of DCA upon the miR34a/SIRT1/p53 pro-apoptotic pathway. Primary rat hepatocytes were incubated with 10 to 400 μ M DCA for 24h. Our results indicated that hepatocytes incubated with 100 μ M DCA showed a progressive and significant decrease in cell viability from ~30 to 60% when incubated with 100 μ M and 400 μ M DCA, respectively ($p < 0.01$) (**Fig. 4.1.A**). Conversely, DCA-induced cell death, as measured by LDH release, increased from ~45% to more than 2-fold ($p < 0.01$) (**Fig. 4.1.B**). We next evaluated whether the miR-34a/SIRT1/p53 pathway was specifically activated by DCA in cultured primary rat hepatocytes. miR-34a induces apoptosis by repressing SIRT1, which then leads to p53 acetylation and activation, with induction of pro-apoptotic genes (Yamakuchi *et al.* 2008). In fact, induction of miR-34a

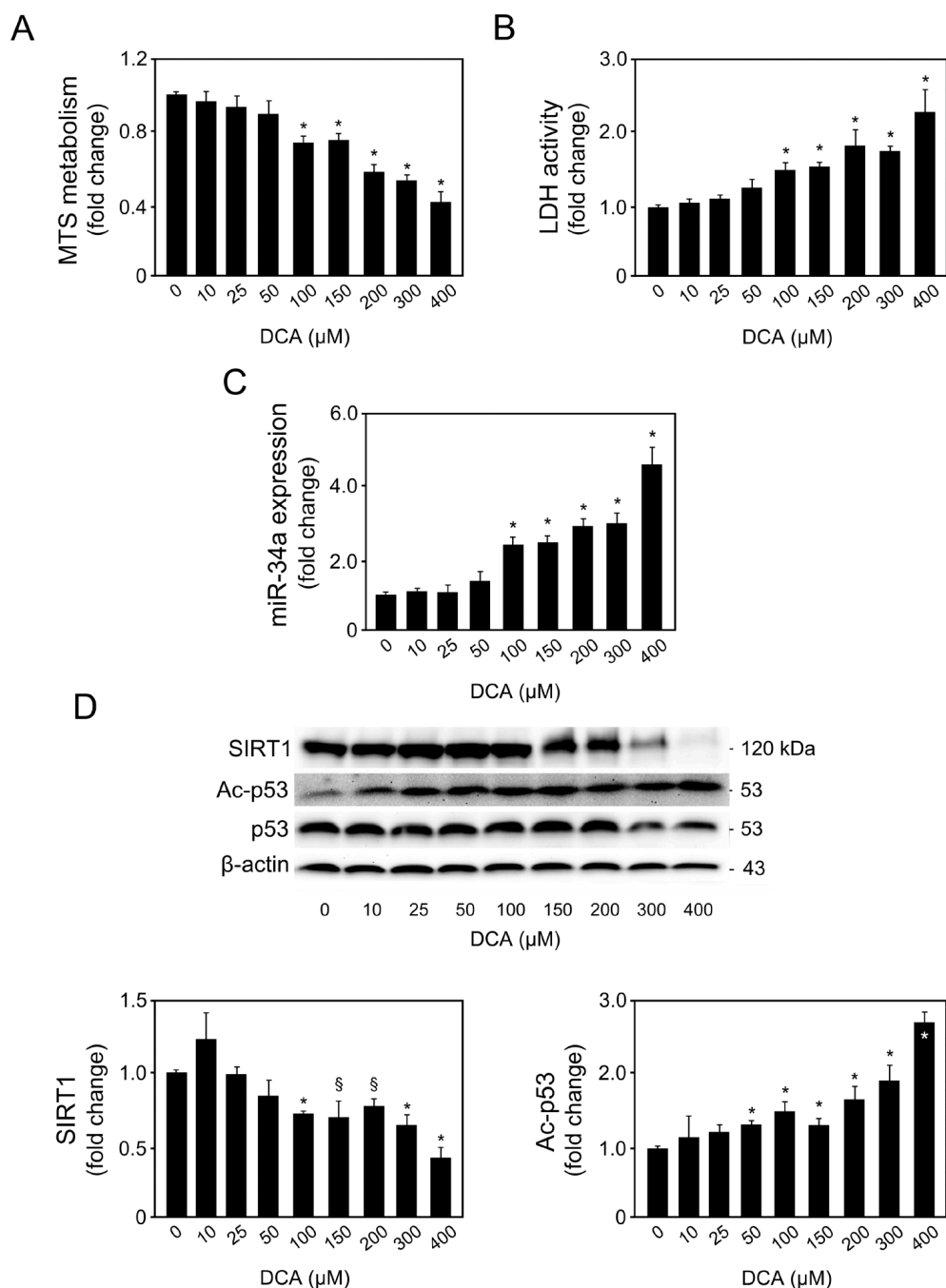


Figure 4.1. DCA induces apoptosis and the miR-34a/SIRT1/p53 pathway in primary rat hepatocytes in a dose-dependent manner. Cells were isolated as described in Material and Methods and treated with 10-400 μM DCA, or no addition (control) for 24 h. **A:** MTS metabolism and **B:** LDH activity. **C:** Real-time RT-PCR analysis of miR-34a. **D:** Immunoblotting of SIRT1 and acetyl-p53. Representative immunoblots are shown. Blots were normalized to endogenous β-actin or total p53, respectively. Results are expressed as mean ± SEM fold change of at least 4 independent experiments. §*p* < 0.05 and **p* < 0.01 from Control.

expression by DCA was also shown to be dose-dependent (**Fig. 4.1.C**). Concentrations as low as 50 μ M DCA resulted in slightly induced miR-34a expression. DCA further, and significantly, induced miR-34a expression levels by almost 2.5 fold at 100 μ M, up to ~5-fold at 400 μ M ($p < 0.01$), comparing to control.

Importantly, DCA also significantly decreased SIRT1 expression, while increasing acetylated p53 levels in a dose-dependent manner, for doses higher than 50 μ M (at least $p < 0.05$) (**Fig. 4.1.D**). Of note, several other miRNAs have been shown to target SIRT1. For instance, miR-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating SIRT1 (Zhu *et al.* 2011). Also, miR-200a has been shown to regulate SIRT1 expression in mammary epithelial cells, regulating epithelial to mesenchymal transition-like transformation (Eades *et al.* 2011). Therefore, to confirm the specific regulation of SIRT1 by DCA *via* miR-34a, modulation of miR195 and miR-200a expressions by DCA were also evaluated (**Suppl. Fig. 4.1.**). Both miRNAs expression levels were unchanged in the presence of 10-300 μ M DCA. Only when in the presence of 400 μ M DCA, miR-195 and miR-200a expression increased, probably reflecting an un-specific effect of this high, non-physiological DCA concentration. All together, these results suggest that the miR-34a/SIRT1/p53 pathway is activated by DCA in primary rat hepatocytes, in a dose-dependent manner. To further explore this notion, cells were incubated with 100 μ M DCA for 16 to 64h (**Fig. 4.2.**). Primary rat hepatocytes displayed a progressive decrease in cell viability from 20%, after 16 h of incubation, to a maximum of ~60%, after 40 to 52 h of incubation ($p < 0.01$) (**Fig. 4.2.A**). This effect was concomitant with a > 60% increase in caspase-3-like activity by DCA up until 52 h of incubation (at least $p < 0.05$), as measured by the ApoTox-GloTM Triplex Assay (**Fig. 4.2.B**) and Western blot analysis (data not shown). Co-incubation of cells with DCA and pan-caspase inhibitor z-VAD.fmk completely abrogated DCA-induced cytotoxicity (**Suppl. Fig. 4.2.**), evidencing that apoptosis is a major cell death pathway induced by DCA. In fact, DCA also increased caspase-8- and -9-like activities, up until 52 h of incubation ($p < 0.05$) (data not shown), implying the engagement of both the death receptor and the mitochondrial pathways of

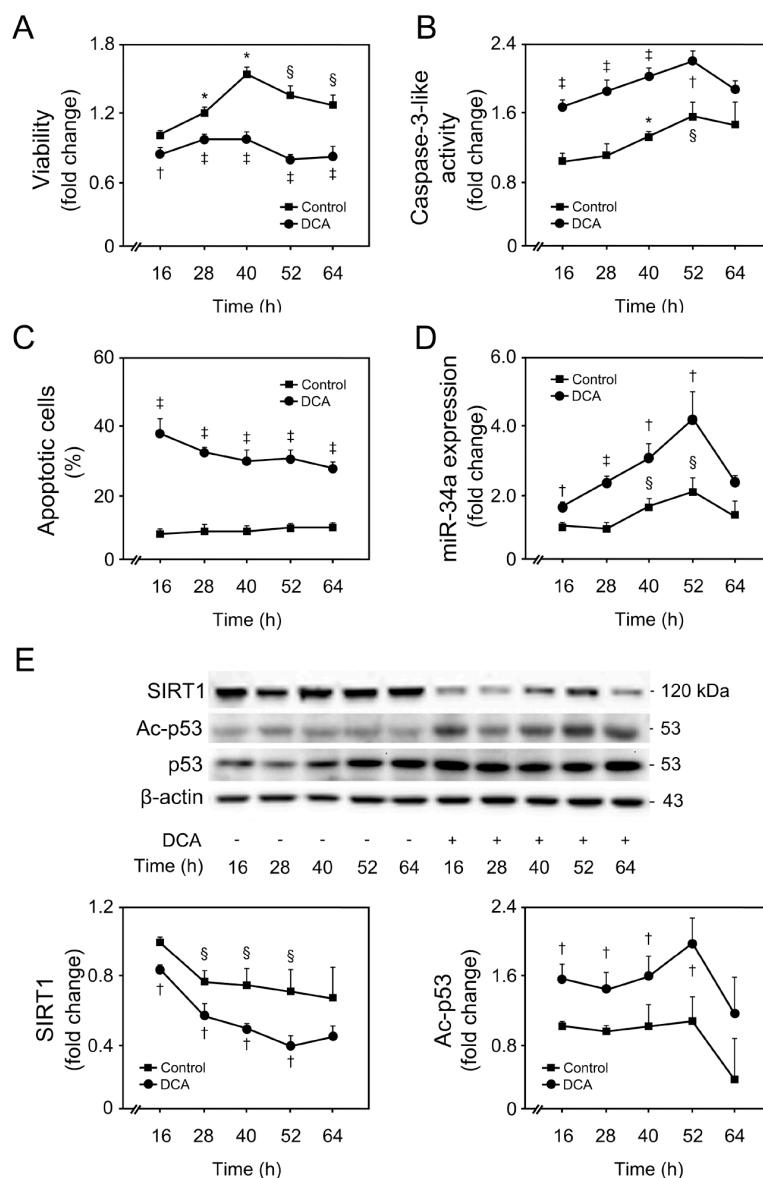


Figure 4.2. DCA induces apoptosis and the miR-34a/SIRT1/p53 pathway in primary rat hepatocytes in a time-dependent manner. Cells were isolated as described in Material and Methods and treated with 100 μ M DCA, or no addition (control) for 16, 28, 40, 52, and 64 h. **A:** Viability and **B:** Caspase-3-like activity measured using the ApoTox-GloTM Triplex Assay. **C:** Apoptotic cells were detected by Hoechst staining and results are expressed as percentage of apoptotic cells. **D:** Real-time RT-PCR analysis of miR-34a. **E:** Immunoblotting of SIRT1 and acetyl-p53. Representative immunoblots are shown. Blots were normalized to endogenous β -actin or total p53, respectively. Results are expressed as mean $\pm$ SEM fold change of at least 4 independent experiments. $^{\S}p < 0.05$ and $^*p < 0.01$ from 16 h Control; $^{\dagger}p < 0.05$ and $^{\ddagger}p < 0.01$ from respective time-point control.

apoptosis (Ignacio Barrasa *et al.* 2011). End-stage apoptosis, evaluated using Hoechst staining, confirmed DCA-induced nuclear fragmentation in ~30% of cultured hepatocytes throughout the time-course ($p < 0.01$) (**Fig. 4.2.C**). For exposure periods longer than 64 hours, DCA-induced cell death shifted more to a necrotic nature, likely reflecting an increase of its toxicity, independently of activation of programmed apoptosis.

miR-34a basal expression levels slightly increased in primary rat hepatocytes with time in culture ($p < 0.05$), whereas DCA significantly induced miR-34a expression up until 52 h of incubation ($p < 0.05$) (**Fig. 4.2.D**), in agreement with our previous results. Both SIRT1 expression and p53 acetylation, normalized with total p53 levels, reflected miR-34a expression changes in control hepatocytes (**Fig. 4.2.E**). Importantly, DCA significantly decreased SIRT1 expression ($p < 0.05$), while increasing acetylated p53 levels ($p < 0.05$) up until 52 h of incubation. Histone H3 acetylation levels were also evaluated, as an additional target of SIRT1 modulation. In fact, DCA also increased acetylated Histone H3 levels but only up until 40h of incubation, suggesting that p53 acetylation is a major effect of DCA-induced inhibition of SIRT1 expression (data not shown).

4.4.2. Activation of miR-34a is an important event during DCA-induced apoptosis

Hydrophilic bile acids, namely ursodeoxycholic and tauroursodeoxycholic acids, significantly inhibit miR-34a/SIRT1/p53-dependent apoptosis (Castro *et al.* 2013). Therefore, our results suggest that activation of the miR-34a/SIRT1/p53 pro-apoptotic pathway is a specific effect of DCA, and not other bile acids, resulting in apoptosis of primary rat hepatocytes, at least in early stages.

To clarify this, and to determine to which extent is miR34a essential to DCA-mediated cell death, primary rat hepatocytes were transfected with a miR-34a inhibitor, in the presence or absence of DCA. As expected, miR-34a was markedly decreased in Anti-miR-34a transfected cells, compared with Anti-miR control ($p < 0.05$) (**Fig. 4.3.A**). DCA significantly counteracted miR-34a downregulation ($p < 0.05$). In addition, miR-34a inhibition increased SIRT1 protein levels by $> 80\%$ ($p < 0.01$), with DCA preventing this increase

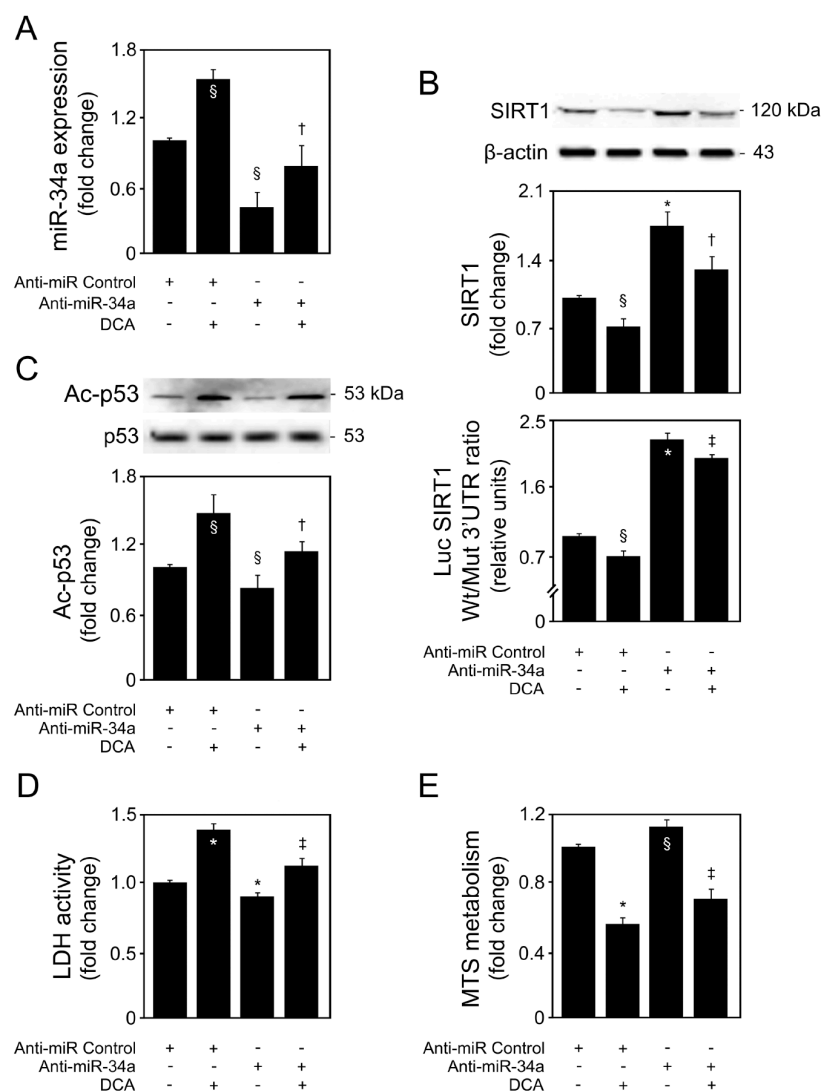


Figure 4.3. miR-34a inhibition impairs the ability of DCA to inhibit SIRT1 and induce Ac-p53. Primary rat hepatocytes were transfected with a miR-34a inhibitor (Anti-miR-34a) or control (Anti-miR-Control) and treated with 100 μ M DCA, or no addition (control) for 40 h, as described in Material and Methods. **A:** Real-time RT-PCR analysis of miR-34a overexpression. **B:** Immunoblotting of SIRT1 in cells transfected with the miR-34a inhibitor (top); and ratio between Wt and Mut miR-34a luciferase activity (bottom). Representative immunoblots are shown. Blots were normalized to endogenous β -actin. Cells were co-transfected with a reporter vector consisting of a luciferase cDNA fused to the 3'-UTR of SIRT1, containing either a wild-type (Wt) or mutant (Mut) miR-34a binding site. The CMV-Renilla luciferase vector served as an internal standard control. **C:** Immunoblotting of acetyl-p53. Representative immunoblots are shown. Blots were normalized to total p53. **D:** LDH activity and **E:** MTS metabolism. Results are expressed as mean $\pm$ SEM fold change

from 6 different experiments. [§] $p < 0.05$ and ^{*} $p < 0.01$ from Anti-miR-Control; [†] $p < 0.05$ and [‡] $p < 0.01$ from Anti-miR-34a.

($p < 0.05$) (**Fig. 4.3.B**, top). These results were validated in cells co-transfected with a luciferase reporter construct containing the wild-type miR-34a binding site within SIRT1 3'UTR (Luc-SIRT1 Wt 3'UTR), or a mutated sequence (Luc-SIRT1 Mut 3'UTR), together with Anti-miR-34a (**Fig. 4.3.B**, bottom). p53 acetylation was decreased by almost 30% following miR-34a inhibition ($p < 0.05$), an effect completely abrogated by DCA ($p < 0.05$) (**Fig. 4.3.C**). Finally, the effects of miR-34a inhibition in DCA-induced cell death were also evaluated. miR-34a inhibition alone slightly, but significantly, inhibited cell death ($p < 0.01$) (**Fig. 4.3.D**) and increased cellular viability ($p < 0.05$) (**Fig. 4.3.E**). More importantly, it significantly impaired the ability of DCA to induce primary hepatocyte cell death ($p < 0.01$) and decrease cellular viability ($p < 0.01$).

To clearly establish that DCA specifically modulates the miR-34a-dependent pro-apoptotic pathway, cells were alternatively transfected with a miR-34a precursor, in the presence or absence of DCA. In agreement with the previous results, DCA potentiated the effects of miR-34a overexpression ($p < 0.05$) (**Fig. 4.4.A**), including on SIRT1 inhibition ($p < 0.05$) (**Fig. 4.4.B**). Curiously, the magnitudes of both effects were quite different, probably indicating that the system becomes so saturated with miR-34a levels that its downstream effects are not further modulated in the same proportion as its increased expression. SIRT1 expression and localization was also assessed by immunocytochemistry, in parallel with Hoechst staining. SIRT1 expression was strong in control cells, being localized in large punctae in both the cytoplasm and the nucleus (**Fig. 4.4.C**). Pre-miR-34a- and DCA-treated cells displayed decreased number and intensity of SIRT1 puncta, with lower accumulation of nuclear SIRT1. Curiously, modulation of SIRT1 by DCA appears to be mostly posttranscriptional, as DCA had little effects in cells transfected with a reporter plasmid harboring the SIRT1 promoter (data not shown). In fact, neither miR-34a overexpression, nor DCA were able to significantly modulate *sirt1* mRNA levels (**Fig. 4.4.D**), further indicating that DCA inhibits SIRT1 protein expression *via* miR-34a. As for p53 acetylation,

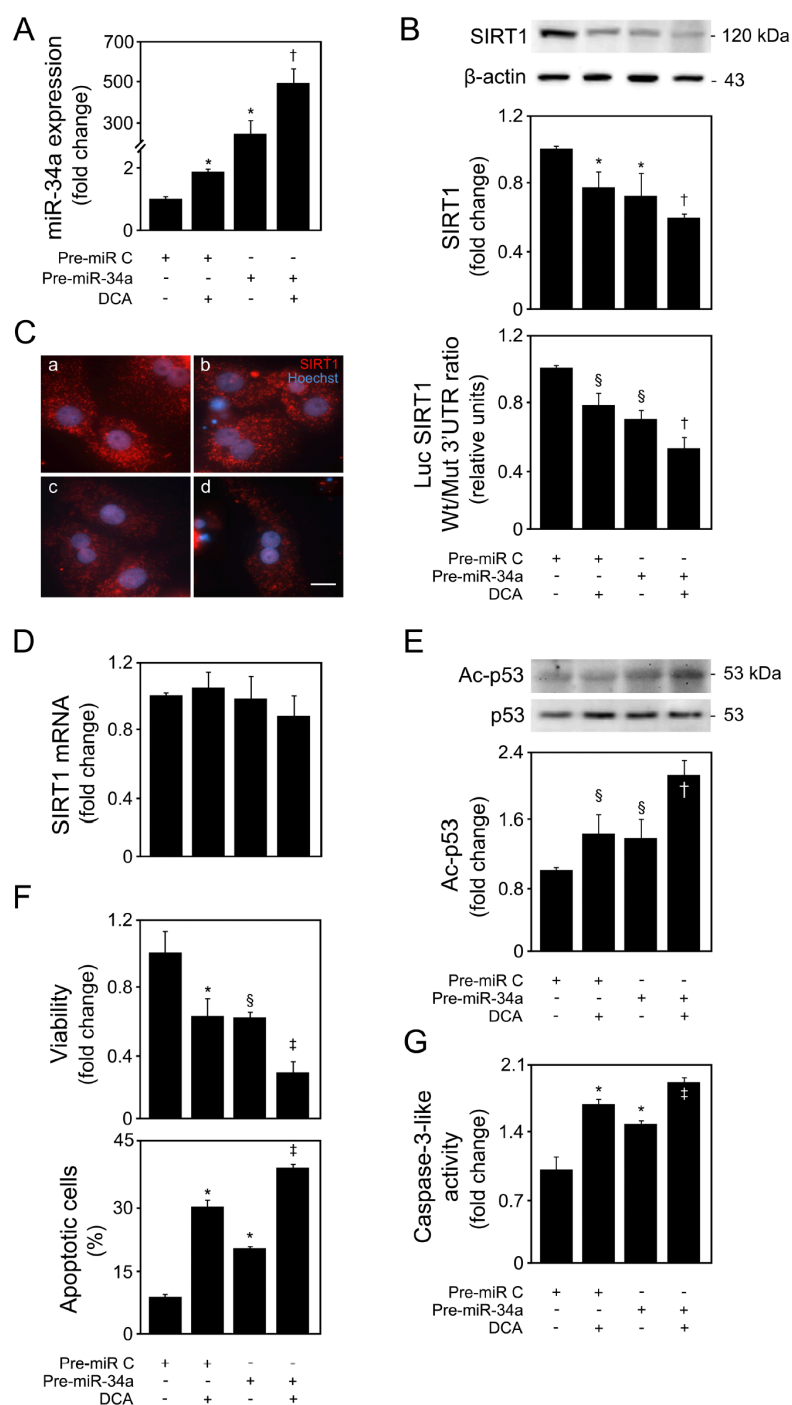


Figure 4.4. DCA exacerbates miR-34a-dependent signalling and apoptosis in primary rat hepatocytes. Cells were transfected with a miR-34a precursor (Pre-miR-34a) or control (Pre-miR-Control) and treated with 100 μ M DCA, or no addition (control) for 40 h, as described in Material and Methods. **A:** Real-time RT-PCR analysis of miR-34a overexpression. **B:** Immunoblotting of SIRT1 in cells transfected with the miR-34a precursor (top); and ratio between Wt and Mut SIRT1 3'UTR luciferase activity (bottom). Representative immunoblots are shown. Blots were normalized to endogenous β -actin. Primary rat

hepatocytes were co-transfected with a reporter vector consisting of a luciferase cDNA fused to the 3'-UTR of SIRT1, containing either a wild-type (Wt) or mutant (Mut) miR-34a binding site. The CMV-Renilla luciferase vector served as an internal standard control. **C:** SIRT1 localization determined by immunocytochemistry. SIRT1 staining (red) and Hoechst staining (blue) is shown as control (a), DCA (b), miR-34a overexpression (c), and miR-34a overexpression with DCA treatment (d). Scale bar, 10 μ m. Magnification, x 630. **D:** SIRT1 mRNA levels were measured by Real Time RT-PCR. **E:** Immunoblotting of acetyl-p53. Representative immunoblots are shown. Blots were normalized to total p53. **F:** Cell viability (top), determined by the ApoTox-Glo™ Triplex Assay and apoptosis (bottom), determined by Hoechst staining. **G:** Caspase-3-like activity determined by the ApoTox-Glo™ Triplex Assay. Results are expressed as mean $\pm$ SEM percentage from 6 different experiments. $^{\S}p < 0.05$ and $^*p < 0.01$ from Pre-miR-Control; $^{\dagger}p < 0.05$ and $^{\ddagger}p < 0.01$ from Pre-miR-34a.

its levels were increased following miR-34a overexpression ($p < 0.05$) and further by DCA ($p < 0.05$) (**Fig. 4.4.E**). Finally, miR-34a overexpression significantly decreased cell viability ($p < 0.05$) (**Fig. 4.4.F**, top), while increasing apoptosis ($p < 0.01$) (**Fig. 4.4.F**, bottom) and caspase-3-like activity ($p < 0.01$) (**Fig. 4.4.G**). miR-34a overexpressing cells treated with DCA were less viable ($p < 0.01$) and had higher levels of caspase-3 activity ($p < 0.01$) and apoptosis ($p < 0.01$), compared with miR-34a overexpression cells alone, further reinforcing the notion that induction of apoptosis by DCA is largely dependent on miR-34a.

4.4.3. Targeting of SIRT1 by DCA via miR-34a plays a key role on its ability to activate p53 and apoptosis

In order to determine to which extent is miR-34a-dependent SIRT1 down-regulation by DCA critical for its apoptotic effects, primary rat hepatocytes were incubated with resveratrol, in the presence or absence of DCA, in order to overexpress SIRT1. Cells incubated with 10 and 50 μ M resveratrol displayed a ~30 ($p < 0.05$) and 65% ($p < 0.01$) increase in SIRT1 protein levels, respectively (**Fig. 4.5.A**). Interestingly, DCA was no longer capable of significantly reducing SIRT1 in the presence of resveratrol

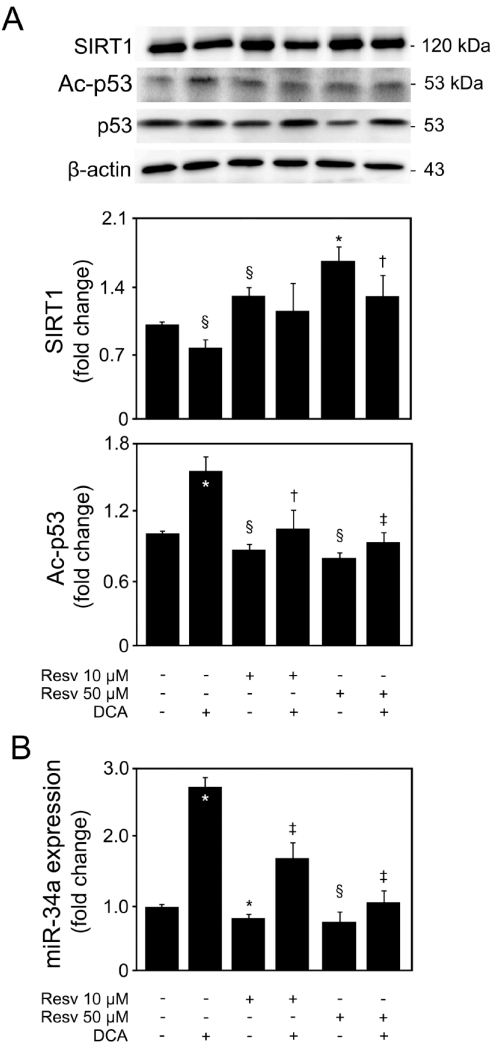


Figure 4.5. Overexpression of SIRT1 impairs DCA induction of the miR-34a/SIRT1/p53 pathway in primary rat hepatocytes. Cells were isolated as described in Material and Methods and treated with 10 μM or 50 μM Resveratrol or no addition (control) for 24 h. When indicated, cells were co-incubated with 100 μM DCA. **A:** Immunoblotting of SIRT1 and acetyl-p53. Representative immunoblots are shown. Blots were normalized to endogenous β-actin or total p53, respectively. **B:** Real-time RT-PCR analysis of miR-34a. Results are expressed as mean ± SEM fold change of at least 4 independent experiments. Resv, Resveratrol. [§] $p < 0.05$ and ^{*} $p < 0.01$ from Control; [†] $p < 0.05$ and [‡] $p < 0.01$ from DCA alone.

($p < 0.05$). Moreover, DCA-induced cell death and caspase-3 activity were also inhibited (data not shown). Importantly, SIRT1 overexpression also impacted on the ability of DCA to acetylate p53 (**Fig. 4.5.A**). In fact, DCA-induced p53 acetylation was abrogated in the presence of resveratrol (at least $p < 0.05$), indicating that targeting of SIRT1 by DCA mediates p53

activation. Curiously, we have recently shown that ursodeoxycholic acid inhibits miR-34a-dependent apoptosis by reducing p53 transactivation (Castro *et al.* 2013) and that DCA-induced apoptosis is associated with p53-dependent mechanisms (Castro *et al.* 2007a). In addition, SIRT1 also regulates p53 dependent apoptosis through deacetylating and stabilizing p53. Furthermore, p53 induces expression of miR-34a, which suppresses SIRT1, resulting in a positive feedback loop (Yamakuchi *et al.* 2008; Yamakuchi *et al.* 2009). Therefore, we next investigated whether SIRT1 overexpression was also affecting miR-34a activation, and whether this impacted on the ability of DCA to induce miR-34a. SIRT1 overexpression decreased miR-34a expression levels ($p < 0.01$) (**Fig. 4.5.B**). More importantly, DCA-induced miR-34a was significantly reduced in the presence of resveratrol ($p < 0.01$), suggesting that DCA-induced p53 activation may, in fact, be an important regulatory step in engaging the miR-34a/SIRT1 pathway of apoptosis in primary rat hepatocytes. Again, this appears to be a specific effect, as miR-195 and miR-200a expressions were not significantly affected by SIRT1 overexpression, either in the presence or absence of DCA (data not shown).

4.4.4. DCA engages the miR-34a/SIRT1-dependent pro-apoptotic pathway via p53

Since p53 arose as a likely target of DCA in activating the miR34a/SIRT1 pathway, we next investigated whether p53-induced miR-34a expression was enhanced by DCA. Primary rat hepatocytes were transfected with a construct overexpressing p53, in the presence or absence of DCA. Both DCA and p53 overexpression led to a 2-fold increase in miR-34a expression ($p < 0.05$) (**Fig. 4.6.A**). Incubation of p53-overexpressing cells with DCA further increased miR-34a expression by almost 3-fold ($p < 0.05$), as compared with empty vector-transfected cells. In agreement, inhibition of SIRT1 by p53 overexpression was potentiated in the presence of DCA ($p < 0.05$) (**Fig. 4.6.B**). To elucidate whether DCA activation of the miR-34a/SIRT1/p53 pro-apoptotic pathway was dependent on p53, we first analysed total p53 levels in cells incubated with DCA with or without p53 overexpression. In control-transfected cells, DCA increased total p53 levels

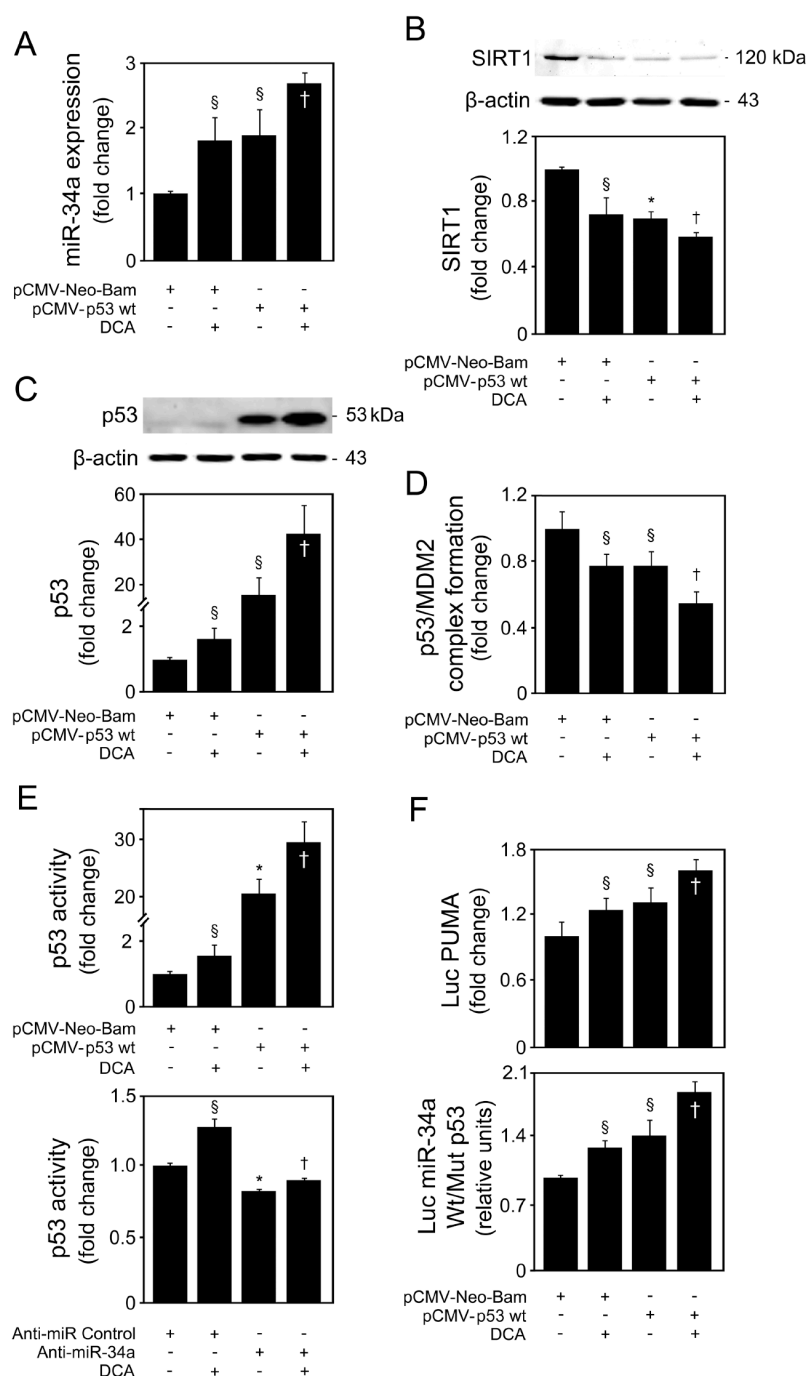


Figure 4.6. DCA induces p53-dependent activation of the miR-34a apoptotic pathway in primary rat hepatocytes. Cells were transfected using a pCMV-Neo-Bam vector harboring the wild-type p53 human sequence (pCMV-p53 Wt), or an empty vector (pCMV-Neo-Bam) and treated with 100 μ M DCA, or no addition (control) for 24 h, as described in Materials and Methods. **A:** Real-time RT-PCR analysis of miR-34a expression. **B:** Immunoblotting of SIRT1 and **C:** p53. Representative immunoblots are shown. Protein blots were normalized to endogenous β -actin. **D:** p53/MDM2 binding as determined by the ImmunoSet™

p53/MDM2 complex-specific immunometric enzyme immunoassay and expressed as -fold change relative to the control. **E:** Levels of nuclear p53 capable to bind to its DNA consensus recognition sequence, as determined by the TransAM™ p53 enzyme-linked immunosorbent assay with p53 overexpression (top) and miR-34a inhibition (bottom). **F:** p53-dependent PUMA and miR-34a promoter activation. Primary rat hepatocytes were co-transfected with a luciferase construct with the PUMA promoter containing consensus p53 binding sites upstream of the transcription start site (Luc PUMA) (top). Alternatively, cells were co-transfected with a pGL4 reporter vector consisting of a luciferase cDNA fused to miR-34a promoter containing either wild-type (Wt) or mutant (Mut) p53 binding sequence (bottom). Cells were also co-transfected with a CMV-Renilla luciferase vector as an internal standard. Results are expressed as mean $\pm$ SEM fold change from 5 different experiments. $^{\S}p < 0.05$ and $^*p < 0.01$ from pCMV-Neo-Bam alone; $^{\dagger}p < 0.05$ from pCMV-Neo-Bam with DCA.

by $> 50\%$ ($p < 0.05$) (**Fig. 4.6.C**) and, more strikingly, further increased p53 levels in p53-overexpressing cells ($p < 0.05$). We also investigated whether DCA was simultaneously activating p53 at the post-transcriptional level by analysing p53/MDM2 complex formation. DCA and p53 overexpression alone inhibited complex formation by almost 30% ($p < 0.05$) (**Fig. 4.6.D**). In addition, both synergistically decreased p53/MDM2 association in $\sim 50\%$ ($p < 0.05$), compared with control-transfected cells. To assess whether the reduced p53/MDM2 complex formation was related with increased p53 transactivity, we used a p53 transcription factor assay kit. DCA was shown to induce p53 activity, either alone ($p < 0.05$) or after p53 overexpression ($p < 0.05$) (**Fig. 4.6.E**, top). In addition, and attesting to the targeting of the p53/miR-34a/SIRT1 positive feedback loop by DCA, our results also showed that DCA-induced p53 activity was completely abrogated in cells transfected with Anti-miR-34a ($p < 0.01$), when comparing with Anti-miR-control treated cells (**Fig. 4.6.E**, bottom).

As an additional measure of p53 activation, we next analysed the ability of DCA to modulate transcriptional activation of p53 target PUMA (**Fig. 4.6.F**, top). In agreement with our previous results, p53 overexpression or DCA treatment alone increased the promoter activity of PUMA ($p < 0.05$), as compared with control. Maximum activation was seen in cells

overexpressing p53 and incubated with DCA ($p < 0.05$). Finally, to clearly establish that activation of the miR-34a/SIRT1/p53 pathway by DCA is largely dependent on its ability to increase p53 expression and activity, cells were co-transfected with luciferase reporter constructs under the transcriptional control of human miR-34a promoter elements containing either wild-type or mutant p53 binding sites. p53 overexpression increased wild-type miR-34a promoter activity by almost 40%, ($p < 0.05$) (**Fig. 4.6.F**, bottom). Significantly, DCA treatment increased wild-type miR-34a promoter activity either alone ($p < 0.05$) or in p53 overexpressing cells ($p < 0.05$). In addition, DCA also increased apoptosis induced by p53 overexpression (data not shown). Altogether, these results indicate that DCA activates p53 by inducing both its expression and transcriptional activity, as well as reducing p53/MDM2 complex formation, resulting in a strong, functional activation of the miR-34a/SIRT1/p53 pro-apoptotic pathway.

4.4.5. p53/miR-34a/SIRT1-dependent apoptosis by DCA is activated by JNK1

Bile acid-induced apoptosis was already shown to involve JNK1/2 activation (Gupta *et al.* 2004; Higuchi *et al.* 2004). In fact, JNK overactivation is one of the most common effector mechanisms of liver injury, including for DCA (Qiao *et al.* 2003). In our model, DCA significantly induced JNK1/2 phosphorylation up until 40 h of incubation ($p < 0.05$) (**Fig. 4.7.A**). Curiously, JNK can also, directly or indirectly, modulate p53 expression and positively influence apoptosis. In fact, JNK signalling may stabilize p53 and enhance its ability to elicit cellular apoptosis by inducing p53 phosphorylation and leading to attenuation of the p53/MDM2 interaction (Fuchs *et al.* 1998; Ljungman 2000). Finally, we have recently shown that JNK phosphorylation levels increase with non-alcoholic fatty liver disease severity, in parallel with miR-34a expression (Ferreira *et al.* 2011; Castro *et al.* 2013). Therefore, we next analysed whether DCA-induced JNK was the mechanistic link responsible for p53/miR-34a activation. Primary rat hepatocytes were transfected with siRNAs targeting either JNK1 or JNK2, as these JNK isoforms appear to have very different and opposite functions in hepatocyte

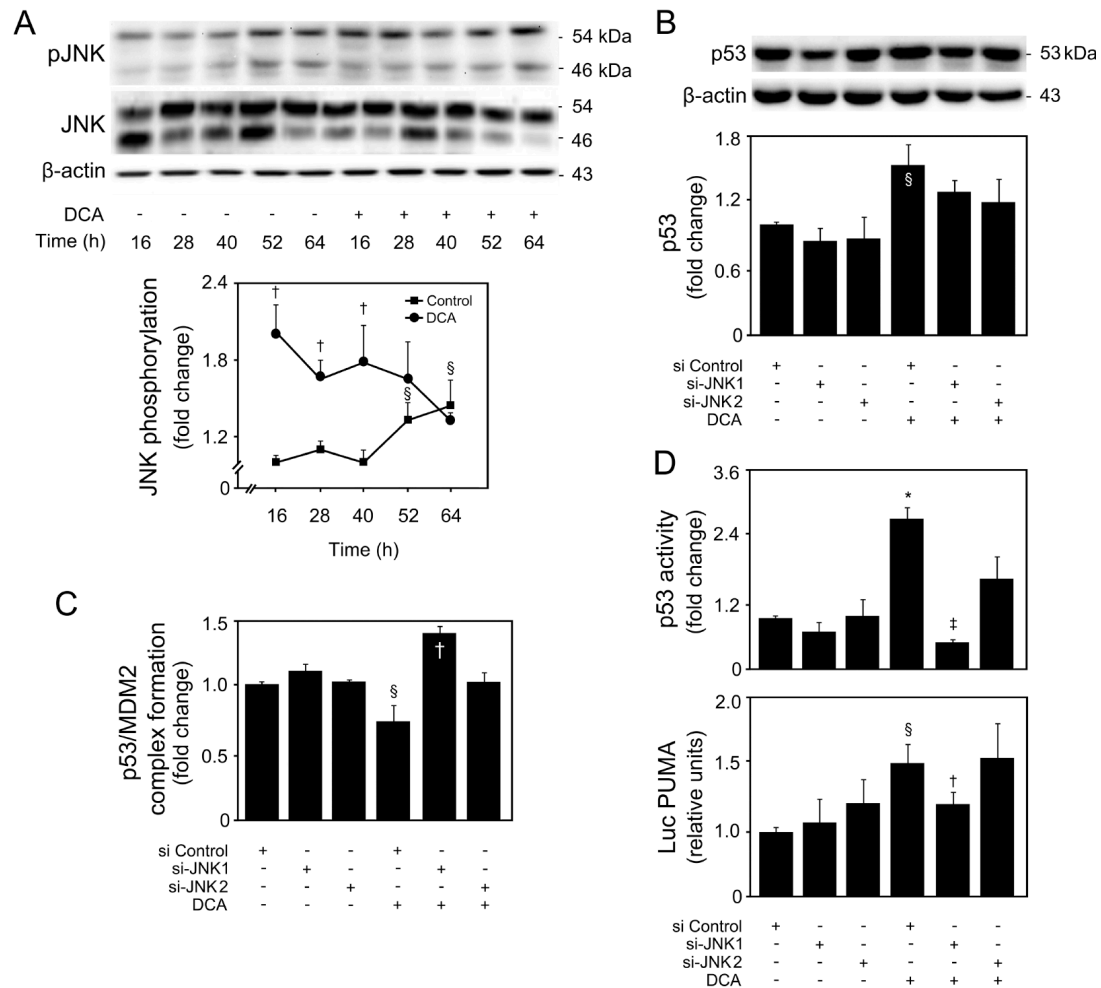


Figure 4.7. JNK1 is responsible for DCA-induced p53 activation in primary rat hepatocytes. Cells were treated with 100 μ M DCA, or no addition (control) for 16, 28, 40, 52, and 64 h. Alternatively, cells were transfected with specific short interference RNA (siRNA) nucleotides designed to knock down *jnk1* or *jnk2* gene expression, and a control siRNA containing a scrambled sequence, and treated with 100 μ M DCA, or no addition (control) for 24 h, as described in Material and Methods. **A:** Immunoblotting of JNK phosphorylation (pJNK) and **B:** p53 expression. Representative immunoblots are shown. Blots were normalized to total JNK or β -actin, respectively. **C:** p53/MDM2 binding as determined by the ImmunoSet™ p53/MDM2 complex-specific immunometric enzyme immunoassay and expressed as fold change relative to control. **D:** Levels of nuclear p53 capable to bind to its DNA consensus recognition sequence, as determined by the TransAM™ p53 enzyme-linked immunosorbent assay (top) and p53-dependent PUMA promoter activation (bottom). Primary rat hepatocytes were co-transfected with a luciferase construct with the PUMA promoter containing consensus p53 binding sites upstream of the transcription start site (Luc PUMA). Results were normalized for the CMV-Renilla luciferase activity. Results are expressed as mean $\pm$ SEM fold change from 7 different experiments. $^{\S}p < 0.05$ and $^*p < 0.01$ from Control or si Control; $^{\dagger}p < 0.05$ and $^{\ddagger}p < 0.01$ from respective time-point control or from si Control with DCA.

death (Amir *et al.* 2012). Upon silencing, JNK1 levels decreased by ~ 75 % ($p < 0.01$), while JNK2 levels were inhibited in ~ 60 % ($p < 0.01$) (data not shown). Interestingly, DCA-induced p53 expression (**Fig. 4.7.B**), but more significantly p53/MDM2 dissociation (**Fig. 4.7.C**), and activation (**Fig. 4.7.D**), were almost completely abolished in the absence of JNK1 ($p < 0.05$), but not when JNK2 was silenced. In fact, DCA also increased JNK1 expression and phosphorylation (data not shown).

When specifically looking at the miR-34a/SIRT1 pro-apoptotic pathway, our results showed that DCA-induced miR-34a expression, as seen in cells transfected with a miR-34a promoter Luc-construct (**Fig. 4.8.A**) and by Real-Time RT-PCR (**Fig. 4.8.B**), was also inhibited by JNK1 silencing ($p < 0.05$). In addition, the loss of SIRT1 (**Fig. 4.8.C**) and cell viability (**Fig. 4.8.D**) induced by DCA was almost completely restored when JNK1, but not JNK2, was silenced ($p < 0.05$). Altogether, these results suggest that activation of JNK1 by DCA is responsible for engaging p53-dependent apoptotic pathways, particularly the miR-34a signalling pathway.

To better clarify the mechanisms by which DCA-induced JNK activation was promoting p53/miR-34a-dependent apoptosis, we additionally used two dominant interfering forms of the JNK signalling pathway, pCMV-Flag-JBD (JIP-1) (Dickens *et al.* 1997) and DN-c-Jun Flag Δ 169 (Ham *et al.* 1995). The binding domain fragment of scaffolding protein JIP-1 has been shown to bind JNK1/2, leading to its cytoplasmic retention and inhibition of JNK-regulated gene expression. As for DN-c-Jun Flag Δ 169, it lacks the c-Jun transactivation domain. When overexpressed, DN-c-Jun Flag Δ 169 competes with endogenous c-Jun for binding to DNA regulatory elements, thus inhibiting c-Jun-dependent transcription. As expected, cells transfected with constructs encoding either pCMV-Flag-JBD (JIP-1) or DN-c-Jun Flag Δ 169, in the presence or absence of DCA, had no effects on JNK levels (data not shown). However, pCMV-Flag-JBD (JIP-1) was able to inhibit DCA-induced p53 activation and miR-34a expression ($p < 0.05$) (**Fig. 4.9.A** and **B**, respectively), as well as caspase-3 activation and cell death ($p < 0.05$) (**Fig. 4.9.C** and **D**, respectively). Interestingly, DN-c-Jun Flag Δ 169 was as effective as pCMV-Flag-JBD (JIP-1) in inhibiting DCA-induced p53/miR-34a-dependent

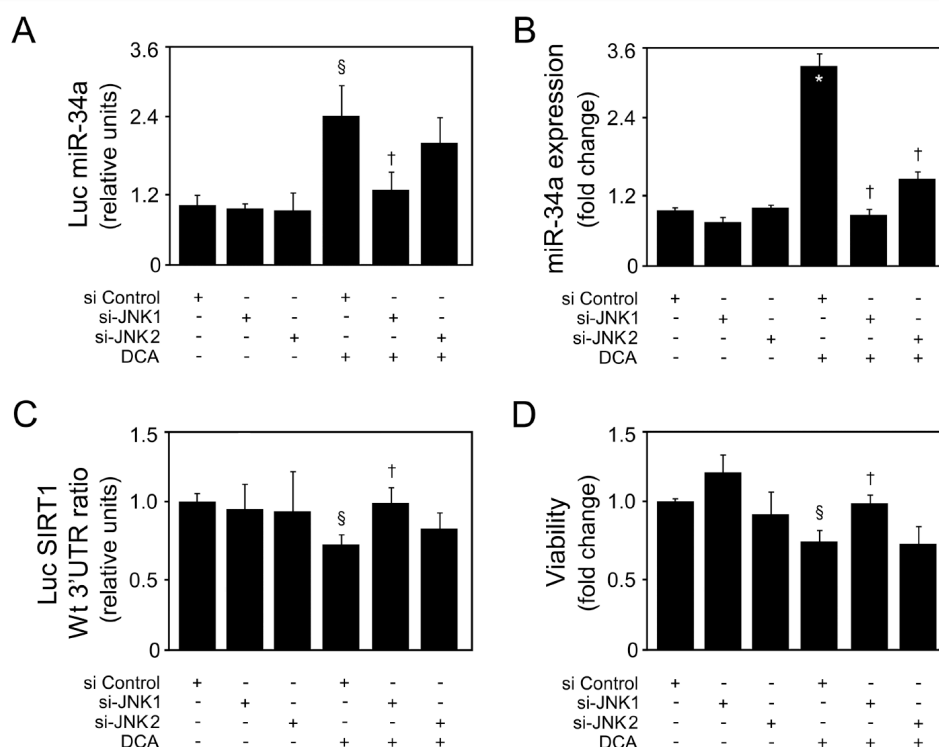


Figure 4.8. DCA-induced p53/miR-34a signalling and apoptosis of primary rat hepatocytes is JNK1-dependent. Cells were transfected with specific short interference RNA (siRNA) nucleotides designed to knock down *jnk1* or *jnk2* gene expression, and a control siRNA containing a scrambled sequence, and treated with 100 μ M DCA, or no addition (control) for 24 h, as described in Material and Methods. **A:** Cells were co-transfected with a pGL4 reporter vector consisting of a luciferase cDNA fused to miR-34a promoter containing either wild-type (Wt) or mutant (Mut) p53 binding sequence. Cells were also co-transfected with a CMV-Renilla luciferase vector as an internal standard. **B:** Real-time RT-PCR analysis of miR-34a expression. **C:** Cells were co-transfected with a reporter vector consisting of a luciferase cDNA fused to the 3'-UTR of SIRT1, containing either a wild-type (Wt) or mutant (Mut) miR-34a binding site. Ratio between Wt and Mut miR-34a luciferase activity are displayed. Cells were also co-transfected with a CMV-Renilla luciferase vector as an internal standard. **D:** Viability was measured using the ApoTox-Glo™ Triplex Assay. Results are expressed as mean $\pm$ SEM fold change from 7 different experiments. $^{\S}p < 0.05$ and $^*p < 0.01$ from si Control; $^{\dagger}p < 0.05$ from si Control with DCA.

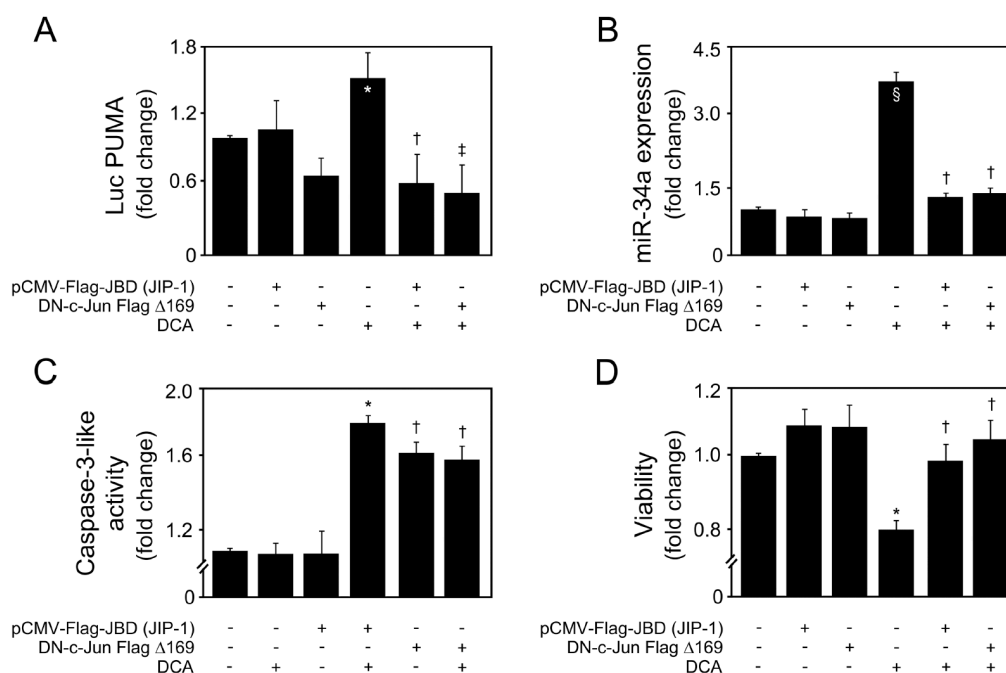


Figure 4.9. JNK and c-Jun act as important triggers of the miR-34a/SIRT1/p53 pro-apoptotic pathway by DCA. Primary rat hepatocytes were transfected with either the binding domain of the JNK interacting protein-1 (JIP-1) (pCMV-Flag-JBD (JIP-1)), or dominant negative DN-c-Jun FlagD169 plasmids and treated with 100 μ M DCA, or no addition (control) for 24 h, as described in Material and Methods. **A:** Cells were co-transfected with a luciferase construct with the PUMA promoter containing consensus p53 binding sites upstream of the transcription start site (Luc PUMA). Results were normalized for the CMV-Renilla luciferase activity. **B:** Real-time RT-PCR analysis of miR-34a expression. **C:** Caspase-3-like activity and **D:** viability measured using the ApoTox-Glo™ Triplex Assay. Results are expressed as mean $\pm$ SEM fold change from 5 different experiments. § p < 0.05 and * p < 0.01 from Control; † p < 0.05 and ‡ p < 0.01 from DCA alone.

signalling and apoptosis. These results confirm that the JNK1 signalling pathway plays a key role in mediating DCA-induced p53/miR-34a/SIRT1, leading to increased cellular caspase activation and apoptosis.

4.5. Discussion

Bile acids are essential to facilitate the digestion and absorption of fat. Despite the physiological properties, excessive accumulation of bile acids is associated with cytotoxic effects. In fact, previous studies have argued that conjugated bile acids, including DCA, may induce apoptosis in hepatocytes upon activation of death receptors (Higuchi *et al.* 2003). In addition, DCA

induces apoptosis by impairing mitochondrial function and leading to cytochrome *c* release into the cytosol (Rodrigues *et al.* 1998b). Both JNK and p53 may also represent important mediators of DCA-induced cytotoxicity (Qiao *et al.* 2003; Castro *et al.* 2007a). However, the exact network of pathways involved in DCA-induced hepatocyte apoptosis remains scant. In particular, no studies have yet explored miRNA expression changes and function during cell death by cytotoxic bile acids. In this study, we investigated whether DCA modulates the miR-34a/SIRT1/p53 pro-apoptotic pathway in primary rat hepatocytes and whether JNK1 may link this pathway to already described mechanistic actions of DCA. Our results show that by activating p53, DCA induces miR-34a transcription and inhibition of SIRT1, which translates into increased caspase activation and apoptosis of primary rat hepatocytes. Importantly, JNK1, but not JNK2, acts as a crucial target of DCA in mediating p53/miR-34a activation and downstream apoptosis.

miR-34a-dependent apoptosis has already been shown to occur through both p53-dependent and -independent mechanisms (Chang *et al.* 2007). In addition, miR-34a inhibits translation of SIRT1, a NAD-dependent deacetylase, with anti-apoptotic properties (Brooks *et al.* 2009). Our results show that DCA induces miR-34a expression, abrogates SIRT1 expression and increases p53 acetylation in primary rat hepatocytes, providing a new mechanistic link for its pro-apoptotic properties. This effect appears to be both dose- and time-dependent, as DCA-induced miR-34a and downstream targets are only significant for concentrations higher than 50 μ M DCA and start to attenuate after 52 h of incubation. This is consistent with the intrinsic nature of miRNA-mediated regulation and with the fact that bile acids, including DCA, sustain apoptosis in primary hepatocytes within a relatively short time (Qiao *et al.* 2001). In fact, after 64h of incubation, DCA-induced cell death was predominantly necrotic in nature.

As we have recently shown, miR-34a overexpression leads to cell death and apoptosis in primary rat hepatocytes (Castro *et al.* 2013). In this study, we unequivocally characterized not only miR-34a, but also its target SIRT1, as determinant players during DCA-induced apoptosis in primary rat hepatocytes. In addition, we have also previously shown that p53-induced apoptosis involves impairment of MDM2-dependent shuttling of p53 to the

cytoplasm in hepatocytes (Sola *et al.* 2003). This results in higher nuclear p53 capable of transactivating target genes, which could include miR-34a. Moreover, p53 acetylation that usually occurs in response to DNA damage and genotoxic stress, and which we show to also occur in hepatocytes in response to DCA, is indispensable for p53 transcriptional activity. Upon p53 acetylation, the p53/MDM2 interaction is disrupted and p53 is able to transcribe genes involved in apoptosis (Brooks *et al.* 2011). Our results show that DCA increases p53 expression, transactivity and DNA binding activities, in parallel with p53 acetylation, in a positive feedback loop contributing for augmented p53 activation. More importantly, it appears that by doing so, DCA is engaging miR-34a-dependent apoptotic signalling. In fact, this may explain why DCA is more capable of inducing miR-34a expression when miR-34a itself is overexpressed, as compared with DCA alone. Curiously, we have previously shown that DCA induces cyclin D1-mediated Bax protein translocation and apoptosis through a p53-dependent mechanism (Castro *et al.* 2007a). In addition, cyclin D1 has been shown to be a direct target of miR-34a in prostate cancer PC3 cells (Fujita *et al.* 2008). Therefore, it is possible that miR-34a activation represents the mechanism behind DCA-induced cyclin D1. As for the mechanisms by which DCA induces p53 expression and transactivity, our results show that they are largely dependent on JNK. In fact, JNK has been previously shown to be able to activate p53 (Fuchs *et al.* 1998; Saha *et al.* 2012) and our results demonstrate that treatment of primary rat hepatocytes with DCA significantly induce JNK phosphorylation. Interestingly, in the absence of DCA, JNK phosphorylation, activation of the miR-34a/SIRT1/p53 pathway and cell death, all increased in primary hepatocytes cultured from 52 to 64 h. This is consistent with the notion that hepatocytes start to dedifferentiate upon isolation and culture. After several days in culture, oxidative stress-induced apoptosis becomes a major event (Elaut *et al.* 2006). In fact, in these conditions, extramitochondrial glutathione depletion may lead to a sustained activation of JNK, which is then capable to activate the p53/miR-34a pro-apoptotic pathway.

JNK has three isoforms: JNK1, 2 and 3. While JNK1 and JNK2 are extensively expressed in mammalian tissues, including hepatocytes, expression of JNK3 is restricted to the brain and testis (Yan *et al.* 2010).

Each isoform has overlapping or distinct roles in liver pathophysiology. In particular, studies using primary mouse hepatocytes have suggested that DCA-induced JNK1 signalling is mostly cytotoxic, while DCA-induced JNK2 plays more of a protective role against apoptosis (Qiao *et al.* 2003). In fact, our results showed that JNK1 silencing abrogated DCA-induced p53/miR34a expression and activation, thereby diminishing apoptosis. On the contrary, JNK2 silencing did not significantly repress the DCA-induced p53/SIRT1/miR34a pro-apoptotic pathway, despite a tendency to inhibit miR-34a expression in cells incubated with DCA. Curiously, under specific settings, JNK2 may activate apoptotic players in the liver (Wang *et al.* 2006b). Therefore, it is possible that JNK2 plays a redundant role in activating p53/miR-34a, but only when in the presence of an apoptotic stimulus. In fact, while its silencing alone results in a very slight inhibition of p53/miR-34a/SIRT1-associated apoptosis, it no longer does so in cells incubated with DCA.

Overall, our findings suggest that DCA-induced JNK1 activation is a critical apoptotic mechanism of DCA, upstream of p53 and miR-34a activation and SIRT1 inhibition. In addition, JNK1, but not JNK2, phosphorylates c-Jun, a critical member of the activator protein 1 transcription factor complex, which can then induce expression of several death mediators (Czaja 2003). In fact, c-Jun appears to be the next immediate target transducing JNK1 effects, as cells transfected with the DN-c-Jun Flag Δ 169 plasmid showed an almost complete abrogation of DCA-induced p53/miR-34a apoptotic signalling. Interestingly, because DCA induces p53 acetylation through the miR-34a/SIRT1 pathway, in a p53/miR-34a/SIRT1 positive feedback loop (Yamakuchi *et al.* 2009), p53 might also be inducing JNK1 phosphorylation, thereby decreasing p53/MDM2 complex formation. It remains possible that JNK1 regulates miR-34a directly, as a recent study has shown that the miR-34a promoter contains an activator protein 1 site, which appears to be required for maximal transactivation of miR-34a (Ichimura *et al.* 2010; Chen *et al.* 2012). Nevertheless, and all together, DCA-induced JNK1 phosphorylation appears to induce both p53 expression and activation, converging in a strong and functional engagement of the miR-34a-dependent apoptotic pathway in primary rat hepatocytes.

Based on our findings, it may be hypothesized that activation of miR-34a-dependent cytotoxicity by DCA may play an important role in liver disease. We have previously shown that patients with steatohepatitis display higher levels of DCA when compared with control patients (Aranha *et al.* 2008). These may arise from a secretory dysfunction associated with hepatic injury in steatohepatitis patients. Whatever the cause, increased bile acids may aggravate injury, thus creating a vicious cycle. Remarkably, in cellular models, free fatty acids activate JNK1/c-Jun pathway inducing PUMA transcriptional up-regulation with subsequent Bax activation as integral steps promoting saturated free fatty acid-mediated apoptosis (Cazanave *et al.* 2009). In addition, we have also shown that apoptosis is a prominent feature in patients with alcoholic steatohepatitis and non-alcoholic steatohepatitis (Ribeiro *et al.* 2004). Thus, hepatocytes in steatohepatitis and many other liver diseases exhibit receptor-dependent and -independent forms of cell death, both of which are associated with mitochondrial dysfunction, in the same fashion of DCA-induced apoptosis in hepatocytes. Furthermore, we have recently demonstrated that JNK could be a mechanistic link between insulin resistance and apoptosis during non-alcoholic fatty liver disease progression (Ferreira *et al.* 2011) and that the miR-34a/SIRT1/p53 pro-apoptotic pathway is increased in more severe stages of non-alcoholic fatty liver disease (Castro *et al.* 2013). In addition, SIRT1 protein is degraded in response to JNK1 activation, thus contributing to hepatic steatosis in obese mice (Gao *et al.* 2011). In the light of this finding, it is possible that DCA-induced SIRT1 degradation is also occurring through JNK1 directly, in parallel with miR-34a-mediated inhibition. Finally, a deficiency of JNK1, but not JNK2, has been shown to improve insulin sensitivity and decrease adiposity in different animal models of obesity (Hirosumi *et al.* 2002). Therefore, the functional relevance of the JNK1/p53/miR-34a/SIRT1 pro-apoptotic pathway in steatohepatitis *in vivo* and other liver diseases should be further exploited. Strategies aimed at antagonizing JNK1/p53- or miR-34a- dependent pathways may prove useful in ameliorating pathologies involving bile acid-associated cytotoxicity.

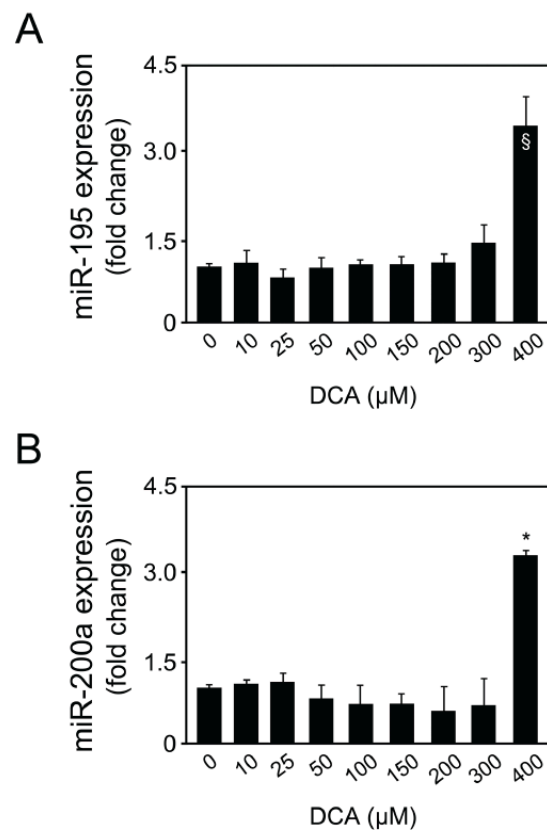
In conclusion, the specific targeting of miR-34a by DCA in primary rat hepatocytes results in decreased SIRT1 expression and increased p53

acetylation and apoptosis. The mechanism by which DCA induces miR-34a expression appears to occur, at least in part, through JNK1 activation that increases p53 expression and transactivity. By adding more pieces to the puzzle, these findings underscore new targets for the development of novel drugs to ameliorate liver diseases, particularly those involving bile acid-, apoptosis- or inflammation-associated cytotoxicity.

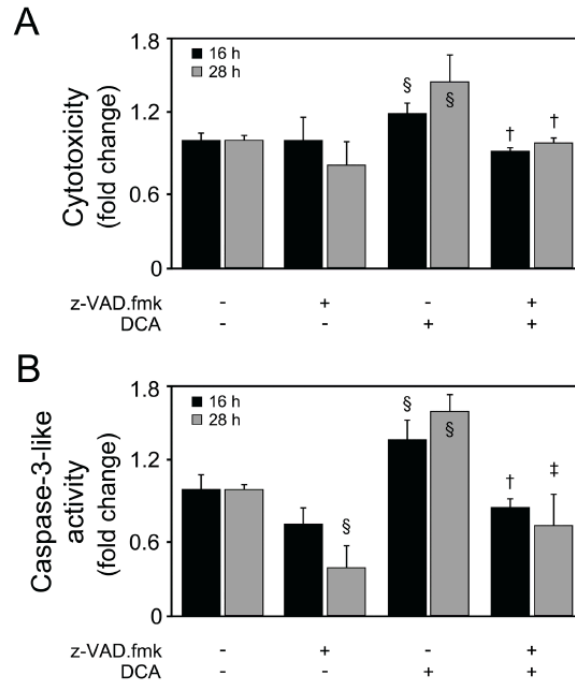
Acknowledgments

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4.6. Supplementary figures



Supplementary Figure 4.1. DCA does not modulate miR-195 and miR-200a expressions in primary rat hepatocytes. Cells were isolated as described in Material and Methods and treated with 10-400 μM DCA, or no addition (control) for 24 h. **A:** Real-time RT-PCR analysis of miR-195 and **B:** miR-200a. Results are expressed as mean $\pm$ SEM fold change of at least 4 independent experiments. [§] $p < 0.05$ and ^{*} $p < 0.01$ from Control.



Supplementary Figure 4.2. DCA induces caspase-dependent cell death in primary rat hepatocytes. Cells were isolated as described in Material and Methods and treated with 100 μ M DCA, or no addition (control) in the presence or absence of 50 μ M z-VAD-fmk for 16 and 28 h. **A:** Cytotoxicity and **B:** caspase-3-like activity measured using the ApoTox-Glo™ Triplex Assay. Results are expressed as mean $\pm$ SEM fold change of 3 independent experiments. $^{\$}p < 0.05$ from Control; $^{\dagger}p < 0.05$ and $^{\ddagger}p < 0.05$ from DCA alone.

CONCLUDING REMARKS

The original work presented in this thesis focused on the molecular pathways governing NAFLD pathogenesis, with the goal of discovering key targets that may contribute for the development of better therapeutic strategies for NAFLD management. We have successfully shown that more severe stages of NAFLD are associated with increased liver caspase-2 and -3 activation, as well as apoptosis. This correlates with a decrease in the insulin signalling pathway and IR in these patients. In addition, JNK appears to function as a mechanistic link between IR and apoptosis during NAFLD progression. Moreover, we demonstrated the existence of a link between hepatocyte apoptosis and the miR-34a/SIRT1/p53 pro-apoptotic pathway during NAFLD progression. In fact, miR-34a expression and p53 acetylation increase with NAFLD severity, while SIRT1 expression decreases. Targeting of the miR-34a/SIRT1/p53 pro-apoptotic pathway by UDCA leads to an increase in SIRT1 expression, and decreased p53 acetylation, miR-34a expression, and apoptosis. On the contrary, cytotoxic DCA specifically activates the miR-34a/SIRT1/p53 pro-apoptotic pathway in primary rat hepatocytes and in the rat liver, in a JNK1-dependent manner (**Figure 5.1.**).

In this chapter, we integrate our new findings on the molecular pathways governing NAFLD pathogenesis in light of the emerging role of bile acids and miRNAs as modulators of these key pathways. We will focus on the role of JNK, miR-34a and caspase-2 as key players involved in NAFLD progression. Questions raised by our studies, particularly whether the efficacy of UDCA for treating NAFLD should be re-evaluated will also be discussed. Undoubtedly, studies exploring the role of UDCA derivatives in modulating key targets and pathways in NAFLD, namely caspase-2 and the JNK1/p53/miR-34a/SIRT1 pro-apoptotic pathway and, ultimately, their efficacy in halting NAFLD progression, are highly desirable.

In our initial studies, we observed for the first time that IR is differentially sensed in the three insulin-sensitive tissues, muscle, liver and adipose tissues, and that apoptosis and IR increase with more severe NAFLD stages, in morbid obese patients. It appears that IR may target primarily the muscle tissue as NAFLD advances to more severe stages, leading to a more

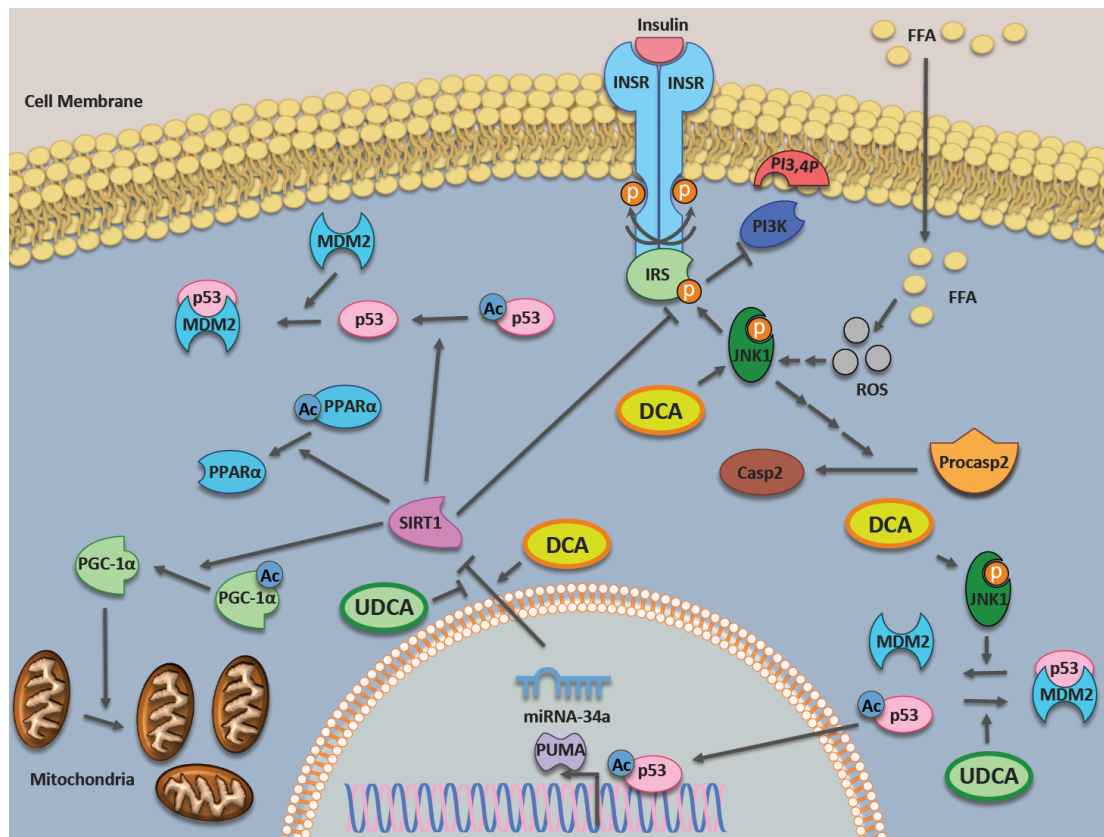


Figure 5.1. Proposed mechanism for bile acids as modulators of cell death, insulin signalling and miR-34a/SIRT1/p53 pro-apoptotic pathway. NAFLD patients have higher levels of FFAs that induce ROS production leading to JNK1 activation. DCA potentiates JNK1 activation, which could induce JNK1-mediated caspase-2 activation. Activation of JNK1 also leads to the suppression of the insulin signalling pathway through JNK1-dependent Ser307 phosphorylation of IRS-1. In addition, DCA-induced JNK1 activation also increases MDM2 phosphorylation and p53/MDM2 complex disruption increasing p53 transcriptional activity by transcribing PUMA and miR-34a. On the contrary, UDCA induces p53/MDM2 complex formation decreasing p53 transcriptional activity. Once in the cytoplasm, miR-34a decreases SIRT1 expression by repressing SIRT1 translation. This process is inhibited by UDCA and induced by DCA. When SIRT1 expression is decreased it cannot inhibit JNK1-dependent Ser307 phosphorylation of IRS-1 and promote its tyrosine phosphorylation. Furthermore, SIRT1 inhibition also increases acetylated p53, which is free from MDM2 and can act as a transcription factor enhancing even more the repression of SIRT1 by increasing miR-34a expression. SIRT1 repression also inhibits PPARα and PGC-1α by increasing their acetylation states. In this way, fatty acid oxidation, gluconeogenesis, and mitochondrial biogenesis are compromised. Altogether, DCA induces a state of apoptosis, FFA intracellular accumulation, increased ROS levels and ER stress, and IR, all characteristic from NAFLD, while UDCA has the opposite effect by improving SIRT1 levels and decreasing apoptosis and p53 transcriptional activity.

systemic IR, where the liver tissue is affected and, to a lesser extent still, the adipose tissue. In fact, it has been described that IR in the skeletal muscle manifests long before hyperglycaemia becomes evident (DeFronzo 2009). Importantly, a blockage in the insulin signalling cascade at the IRS-1 level is thought to be the primary defect leading to IR in the muscle. One of the key molecules responsible for this decreased IRS-1 tyrosine phosphorylation may turn out to be JNK, as our results showed that its phosphorylation increases in more severe stages of NAFLD. In fact, obesity has been described to increase TNF- α expression which, together with FFAs, induces ROS modulation and JNK activation. Activated JNK would then be able to increase IRS-1 serine phosphorylation, thus preventing its interaction with INSR. In agreement, disruption of the JNK-binding motif in IRS-1 significantly reduces IRS-1 serine phosphorylation and increases insulin-stimulated tyrosine phosphorylation and AKT activation (Lee *et al.* 2003). In addition, JNK activation has been previously described in human NASH (Puri *et al.* 2008) and has a crucial role in inducing IR and/or steatohepatitis in rodents (Wang *et al.* 2008; Singh *et al.* 2009).

Therefore, and all together, our results go further in strongly suggesting that JNK phosphorylation may be responsible for aggravating IR, as NAFLD advances to more severe stages. Interestingly, JNK activation may also result in apoptosis, thus providing a link between IR and cell death (Schattenberg *et al.* 2006). In that regard, we have also shown that caspase-2 is increased in the liver of NAFLD patients, correlating with disease pathogenesis. Interestingly, it has been described that caspase-2 deficient mice display reduced body fat content (Zhang *et al.* 2007) and that diet-induced obese rats gain much less white-fat with reduced mRNA levels of caspase-2 (Jobgen *et al.* 2009). Therefore, it would seem that caspase-2 activation might increase accumulation of liver fat and/or induce apoptosis during NAFLD progression. Importantly, a link between caspase-2- and JNK-mediated signalling has been described in age-related muscle cell apoptosis and in amyloid β -induced apoptosis (Braga *et al.* 2008; Viana *et al.* 2010), further giving strength to our hypothesis that JNK acts as a mechanistic link between IR, caspase-2 and apoptosis during NAFLD progression. Although these results have unveiled the importance of

caspase-2 and JNK signalling in NAFLD pathogenesis, a more mechanistic approach should be taken to confirm its effective role in NAFLD.

With that in mind, we next aimed to identify mechanistic players in linking JNK, the insulin signalling pathway and apoptosis, during NAFLD progression. Our attention was directed toward miR-34a, and in particular the miR-34a/SIRT1/p53 pro-apoptotic pathway, as a top pick candidate. Firstly, miR-34a had already been described as upregulated in the livers of mice fed HFD, as well as in patients with metabolic syndrome and NASH (Cheung *et al.* 2008); secondly, NAFLD-associated inflammation increases p53 expression levels (Panasiuk *et al.* 2006), which engage different mitochondrial pathways of apoptosis in an animal model of NASH (Farrell *et al.* 2009); thirdly, SIRT1 has a central role in regulating hepatic fatty acid metabolism (Purushotham *et al.* 2009); and finally, SIRT1 also modulates the insulin signalling pathway by repressing PTPN1 (Sun *et al.* 2007). In fact, SIRT1 activation increases both glucose uptake and insulin signalling, while decreasing IRS-1 serine phosphorylation (Yoshizaki *et al.* 2009). As a proof-of-principle, we first showed that, in the liver of morbidly obese NAFLD patients, miR-34a expression and p53 acetylation are increased, while SIRT1 decreases, from less- to more-advanced stages of NAFLD. With this new piece of information in mind, we then hypothesized that deregulation of p53 in the liver, during obesity, could favour excessive lipid accumulation by activating miR-34a and altering SIRT1 expression. In fact, it is well known that steatosis promotes oxidative stress and increases the vulnerability of hepatocytes to acute injury (Fulop *et al.* 2006). More importantly, increased oxidative stress acts to stabilize active p53 in a feed-forward regulatory mechanism, thus activating downstream genes that are involved in apoptosis, oxidative stress and IR (Stambolic *et al.* 2001; Derdak *et al.* 2011), while p53 knockout mice develop less steatosis and liver injury than wild-type mice (Tomita *et al.* 2012). At the same time, increasing SIRT1 expression in hepatocytes, using for instance resveratrol, reduces triacylglycerol levels and improves IR in NAFLD (Shang *et al.* 2008). Our results linked these previous isolated effects, further showing that they correlate with NAFLD progression; more severe stages of NAFLD display increased miR-34a expression, p53 acetylation and apoptosis, and decreased SIRT1 expression.

UDCA is a well characterized inhibitor of apoptosis (Rodrigues *et al.* 1998a; Rodrigues *et al.* 1999). In particular, we had shown that UDCA inhibits E2F1-mediated apoptosis with decreased MDM2 degradation, allowing MDM2/p53 complex formation (Sola *et al.* 2003; Amaral *et al.* 2010), and modulating p53-induced apoptosis by altering p53 transactivation and DNA binding activity (Amaral *et al.* 2007). Thus, we next investigated whether UDCA could also improve molecular pathways involved in NAFLD pathogenesis. We showed that UDCA decreases miR-34a expression and p53 acetylation, while increasing SIRT1 expression. This new finding may contribute to ascertain the still controversial role of UDCA as a therapeutic tool for NAFLD. In fact, by inducing SIRT1, UDCA may contribute for attenuation of steatosis in human patients, as high levels of hepatic SIRT1 have been shown to decrease *de novo* fatty acid synthesis in the liver *via* SREBP1c (Ponugoti *et al.* 2010). In addition, SIRT1-induced expression can also deacetylate and activate PGC1 α (Rodgers *et al.* 2005), which may increase the expression of fatty acid oxidizing enzymes *via* PPAR α (Lee *et al.* 2004; Purushotham *et al.* 2009). And at its core, UDCA may have some beneficial activity against hepatocyte apoptosis, common during NAFLD pathogenesis.

Interestingly, we have previously shown that patients with NASH display higher levels of DCA when compared with control patients (Aranha *et al.* 2008). In addition, it was recently shown that dietary or genetic obesity induces alterations in gut microbiota, resulting in increased levels of DCA. Then, enterohepatic circulation of DCA activates hepatic stellate cells, which in turn secrete various inflammatory and tumour-promoting factors in the liver (Yoshimoto *et al.* 2013). Given this potential pathogenic role of DCA in NAFLD, we decided to explore whether it was also capable of modulating the miR-34a/SIRT1/p53 pathway in the liver. This would be particularly relevant, given the inhibitory role of UDCA in this pathway. In fact, UDCA has already been established as a strong inhibitor of DCA-induced cytotoxicity; rats fed with DCA display significant mitochondrial changes, including increased levels of BAX and lower levels of BAD at the mitochondrial membrane. Strikingly, UDCA co-feeding completely abolishes these changes (Rodrigues *et al.* 1998b). Furthermore, isolated mitochondria or cultured hepatocytes

treated with DCA display significant cytochrome *c* release from the mitochondria, while co-incubation with UDCA almost completely abolishes these changes (Rodrigues *et al.* 1999). Interestingly, and as stated before, mitochondrial dysfunction is also a key feature in NAFLD, characterized by enhanced ROS production and mitochondrial cytochrome *c* (Caldwell *et al.* 2004).

Our results established that DCA acts as a strong activator of the miR-34a/SIRT1/p53 pathway, representing a new route by which it induces apoptosis in rat hepatocytes both *in vitro* and *in vivo*. In particular, DCA increases p53 transcriptional activity by decreasing p53/MDM2 complex formation and engaging p53-dependent miR-34a expression. Because DCA is increased in obesity and in NASH patients, it may be acting as one of the key factors inducing liver cytotoxicity and apoptosis during NAFLD pathogenesis. Moreover, DCA-induced activation of the miR-34a/SIRT1/p53 pathway may be impaired by UDCA, further reinforcing the need to re-evaluate UDCA's role in NAFLD management. Furthermore, TNF-R1, Fas and TRAIL, all found to be increased at some point during NAFLD, have also been described to activate JNK1, through the action of either FFAs or bile acids, among which DCA (Higuchi *et al.* 2004; Malhi *et al.* 2006). Curiously, our results confirm that DCA-induced JNK1 is crucial for its ability to activate apoptosis, upstream of the miR-34a/SIRT1/p53 pathway. It would be interesting to evaluate the ability of UDCA in inhibiting JNK1 activity, as a key mechanism during inhibition of DCA-induced cytotoxicity and, at the same time, in order to potentiate the beneficial effects of SIRT1 during the metabolic syndrome in hepatocytes. In addition, because caspase-2 may be also an important player during NAFLD-associated apoptosis in our studies, and is itself induced by JNK, it would be important to explore the role of UDCA in inhibiting caspase-2 activation, as well as to understand whether JNK1 and JNK2 have a differential ability in activating caspase-2, in a similar way as observed for the miR-34a/SIRT1/p53 pro-apoptotic pathway.

Altogether, the results presented in this thesis served their purpose in better characterizing key molecular steps during NAFLD pathogenesis, while further reinforcing the notion that UDCA might have a beneficial effect in treating NAFLD patients. However, several clinical trials have failed to

unequivocally demonstrate a substantial therapeutic effect of UDCA in NAFLD treatment and, until now, it remains absent from clinical practice (Liechti *et al.* 2012). Still, in a recent clinical trial, NASH patients treated with high doses of UDCA showed improvement in metabolic and fibrosis parameters (Ratziu *et al.* 2011). One can argue that, until now, a significant factor dictating the failure of UDCA in NAFLD clinical trials is likely related with the reduced number of patients and/or duration of treatment. In addition, the selected patients were already in a more or less advanced stage of the disease; because one of the main key actions of UDCA is its ability to inhibit key players in the apoptotic process, it could simply be too late for UDCA to act and prevent disease evolution. Ideally, more clinical trials should be performed, with a higher number of patients, for longer periods of time and with patients displaying signs of simple steatosis and/or less severe stages of NASH. One must also consider the possibility that, even so, and despite being currently used to treat cholestatic diseases, in parallel to our encouraging animal studies presented herein, UDCA alone may never be significantly effective in human NAFLD patients. Nevertheless, the use of UDCA-derived molecules for NAFLD treatment may represent a different strategy worth pursuing. TUDCA, for instance, down-regulates *de novo* lipogenesis genes in a model of *ob/ob* mice (Yang *et al.* 2010). More recently, norUDCA was shown to reverse liver injury and fibrosis, as well as decrease serum lipids, in an animal model of inflammation and cholestatic liver (Moustafa *et al.* 2012). To improve the efficacy of UDCA for diseases like NAFLD, several chemical alterations to its structure have been performed and evaluated. In one of those studies, highly water-soluble prodrugs of UDCA were synthesized. Phosphate ester prodrugs present a modification that increases their aqueous solubility. In addition, these prodrugs are activated *in vivo* by ubiquitous endogenous phosphatases. Interestingly, the phosphate ester prodrugs of UDCA were shown to possess similar anti-apoptotic effects as UDCA alone, with the benefit that they can be used in aqueous solutions, contrary to UDCA alone (Dosa *et al.* 2013). Because we now showed that IR, DCA-induced apoptosis and activation of the JNK1/p53/miR-34a/SIRT1 pro-apoptotic pathway appear to represent crucial steps during NAFLD pathogenesis, it would be extremely valuable to

investigate whether TUDCA, norUDCA, or even other UDCA-derivatives are also capable of inhibiting these pathways and, by doing so, ameliorate NAFLD pathogenesis. In the same line of thought, results from clinical trials using TUDCA or norUDCA are eagerly waited. This will surely ascertain the potential role of these molecules as attractive new drugs to arrest the metabolic and apoptotic liver features in NAFLD patients and hopefully halt NAFLD progression by doing so.

REFERENCES

- Akgul, C. Mcl-1 is a potential therapeutic target in multiple types of cancer. *Cell Mol Life Sci* (2009) **66**: 1326-1336.
- Alisi, A., Da Sacco, L., Bruscalupi, G., Piemonte, F., Panera, N., De Vito, R., Leoni, S., Bottazzo, G. F., Masotti, A., Nobili, V. Mirnome analysis reveals novel molecular determinants in the pathogenesis of diet-induced nonalcoholic fatty liver disease. *Lab Invest* (2011) **91**: 283-293.
- Allen, R. M., Marquart, T. J., Albert, C. J., Suchy, F. J., Wang, D. Q., Ananthanarayanan, M., Ford, D. A., Baldan, A. miR-33 controls the expression of biliary transporters, and mediates statin- and diet-induced hepatotoxicity. *EMBO Mol Med* (2012) **4**: 882-895.
- Amaral, J. D., Castro, R. E., Sola, S., Steer, C. J., Rodrigues, C. M. p53 is a key molecular target of ursodeoxycholic acid in regulating apoptosis. *J Biol Chem* (2007) **282**: 34250-34259.
- Amaral, J. D., Castro, R. E., Sola, S., Steer, C. J., Rodrigues, C. M. Ursodeoxycholic acid modulates the ubiquitin-proteasome degradation pathway of p53. *Biochem Biophys Res Commun* (2010) **400**: 649-654.
- Amaral, J. D., Viana, R. J., Ramalho, R. M., Steer, C. J., Rodrigues, C. M. Bile acids: regulation of apoptosis by ursodeoxycholic acid. *J Lipid Res* (2009) **50**: 1721-1734.
- Amir, M., Liu, K., Zhao, E., Czaja, M. J. Distinct functions of JNK and c-Jun in oxidant-induced hepatocyte death. *J Cell Biochem* (2012) **113**: 3254-3265.
- Anthonsen, M. W., Ronnstrand, L., Wernstedt, C., Degerman, E., Holm, C. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J Biol Chem* (1998) **273**: 215-221.
- Aranha, M. M., Cortez-Pinto, H., Costa, A., da Silva, I. B., Camilo, M. E., de Moura, M. C., Rodrigues, C. M. Bile acid levels are increased in the liver of patients with steatohepatitis. *Eur J Gastroenterol Hepatol* (2008) **20**: 519-525.
- Arden, K. C., Biggs, W. H., 3rd. Regulation of the FoxO family of transcription factors by phosphatidylinositol-3 kinase-activated signaling. *Arch Biochem Biophys* (2002) **403**: 292-298.
- Arner, P. Insulin resistance in type 2 diabetes: role of fatty acids. *Diabetes Metab Res Rev* (2002) **18 Suppl 2**: S5-9.
- Athyros, V. G., Tziomalos, K., Gossios, T. D., Griva, T., Anagnostis, P., Kargiotis, K., Pagourelas, E. D., Theocharidou, E., Karagiannis, A., Mikhailidis, D. P. Safety and efficacy of long-term statin treatment for cardiovascular events in patients with coronary heart disease and abnormal liver tests in the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) Study: a post-hoc analysis. *Lancet* (2010) **376**: 1916-1922.
- ATPIII. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *Jama* (2001) **285**: 2486-2497.

References

- Audrito, V., Vaisitti, T., Rossi, D., Gottardi, D., D'Arena, G., Laurenti, L., Gaidano, G., Malavasi, F., Deaglio, S. Nicotinamide blocks proliferation and induces apoptosis of chronic lymphocytic leukemia cells through activation of the p53/miR-34a/SIRT1 tumor suppressor network. *Cancer Res* (2011) **71**: 4473-4483.
- Ayonrinde, O. T., Olynyk, J. K., Beilin, L. J., Mori, T. A., Pennell, C. E., de Klerk, N., Oddy, W. H., Shipman, P., Adams, L. A. Gender-specific differences in adipose distribution and adipocytokines influence adolescent nonalcoholic fatty liver disease. *Hepatology* (2011) **53**: 800-809.
- Bagchi, A., Mills, A. A. The quest for the 1p36 tumor suppressor. *Cancer Res* (2008) **68**: 2551-2556.
- Bajaj, M., Suraamornkul, S., Hardies, L. J., Pratipanawatr, T., DeFronzo, R. A. Plasma resistin concentration, hepatic fat content, and hepatic and peripheral insulin resistance in pioglitazone-treated type II diabetic patients. *Int J Obes Relat Metab Disord* (2004) **28**: 783-789.
- Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* (1990) **249**: 912-915.
- Bandyopadhyay, G. K., Yu, J. G., Ofrecio, J., Olefsky, J. M. Increased p85/55/50 expression and decreased phosphatidylinositol 3-kinase activity in insulin-resistant human skeletal muscle. *Diabetes* (2005) **54**: 2351-2359.
- Barreiro, F. J., Kobayashi, S., Bronk, S. F., Werneburg, N. W., Malhi, H., Gores, G. J. Transcriptional regulation of Bim by FoxO3A mediates hepatocyte lipoapoptosis. *J Biol Chem* (2007) **282**: 27141-27154.
- Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* (2009) **136**: 215-233.
- Baumeister, S. E., Volzke, H., Marschall, P., John, U., Schmidt, C. O., Flessa, S., Alte, D. Impact of fatty liver disease on health care utilization and costs in a general population: a 5-year observation. *Gastroenterology* (2008) **134**: 85-94.
- Bayascas, J. R. PDK1: the major transducer of PI 3-kinase actions. *Curr Top Microbiol Immunol* (2010) **346**: 9-29.
- Bedogni, G., Miglioli, L., Masutti, F., Castiglione, A., Croce, L. S., Tiribelli, C., Bellentani, S. Incidence and natural course of fatty liver in the general population: the Dionysos study. *Hepatology* (2007) **46**: 1387-1391.
- Belfort, R., Mandarino, L., Kashyap, S., Wirfel, K., Pratipanawatr, T., Berria, R., DeFronzo, R. A., Cusi, K. Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* (2005) **54**: 1640-1648.
- Bellacosa, A., Testa, J. R., Staal, S. P., Tsichlis, P. N. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* (1991) **254**: 274-277.
- Bellentani, S., Scaglioni, F., Marino, M., Bedogni, G. Epidemiology of non-alcoholic fatty liver disease. *Dig Dis* (2010) **28**: 155-161.

- Benz, C., Angermuller, S., Otto, G., Sauer, P., Stremmel, W., Stiehl, A. Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. *Eur J Clin Invest* (2000) **30**: 203-209.
- Beraza, N., Ofner-Ziegenfuss, L., Ehedego, H., Boekschoten, M., Bischoff, S. C., Mueller, M., Trauner, M., Trautwein, C. Nor-ursodeoxycholic acid reverses hepatocyte-specific nemo-dependent steatohepatitis. *Gut* (2011) **60**: 387-396.
- Berdichevsky, A., Guarente, L., Bose, A. Acute oxidative stress can reverse insulin resistance by inactivation of cytoplasmic JNK. *J Biol Chem* (2010) **285**: 21581-21589.
- Berube, C., Boucher, L. M., Ma, W., Wakeham, A., Salmena, L., Hakem, R., Yeh, W. C., Mak, T. W., Benchimol, S. Apoptosis caused by p53-induced protein with death domain (PIDD) depends on the death adapter protein RAIDD. *Proc Natl Acad Sci U S A* (2005) **102**: 14314-14320.
- Berwick, D. C., Hers, I., Heesom, K. J., Moule, S. K., Tavaré, J. M. The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. *J Biol Chem* (2002) **277**: 33895-33900.
- Bethune, J., Artus-Revel, C. G., Filipowicz, W. Kinetic analysis reveals successive steps leading to miRNA-mediated silencing in mammalian cells. *EMBO Rep* (2012) **13**: 716-723.
- Beuers, U., Boyer, J. L., Paumgartner, G. Ursodeoxycholic acid in cholestasis: potential mechanisms of action and therapeutic applications. *Hepatology* (1998) **28**: 1449-1453.
- Bogoyevitch, M. A., Kobe, B. Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. *Microbiol Mol Biol Rev* (2006) **70**: 1061-1095.
- Bommer, G. T., Gerin, I., Feng, Y., Kaczorowski, A. J., Kuick, R., Love, R. E., Zhai, Y., Giordano, T. J., Qin, Z. S., Moore, B. B., MacDougald, O. A., Cho, K. R., Fearon, E. R. p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* (2007) **17**: 1298-1307.
- Borra, M. T., Langer, M. R., Slama, J. T., Denu, J. M. Substrate specificity and kinetic mechanism of the Sir2 family of NAD⁺-dependent histone/protein deacetylases. *Biochemistry* (2004) **43**: 9877-9887.
- Borradaile, N. M., Han, X., Harp, J. D., Gale, S. E., Ory, D. S., Schaffer, J. E. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J Lipid Res* (2006) **47**: 2726-2737.
- Boura-Halfon, S., Zick, Y. Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab* (2009) **296**: E581-591.
- Braga, M., Sinha Hikim, A. P., Datta, S., Ferrini, M. G., Brown, D., Kovacheva, E. L., Gonzalez-Cadavid, N. F., Sinha-Hikim, I. Involvement of oxidative stress and caspase 2-mediated intrinsic pathway signaling in age-related increase in muscle cell apoptosis in mice. *Apoptosis* (2008) **13**: 822-832.
- Bravo, R., Vicencio, J. M., Parra, V., Troncoso, R., Munoz, J. P., Bui, M., Quiroga, C., Rodriguez, A. E., Verdejo, H. E., Ferreira, J., Iglewski, M., Chiong, M., Simmen, T., Zorzano, A., Hill, J. A., Rothermel, B. A., Szabadkai, G., Lavandero, S. Increased ER-

-
- mitochondrial coupling promotes mitochondrial respiration and bioenergetics during early phases of ER stress. *J Cell Sci* (2011) **124**: 2143-2152.
- Brodeur, G. M. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* (2003) **3**: 203-216.
- Brooks, C. L., Gu, W. How does SIRT1 affect metabolism, senescence and cancer? *Nat Rev Cancer* (2009) **9**: 123-128.
- Brooks, C. L., Gu, W. The impact of acetylation and deacetylation on the p53 pathway. *Protein Cell* (2011) **2**: 456-462.
- Bruce, C. R., Dyck, D. J. Cytokine regulation of skeletal muscle fatty acid metabolism: effect of interleukin-6 and tumor necrosis factor- α . *Am J Physiol Endocrinol Metab* (2004) **287**: E616-621.
- Bugianesi, E., Gastaldelli, A., Vanni, E., Gambino, R., Cassader, M., Baldi, S., Ponti, V., Pagano, G., Ferrannini, E., Rizzetto, M. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* (2005a) **48**: 634-642.
- Bugianesi, E., McCullough, A. J., Marchesini, G. Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatology* (2005b) **42**: 987-1000.
- Bugianesi, E., Pagotto, U., Manini, R., Vanni, E., Gastaldelli, A., de lasio, R., Gentilcore, E., Natale, S., Cassader, M., Rizzetto, M., Pasquali, R., Marchesini, G. Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. *J Clin Endocrinol Metab* (2005c) **90**: 3498-3504.
- Bugianesi, E., Zannoni, C., Vanni, E., Marzocchi, R., Marchesini, G. Non-alcoholic fatty liver and insulin resistance: a cause-effect relationship? *Dig Liver Dis* (2004) **36**: 165-173.
- Buko, V. U., Kuzmitskaya-Nikolaeva, I. A., Naruta, E. E., Lukivskaya, O. Y., Kirko, S. N., Tauschel, H. D. Ursodeoxycholic acid dose-dependently improves liver injury in rats fed a methionine- and choline-deficient diet. *Hepatol Res* (2011) **41**: 647-659.
- Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J., Shoelson, S. E. Local and systemic insulin resistance resulting from hepatic activation of IKK- β and NF- κ B. *Nat Med* (2005) **11**: 183-190.
- Caldwell, S. H., Chang, C. Y., Nakamoto, R. K., Krugner-Higby, L. Mitochondria in nonalcoholic fatty liver disease. *Clin Liver Dis* (2004) **8**: 595-617, x.
- Canbay, A., Feldstein, A. E., Higuchi, H., Werneburg, N., Grambihler, A., Bronk, S. F., Gores, G. J. Kupffer cell engulfment of apoptotic bodies stimulates death ligand and cytokine expression. *Hepatology* (2003a) **38**: 1188-1198.
- Canbay, A., Friedman, S., Gores, G. J. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* (2004) **39**: 273-278.
- Canbay, A., Taimr, P., Torok, N., Higuchi, H., Friedman, S., Gores, G. J. Apoptotic body engulfment by a human stellate cell line is profibrogenic. *Lab Invest* (2003b) **83**: 655-663.
- Canto, C., Auwerx, J. Glucose restriction: longevity SIRTainly, but without building muscle? *Dev Cell* (2008) **14**: 642-644.

- Canto, C., Auwerx, J. PGC-1 α , SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Curr Opin Lipidol* (2009) **20**: 98-105.
- Carthew, R. W., Sontheimer, E. J. Origins and Mechanisms of miRNAs and siRNAs. *Cell* (2009) **136**: 642-655.
- Castro, R. E., Amaral, J. D., Sola, S., Kren, B. T., Steer, C. J., Rodrigues, C. M. Differential regulation of cyclin D1 and cell death by bile acids in primary rat hepatocytes. *Am J Physiol Gastrointest Liver Physiol* (2007a) **293**: G327-334.
- Castro, R. E., Ferreira, D. M., Afonso, M. B., Borralho, P. M., Machado, M. V., Cortez-Pinto, H., Rodrigues, C. M. miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease. *J Hepatol* (2013) **58**: 119-125.
- Castro, R. E., Ferreira, D. M., Zhang, X., Borralho, P. M., Sarver, A. L., Zeng, Y., Steer, C. J., Kren, B. T., Rodrigues, C. M. Identification of microRNAs during rat liver regeneration after partial hepatectomy and modulation by ursodeoxycholic acid. *Am J Physiol Gastrointest Liver Physiol* (2010) **299**: G887-897.
- Castro, R. E., Sola, S., Ma, X., Ramalho, R. M., Kren, B. T., Steer, C. J., Rodrigues, C. M. A distinct microarray gene expression profile in primary rat hepatocytes incubated with ursodeoxycholic acid. *J Hepatol* (2005) **42**: 897-906.
- Castro, R. E., Solá, S., Steer, C. J., Rodrigues, C. M. P. (2007b). Bile acids as modulators of apoptosis. *Hepatotoxicity: From Genomics to in-vitro and in-vivo*, John Willey & Sons, Ltd: 391-419.
- Cazanave, S. C., Mott, J. L., Elmi, N. A., Bronk, S. F., Masuoka, H. C., Charlton, M. R., Gores, G. J. A role for miR-296 in the regulation of lipoapoptosis by targeting PUMA. *J Lipid Res* (2011) **52**: 1517-1525.
- Cazanave, S. C., Mott, J. L., Elmi, N. A., Bronk, S. F., Werneburg, N. W., Akazawa, Y., Kahraman, A., Garrison, S. P., Zambetti, G. P., Charlton, M. R., Gores, G. J. JNK1-dependent PUMA expression contributes to hepatocyte lipoapoptosis. *J Biol Chem* (2009) **284**: 26591-26602.
- Cermelli, S., Ruggieri, A., Marrero, J. A., Ioannou, G. N., Beretta, L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. *PLoS One* (2011) **6**: e23937.
- Chalasani, N., Wilson, L., Kleiner, D. E., Cummings, O. W., Brunt, E. M., Unalp, A. Relationship of steatosis grade and zonal location to histological features of steatohepatitis in adult patients with non-alcoholic fatty liver disease. *J Hepatol* (2008) **48**: 829-834.
- Chalasani, N., Younossi, Z., Lavine, J. E., Diehl, A. M., Brunt, E. M., Cusi, K., Charlton, M., Sanyal, A. J. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* (2012) **55**: 2005-2023.

References

- Chang, L., Kamata, H., Solinas, G., Luo, J. L., Maeda, S., Venuprasad, K., Liu, Y. C., Karin, M. The E3 ubiquitin ligase itch couples JNK activation to TNF α -induced cell death by inducing c-FLIP(L) turnover. *Cell* (2006) **124**: 601-613.
- Chang, L., Karin, M. Mammalian MAP kinase signalling cascades. *Nature* (2001) **410**: 37-40.
- Chang, T. C., Wentzel, E. A., Kent, O. A., Ramachandran, K., Mullendore, M., Lee, K. H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C. J., Arking, D. E., Beer, M. A., Maitra, A., Mendell, J. T. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* (2007) **26**: 745-752.
- Chappell, W. H., Steelman, L. S., Long, J. M., Kempf, R. C., Abrams, S. L., Franklin, R. A., Basecke, J., Stivala, F., Donia, M., Fagone, P., Malaponte, G., Mazzarino, M. C., Nicoletti, F., Libra, M., Maksimovic-Ivanic, D., Mijatovic, S., Montalto, G., Cervello, M., Laidler, P., Milella, M., Tafuri, A., Bonati, A., Evangelisti, C., Cocco, L., Martelli, A. M., McCubrey, J. A. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. *Oncotarget* (2011) **2**: 135-164.
- Charlotte, F., L'Hermine, A., Martin, N., Geleyn, Y., Nollet, M., Gaulard, P., Zafrani, E. S. Immunohistochemical detection of bcl-2 protein in normal and pathological human liver. *Am J Pathol* (1994) **144**: 460-465.
- Chavez-Tapia, N. C., Tellez-Avila, F. I., Barrientos-Gutierrez, T., Mendez-Sanchez, N., Lizardi-Cervera, J., Uribe, M. Bariatric surgery for non-alcoholic steatohepatitis in obese patients. *Cochrane Database Syst Rev* (2010): CD007340.
- Chekulaeva, M., Filipowicz, W. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol* (2009) **21**: 452-460.
- Chen, F., Hu, S. J. Effect of microRNA-34a in cell cycle, differentiation, and apoptosis: a review. *J Biochem Mol Toxicol* (2012) **26**: 79-86.
- Chen, H., Sun, Y., Dong, R., Yang, S., Pan, C., Xiang, D., Miao, M., Jiao, B. Mir-34a is upregulated during liver regeneration in rats and is associated with the suppression of hepatocyte proliferation. *PLoS One* (2011) **6**: e20238.
- Chen, J., Petersen, D. R., Schenker, S., Henderson, G. I. Formation of malondialdehyde adducts in livers of rats exposed to ethanol: role in ethanol-mediated inhibition of cytochrome c oxidase. *Alcohol Clin Exp Res* (2000) **24**: 544-552.
- Cheung, H. H., Lynn Kelly, N., Liston, P., Korneluk, R. G. Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp Cell Res* (2006) **312**: 2347-2357.
- Cheung, O., Puri, P., Eicken, C., Contos, M. J., Mirshahi, F., Maher, J. W., Kellum, J. M., Min, H., Luketic, V. A., Sanyal, A. J. Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* (2008) **48**: 1810-1820.
- Cheung, O., Sanyal, A. J. Recent advances in nonalcoholic fatty liver disease. *Curr Opin Gastroenterol* (2009) **25**: 230-237.
- Chiang, J. Y. Regulation of bile acid synthesis. *Front Biosci* (1998) **3**: d176-193.

- Chiang, J. Y. Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* (2002) **23**: 443-463.
- Chiarini, F., Fala, F., Tazzari, P. L., Ricci, F., Astolfi, A., Pession, A., Pagliaro, P., McCubrey, J. A., Martelli, A. M. Dual inhibition of class IA phosphatidylinositol 3-kinase and mammalian target of rapamycin as a new therapeutic option for T-cell acute lymphoblastic leukemia. *Cancer Res* (2009) **69**: 3520-3528.
- Chipuk, J. E., Green, D. R. Dissecting p53-dependent apoptosis. *Cell Death Differ* (2006) **13**: 994-1002.
- Chitturi, S., Abeygunasekera, S., Farrell, G. C., Holmes-Walker, J., Hui, J. M., Fung, C., Karim, R., Lin, R., Samarasinghe, D., Liddle, C., Weltman, M., George, J. NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* (2002a) **35**: 373-379.
- Chitturi, S., Farrell, G., Frost, L., Kriketos, A., Lin, R., Fung, C., Liddle, C., Samarasinghe, D., George, J. Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: a manifestation of lipotoxicity? *Hepatology* (2002b) **36**: 403-409.
- Chitturi, S., Farrell, G. C. Etiopathogenesis of nonalcoholic steatohepatitis. *Semin Liver Dis* (2001) **21**: 27-41.
- Clark, J. M., Diehl, A. M. Nonalcoholic fatty liver disease: an underrecognized cause of cryptogenic cirrhosis. *Jama* (2003) **289**: 3000-3004.
- Cohen, B. I., Hofmann, A. F., Mosbach, E. H., Stenger, R. J., Rothschild, M. A., Hagey, L. R., Yoon, Y. B. Differing effects of nor-ursodeoxycholic or ursodeoxycholic acid on hepatic histology and bile acid metabolism in the rabbit. *Gastroenterology* (1986) **91**: 189-197.
- Cohen, J. C., Horton, J. D., Hobbs, H. H. Human fatty liver disease: old questions and new insights. *Science* (2011) **332**: 1519-1523.
- Colak, Y., Ozturk, O., Senates, E., Tuncer, I., Yorulmaz, E., Adali, G., Doganay, L., Enc, F. Y. SIRT1 as a potential therapeutic target for treatment of nonalcoholic fatty liver disease. *Med Sci Monit* (2011) **17**: HY5-9.
- Cortez-Pinto, H., Chatham, J., Chacko, V. P., Arnold, C., Rashid, A., Diehl, A. M. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *Jama* (1999) **282**: 1659-1664.
- Crespo, J., Cayon, A., Fernandez-Gil, P., Hernandez-Guerra, M., Mayorga, M., Dominguez-Diez, A., Fernandez-Escalante, J. C., Pons-Romero, F. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* (2001) **34**: 1158-1163.
- Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* (1995) **378**: 785-789.
- Cuny, G. D. Kinase inhibitors as potential therapeutics for acute and chronic neurodegenerative conditions. *Curr Pharm Des* (2009) **15**: 3919-3939.

-
- Czaja, M. J. The future of GI and liver research: editorial perspectives. III. JNK/AP-1 regulation of hepatocyte death. *Am J Physiol Gastrointest Liver Physiol* (2003) **284**: G875-879.
- Dai, B. H., Geng, L., Wang, Y., Sui, C. J., Xie, F., Shen, R. X., Shen, W. F., Yang, J. M. microRNA-199a-5p protects hepatocytes from bile acid-induced sustained endoplasmic reticulum stress. *Cell Death Dis* (2013) **4**: e604.
- Dang, Y., Luo, D., Rong, M., Chen, G. Underexpression of miR-34a in Hepatocellular Carcinoma and Its Contribution towards Enhancement of Proliferating Inhibitory Effects of Agents Targeting c-MET. *PLoS One* (2013) **8**: e61054.
- Datta, S. R., Brunet, A., Greenberg, M. E. Cellular survival: a play in three Akts. *Genes Dev* (1999) **13**: 2905-2927.
- Datta, S. R., Ranger, A. M., Lin, M. Z., Sturgill, J. F., Ma, Y. C., Cowan, C. W., Dikkes, P., Korsmeyer, S. J., Greenberg, M. E. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. *Dev Cell* (2002) **3**: 631-643.
- Day, C. P., James, O. F. Steatohepatitis: a tale of two "hits"? *Gastroenterology* (1998) **114**: 842-845.
- De Luca, A., Maiello, M. R., D'Alessio, A., Pergameno, M., Normanno, N. The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches. *Expert Opin Ther Targets* (2012) **16 Suppl 2**: S17-27.
- Defronzo, R. A. Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* (2009) **58**: 773-795.
- Demeilliers, C., Maisonneuve, C., Grodet, A., Mansouri, A., Nguyen, R., Tinel, M., Letteron, P., Degott, C., Feldmann, G., Pessayre, D., Fromenty, B. Impaired adaptive resynthesis and prolonged depletion of hepatic mitochondrial DNA after repeated alcohol binges in mice. *Gastroenterology* (2002) **123**: 1278-1290.
- Deng, Y., Ren, X., Yang, L., Lin, Y., Wu, X. A JNK-dependent pathway is required for TNF α -induced apoptosis. *Cell* (2003) **115**: 61-70.
- Dent, P., Han, S. I., Mitchell, C., Studer, E., Yacoub, A., Grandis, J., Grant, S., Krystal, G. W., Hylemon, P. B. Inhibition of insulin/IGF-1 receptor signaling enhances bile acid toxicity in primary hepatocytes. *Biochem Pharmacol* (2005) **70**: 1685-1696.
- Derdak, Z., Lang, C. H., Villegas, K. A., Tong, M., Mark, N. M., de la Monte, S. M., Wands, J. R. Activation of p53 enhances apoptosis and insulin resistance in a rat model of alcoholic liver disease. *J Hepatol* (2011) **54**: 164-172.
- Derdak, Z., Villegas, K. A., Harb, R., Wu, A. M., Sousa, A., Wands, J. R. Inhibition of p53 attenuates steatosis and liver injury in a mouse model of non-alcoholic fatty liver disease. *J Hepatol* (2012).
- Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., Davis, R. J. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* (1994) **76**: 1025-1037.

- Dhanasekaran, D. N., Reddy, E. P. JNK signaling in apoptosis. *Oncogene* (2008) **27**: 6245-6251.
- Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., Davis, R. J. A cytoplasmic inhibitor of the JNK signal transduction pathway. *Science* (1997) **277**: 693-696.
- Dongiovanni, P., Valenti, L., Rametta, R., Daly, A. K., Nobili, V., Mozzi, E., Leathart, J. B., Pietrobattista, A., Burt, A. D., Maggioni, M., Fracanzani, A. L., Lattuada, E., Zappa, M. A., Roviato, G., Marchesini, G., Day, C. P., Fargion, S. Genetic variants regulating insulin receptor signalling are associated with the severity of liver damage in patients with non-alcoholic fatty liver disease. *Gut* (2010) **59**: 267-273.
- Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., Parks, E. J. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* (2005) **115**: 1343-1351.
- Dosa, P. I., Ward, T., Castro, R. E., Rodrigues, C. M., Steer, C. J. Synthesis and Evaluation of Water-Soluble Prodrugs of Ursodeoxycholic Acid (UDCA), an Anti-apoptotic Bile Acid. *ChemMedChem* (2013).
- Duan, H., Dixit, V. M. RAIDD is a new 'death' adaptor molecule. *Nature* (1997) **385**: 86-89.
- Dufour, J. F., Oneta, C. M., Gonvers, J. J., Bihl, F., Cerny, A., Cereda, J. M., Zala, J. F., Helbling, B., Steuerwald, M., Zimmermann, A. Randomized placebo-controlled trial of ursodeoxycholic acid with vitamin e in nonalcoholic steatohepatitis. *Clin Gastroenterol Hepatol* (2006) **4**: 1537-1543.
- Eades, G., Yao, Y., Yang, M., Zhang, Y., Chumsri, S., Zhou, Q. miR-200a regulates SIRT1 expression and epithelial to mesenchymal transition (EMT)-like transformation in mammary epithelial cells. *J Biol Chem* (2011) **286**: 25992-26002.
- Elaut, G., Henkens, T., Papeleu, P., Snykers, S., Vinken, M., Vanhaecke, T., Rogiers, V. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Curr Drug Metab* (2006) **7**: 629-660.
- Elliott, M. R., Cheken, F. B., Trampont, P. C., Lazarowski, E. R., Kadl, A., Walk, S. F., Park, D., Woodson, R. I., Ostankovich, M., Sharma, P., Lysiak, J. J., Harden, T. K., Leitinger, N., Ravichandran, K. S. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* (2009) **461**: 282-286.
- Erster, S., Mihara, M., Kim, R. H., Petrenko, O., Moll, U. M. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol* (2004) **24**: 6728-6741.
- Eulalio, A., Trieschler, F., Izauralde, E. The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. *Rna* (2009) **15**: 1433-1442.
- Ewerth, S., Angelin, B., Einarsson, K., Nilsell, K., Bjorkhem, I. Serum concentrations of ursodeoxycholic acid in portal venous and systemic venous blood of fasting humans as determined by isotope dilution-mass spectrometry. *Gastroenterology* (1985) **88**: 126-133.

- Eyrisch, S., Medina-Franco, J. L., Helms, V. Transient pockets on XIAP-BIR2: toward the characterization of putative binding sites of small-molecule XIAP inhibitors. *J Mol Model* (2012) **18**: 2031-2042.
- Fabbrini, E., Magkos, F., Mohammed, B. S., Pietka, T., Abumrad, N. A., Patterson, B. W., Okunade, A., Klein, S. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proc Natl Acad Sci U S A* (2009) **106**: 15430-15435.
- Fabian, M. R., Sonenberg, N., Filipowicz, W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* (2010) **79**: 351-379.
- Fan, M. Q., Huang, C. B., Gu, Y., Xiao, Y., Sheng, J. X., Zhong, L. Decrease expression of microRNA-20a promotes cancer cell proliferation and predicts poor survival of hepatocellular carcinoma. *J Exp Clin Cancer Res* (2013) **32**: 21.
- Fang, Y., Han, S. I., Mitchell, C., Gupta, S., Studer, E., Grant, S., Hylemon, P. B., Dent, P. Bile acids induce mitochondrial ROS, which promote activation of receptor tyrosine kinases and signaling pathways in rat hepatocytes. *Hepatology* (2004) **40**: 961-971.
- Farrell, G. C., Larter, C. Z., Hou, J. Y., Zhang, R. H., Yeh, M. M., Williams, J., dela Pena, A., Francisco, R., Osvath, S. R., Brooling, J., Teoh, N., Sedger, L. M. Apoptosis in experimental NASH is associated with p53 activation and TRAIL receptor expression. *J Gastroenterol Hepatol* (2009) **24**: 443-452.
- Feldstein, A. E., Canbay, A., Angulo, P., Tanai, M., Burgart, L. J., Lindor, K. D., Gores, G. J. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* (2003) **125**: 437-443.
- Feldstein, A. E., Gores, G. J. Apoptosis in alcoholic and nonalcoholic steatohepatitis. *Front Biosci* (2005) **10**: 3093-3099.
- Feldstein, A. E., Werneburg, N. W., Canbay, A., Guicciardi, M. E., Bronk, S. F., Rydzewski, R., Burgart, L. J., Gores, G. J. Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway. *Hepatology* (2004) **40**: 185-194.
- Feng, J., Tamaskovic, R., Yang, Z., Brazil, D. P., Merlo, A., Hess, D., Hemmings, B. A. Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation. *J Biol Chem* (2004) **279**: 35510-35517.
- Ferreira, D. M., Castro, R. E., Machado, M. V., Evangelista, T., Silvestre, A., Costa, A., Coutinho, J., Carepa, F., Cortez-Pinto, H., Rodrigues, C. M. Apoptosis and insulin resistance in liver and peripheral tissues of morbidly obese patients is associated with different stages of non-alcoholic fatty liver disease. *Diabetologia* (2011) **54**: 1788-1798.
- Ferreira, K. S., Kreutz, C., Macnelly, S., Neubert, K., Haber, A., Bogyo, M., Timmer, J., Borner, C. Caspase-3 feeds back on caspase-8, Bid and XIAP in type I Fas signaling in primary mouse hepatocytes. *Apoptosis* (2012) **17**: 503-515.
- Ferret, P. J., Hammoud, R., Tulliez, M., Tran, A., Trebeden, H., Jaffray, P., Malassagne, B., Calmus, Y., Weill, B., Batteux, F. Detoxification of reactive oxygen species by a nonpeptidyl mimic of superoxide dismutase cures acetaminophen-induced acute liver failure in the mouse. *Hepatology* (2001) **33**: 1173-1180.

- Fleischer, B., Schulze-Bergkamen, H., Schuchmann, M., Weber, A., Biesterfeld, S., Muller, M., Krammer, P. H., Galle, P. R. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. *Int J Oncol* (2006) **28**: 25-32.
- Frith, J., Day, C. P., Henderson, E., Burt, A. D., Newton, J. L. Non-alcoholic fatty liver disease in older people. *Gerontology* (2009) **55**: 607-613.
- Fuchs, S. Y., Adler, V., Pincus, M. R., Ronai, Z. MEKK1/JNK signaling stabilizes and activates p53. *Proc Natl Acad Sci U S A* (1998) **95**: 10541-10546.
- Fujita, E., Egashira, J., Urase, K., Kuida, K., Momoi, T. Caspase-9 processing by caspase-3 via a feedback amplification loop in vivo. *Cell Death Differ* (2001) **8**: 335-344.
- Fujita, Y., Kojima, K., Hamada, N., Ohhashi, R., Akao, Y., Nozawa, Y., Deguchi, T., Ito, M. Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells. *Biochem Biophys Res Commun* (2008) **377**: 114-119.
- Fulop, P., Derdak, Z., Sheets, A., Sabo, E., Berthiaume, E. P., Resnick, M. B., Wands, J. R., Paragh, G., Baffy, G. Lack of UCP2 reduces Fas-mediated liver injury in ob/ob mice and reveals importance of cell-specific UCP2 expression. *Hepatology* (2006) **44**: 592-601.
- Furstova, V., Kopska, T., James, R. F., Kovar, J. Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic beta-cell line NES2Y. *Life Sci* (2008) **82**: 684-691.
- Galle, P. R., Krammer, P. H. CD95-induced apoptosis in human liver disease. *Semin Liver Dis* (1998) **18**: 141-151.
- Gao, Z., Hwang, D., Bataille, F., Lefevre, M., York, D., Quon, M. J., Ye, J. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J Biol Chem* (2002) **277**: 48115-48121.
- Gao, Z., Zhang, J., Kheterpal, I., Kennedy, N., Davis, R. J., Ye, J. Sirtuin 1 (SIRT1) protein degradation in response to persistent c-Jun N-terminal kinase 1 (JNK1) activation contributes to hepatic steatosis in obesity. *J Biol Chem* (2011) **286**: 22227-22234.
- Garcia-Ruiz, I., Rodriguez-Juan, C., Diaz-Sanjuan, T., del Hoyo, P., Colina, F., Munoz-Yague, T., Solis-Herruzo, J. A. Uric acid and anti-TNF antibody improve mitochondrial dysfunction in ob/ob mice. *Hepatology* (2006) **44**: 581-591.
- Garofalo, M., Di Leva, G., Romano, G., Nuovo, G., Suh, S. S., Ngankeu, A., Taccioli, C., Pichiorri, F., Alder, H., Secchiero, P., Gasparini, P., Gonelli, A., Costinean, S., Acunzo, M., Condorelli, G., Croce, C. M. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell* (2009) **16**: 498-509.
- Gastaldelli, A., Cusi, K., Pettiti, M., Hardies, J., Miyazaki, Y., Berria, R., Buzzigoli, E., Sironi, A. M., Cersosimo, E., Ferrannini, E., Defronzo, R. A. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology* (2007) **133**: 496-506.
- Gastaldelli, A., Natali, A., Vettor, R., Corradini, S. G. Insulin resistance, adipose depots and gut: interactions and pathological implications. *Dig Liver Dis* (2010) **42**: 310-319.

References

- Gerin, I., Clerbaux, L. A., Haumont, O., Lanthier, N., Das, A. K., Burant, C. F., Leclercq, I. A., MacDougald, O. A., Bommer, G. T. Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. *J Biol Chem* (2010) **285**: 33652-33661.
- Ghildiyal, M., Xu, J., Seitz, H., Weng, Z., Zamore, P. D. Sorting of Drosophila small silencing RNAs partitions microRNA* strands into the RNA interference pathway. *Rna* (2010) **16**: 43-56.
- Goldstein, B. J., Bittner-Kowalczyk, A., White, M. F., Harbeck, M. Tyrosine dephosphorylation and deactivation of insulin receptor substrate-1 by protein-tyrosine phosphatase 1B. Possible facilitation by the formation of a ternary complex with the Grb2 adaptor protein. *J Biol Chem* (2000) **275**: 4283-4289.
- Gong, J., Zhang, J. P., Li, B., Zeng, C., You, K., Chen, M. X., Yuan, Y., Zhuang, S. M. MicroRNA-125b promotes apoptosis by regulating the expression of Mcl-1, Bcl-w and IL-6R. *Oncogene* (2012).
- Goodyear, L. J., Giorgino, F., Sherman, L. A., Carey, J., Smith, R. J., Dohm, G. L. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* (1995) **95**: 2195-2204.
- Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B., Hay, N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev* (2001) **15**: 1406-1418.
- Gramantieri, L., Ferracin, M., Fornari, F., Veronese, A., Sabbioni, S., Liu, C. G., Calin, G. A., Giovannini, C., Ferrazzi, E., Grazi, G. L., Croce, C. M., Bolondi, L., Negrini, M. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* (2007) **67**: 6092-6099.
- Green, D. R. Apoptotic pathways: ten minutes to dead. *Cell* (2005) **121**: 671-674.
- Green, D. R., Kroemer, G. The pathophysiology of mitochondrial cell death. *Science* (2004) **305**: 626-629.
- Green, N. Bariatric surgery: an overview. *Nurs Stand* (2012) **26**: 48-56: quiz 58, 60.
- Gregor, M. F., Yang, L., Fabbrini, E., Mohammed, B. S., Eagon, J. C., Hotamisligil, G. S., Klein, S. Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. *Diabetes* (2009) **58**: 693-700.
- Gu, H., Guo, X., Zou, L., Zhu, H., Zhang, J. Upregulation of microRNA-372 associates with tumor progression and prognosis in hepatocellular carcinoma. *Mol Cell Biochem* (2013) **375**: 23-30.
- Guo, Y., Srinivasula, S. M., Druilhe, A., Fernandes-Alnemri, T., Alnemri, E. S. Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J Biol Chem* (2002) **277**: 13430-13437.
- Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., Davis, R. J. Selective interaction of JNK protein kinase isoforms with transcription factors. *Embo J* (1996) **15**: 2760-2770.

- Gupta, S., Natarajan, R., Payne, S. G., Studer, E. J., Spiegel, S., Dent, P., Hylemon, P. B. Deoxycholic acid activates the c-Jun N-terminal kinase pathway via FAS receptor activation in primary hepatocytes. Role of acidic sphingomyelinase-mediated ceramide generation in FAS receptor activation. *J Biol Chem* (2004) **279**: 5821-5828.
- Haigis, M. C., Guarente, L. P. Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. *Genes Dev* (2006) **20**: 2913-2921.
- Ham, J., Babij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M., Rubin, L. L. A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* (1995) **14**: 927-939.
- Han, S. I., Studer, E., Gupta, S., Fang, Y., Qiao, L., Li, W., Grant, S., Hylemon, P. B., Dent, P. Bile acids enhance the activity of the insulin receptor and glycogen synthase in primary rodent hepatocytes. *Hepatology* (2004) **39**: 456-463.
- Hanada, M., Feng, J., Hemmings, B. A. Structure, regulation and function of PKB/AKT--a major therapeutic target. *Biochim Biophys Acta* (2004) **1697**: 3-16.
- Harrison, S. A., Torgerson, S., Hayashi, P., Ward, J., Schenker, S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* (2003) **98**: 2485-2490.
- Haufe, S., Engeli, S., Kast, P., Bohnke, J., Utz, W., Haas, V., Hermsdorf, M., Mahler, A., Wiesner, S., Birkenfeld, A. L., Sell, H., Otto, C., Mehling, H., Luft, F. C., Eckel, J., Schulz-Menger, J., Boschmann, M., Jordan, J. Randomized comparison of reduced fat and reduced carbohydrate hypocaloric diets on intrahepatic fat in overweight and obese human subjects. *Hepatology* (2011) **53**: 1504-1514.
- He, L., He, X., Lim, L. P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A. L., Linsley, P. S., Chen, C., Lowe, S. W., Cleary, M. A., Hannon, G. J. A microRNA component of the p53 tumour suppressor network. *Nature* (2007) **447**: 1130-1134.
- Hengartner, M. O. The biochemistry of apoptosis. *Nature* (2000) **407**: 770-776.
- Hermeking, H. The miR-34 family in cancer and apoptosis. *Cell Death Differ* (2010) **17**: 193-199.
- Hers, I., Vincent, E. E., Tavaré, J. M. Akt signalling in health and disease. *Cell Signal* (2011) **23**: 1515-1527.
- Hibi, M., Lin, A., Smeal, T., Minden, A., Karin, M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev* (1993) **7**: 2135-2148.
- Higuchi, H., Gores, G. J. Bile acid regulation of hepatic physiology: IV. Bile acids and death receptors. *Am J Physiol Gastrointest Liver Physiol* (2003) **284**: G734-738.
- Higuchi, H., Grambihler, A., Canbay, A., Bronk, S. F., Gores, G. J. Bile acids up-regulate death receptor 5/TRAIL-receptor 2 expression via a c-Jun N-terminal kinase-dependent pathway involving Sp1. *J Biol Chem* (2004) **279**: 51-60.

References

- Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M., Hotamisligil, G. S. A central role for JNK in obesity and insulin resistance. *Nature* (2002) **420**: 333-336.
- Hofmann, A. F. The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med* (1999) **159**: 2647-2658.
- Hofmann, A. F., Roda, A. Physicochemical properties of bile acids and their relationship to biological properties: an overview of the problem. *J Lipid Res* (1984) **25**: 1477-1489.
- Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F., Spiegelman, B. M. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α - and obesity-induced insulin resistance. *Science* (1996) **271**: 665-668.
- Hou, X., Xu, S., Maitland-Toolan, K. A., Sato, K., Jiang, B., Ido, Y., Lan, F., Walsh, K., Wierzbicki, M., Verbeuren, T. J., Cohen, R. A., Zang, M. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* (2008) **283**: 20015-20026.
- Houstis, N., Rosen, E. D., Lander, E. S. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* (2006) **440**: 944-948.
- Hsieh, A. C., Truitt, M. L., Ruggero, D. Oncogenic AKTivation of translation as a therapeutic target. *Br J Cancer* (2011) **105**: 329-336.
- Hsu, S. H., Wang, B., Kota, J., Yu, J., Costinean, S., Kutay, H., Yu, L., Bai, S., La Perle, K., Chivukula, R. R., Mao, H., Wei, M., Clark, K. R., Mendell, J. R., Caligiuri, M. A., Jacob, S. T., Mendell, J. T., Ghoshal, K. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J Clin Invest* (2012) **122**: 2871-2883.
- Hu, J., Xu, Y., Hao, J., Wang, S., Li, C., Meng, S. MiR-122 in hepatic function and liver diseases. *Protein Cell* (2012) **3**: 364-371.
- Hu, P., Han, Z., Couvillon, A. D., Kaufman, R. J., Exton, J. H. Autocrine tumor necrosis factor α links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1 α -mediated NF- κ B activation and down-regulation of TRAF2 expression. *Mol Cell Biol* (2006) **26**: 3071-3084.
- Huang, T. T., Wuerzberger-Davis, S. M., Wu, Z. H., Miyamoto, S. Sequential modification of NEMO/IKK γ by SUMO-1 and ubiquitin mediates NF- κ B activation by genotoxic stress. *Cell* (2003) **115**: 565-576.
- Hui, J. M., Hodge, A., Farrell, G. C., Kench, J. G., Kriketos, A., George, J. Beyond insulin resistance in NASH: TNF- α or adiponectin? *Hepatology* (2004) **40**: 46-54.
- Hwang, J. H., Stein, D. T., Barzilai, N., Cui, M. H., Tonelli, J., Kishore, P., Hawkins, M. Increased intrahepatic triglyceride is associated with peripheral insulin resistance: in vivo MR imaging and spectroscopy studies. *Am J Physiol Endocrinol Metab* (2007) **293**: E1663-1669.
- Ichimura, A., Ruike, Y., Terasawa, K., Shimizu, K., Tsujimoto, G. MicroRNA-34a inhibits cell proliferation by repressing mitogen-activated protein kinase kinase 1 during megakaryocytic differentiation of K562 cells. *Mol Pharmacol* (2010) **77**: 1016-1024.

- Ignacio Barrasa, J., Olmo, N., Perez-Ramos, P., Santiago-Gomez, A., Lecona, E., Turnay, J., Antonia Lizarbe, M. Deoxycholic and chenodeoxycholic bile acids induce apoptosis via oxidative stress in human colon adenocarcinoma cells. *Apoptosis* (2011) **16**: 1054-1067.
- Janssens, S., Tinel, A., Lippens, S., Tschopp, J. PIDD mediates NF-kappaB activation in response to DNA damage. *Cell* (2005) **123**: 1079-1092.
- Jin, X., Chen, Y. P., Kong, M., Zheng, L., Yang, Y. D., Li, Y. M. Transition from hepatic steatosis to steatohepatitis: Unique microRNA patterns and potential downstream functions and pathways. *J Gastroenterol Hepatol* (2011).
- Jobgen, W., Fu, W. J., Gao, H., Li, P., Meininger, C. J., Smith, S. B., Spencer, T. E., Wu, G. High fat feeding and dietary L-arginine supplementation differentially regulate gene expression in rat white adipose tissue. *Amino Acids* (2009) **37**: 187-198.
- Jost, P. J., Grabow, S., Gray, D., McKenzie, M. D., Nachbur, U., Huang, D. C., Bouillet, P., Thomas, H. E., Borner, C., Silke, J., Strasser, A., Kaufmann, T. XIAP discriminates between type I and type II FAS-induced apoptosis. *Nature* (2009) **460**: 1035-1039.
- Kahn, B. B., Flier, J. S. Obesity and insulin resistance. *J Clin Invest* (2000) **106**: 473-481.
- Kakisaka, K., Cazanave, S. C., Fingas, C. D., Guicciardi, M. E., Bronk, S. F., Werneburg, N. W., Mott, J. L., Gores, G. J. Mechanisms of lysophosphatidylcholine-induced hepatocyte lipoapoptosis. *Am J Physiol Gastrointest Liver Physiol* (2012) **302**: G77-84.
- Katagiri, H., Yamada, T., Oka, Y. Adiposity and cardiovascular disorders: disturbance of the regulatory system consisting of humoral and neuronal signals. *Circ Res* (2007) **101**: 27-39.
- Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., Wahli, W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* (1999) **103**: 1489-1498.
- Kim, J. K., Fillmore, J. J., Sunshine, M. J., Albrecht, B., Higashimori, T., Kim, D. W., Liu, Z. X., Soos, T. J., Cline, G. W., O'Brien, W. R., Littman, D. R., Shulman, G. I. PKC-theta knockout mice are protected from fat-induced insulin resistance. *J Clin Invest* (2004) **114**: 823-827.
- Kitamura, T., Kitamura, Y., Kuroda, S., Hino, Y., Ando, M., Kotani, K., Konishi, H., Matsuzaki, H., Kikkawa, U., Ogawa, W., Kasuga, M. Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt. *Mol Cell Biol* (1999) **19**: 6286-6296.
- Klein, E. A., Thompson, I. M., Jr., Tangen, C. M., Crowley, J. J., Lucia, M. S., Goodman, P. J., Minasian, L. M., Ford, L. G., Parnes, H. L., Gaziano, J. M., Karp, D. D., Lieber, M. M., Walther, P. J., Klotz, L., Parsons, J. K., Chin, J. L., Darke, A. K., Lippman, S. M., Goodman, G. E., Meyskens, F. L., Jr., Baker, L. H. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *Jama* (2011) **306**: 1549-1556.
- Kleiner, D. E., Brunt, E. M. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. *Semin Liver Dis* (2012) **32**: 3-13.

References

- Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., Ferrell, L. D., Liu, Y. C., Torbenson, M. S., Unalp-Arida, A., Yeh, M., McCullough, A. J., Sanyal, A. J., Nonalcoholic Steatohepatitis Clinical Research, N. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* (2005) **41**: 1313-1321.
- Knights, C. D., Catania, J., Di Giovanni, S., Muratoglu, S., Perez, R., Swartzbeck, A., Quong, A. A., Zhang, X., Beerman, T., Pestell, R. G., Avantiaggiati, M. L. Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate. *J Cell Biol* (2006) **173**: 533-544.
- Koehler, E. M., Schouten, J. N., Hansen, B. E., van Rooij, F. J., Hofman, A., Stricker, B. H., Janssen, H. L. Prevalence and risk factors of non-alcoholic fatty liver disease in the elderly: results from the Rotterdam study. *J Hepatol* (2012) **57**: 1305-1311.
- Kroemer, G., Galluzzi, L., Brenner, C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* (2007) **87**: 99-163.
- Kubbutat, M. H., Jones, S. N., Vousden, K. H. Regulation of p53 stability by Mdm2. *Nature* (1997) **387**: 299-303.
- Kumar, R., Thompson, E. B. The structure of the nuclear hormone receptors. *Steroids* (1999) **64**: 310-319.
- Kurosawa, H., Que, F. G., Roberts, L. R., Fesmier, P. J., Gores, G. J. Hepatocytes in the bile duct-ligated rat express Bcl-2. *Am J Physiol* (1997) **272**: G1587-1593.
- Kusminski, C. M., Shetty, S., Orci, L., Unger, R. H., Scherer, P. E. Diabetes and apoptosis: lipotoxicity. *Apoptosis* (2009) **14**: 1484-1495.
- Lahav, G., Rosenfeld, N., Sigal, A., Geva-Zatorsky, N., Levine, A. J., Elowitz, M. B., Alon, U. Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nat Genet* (2004) **36**: 147-150.
- Lan, F., Cacicedo, J. M., Ruderman, N., Ido, Y. SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation. *J Biol Chem* (2008) **283**: 27628-27635.
- Lee, G. Y., Kim, N. H., Zhao, Z. S., Cha, B. S., Kim, Y. S. Peroxisomal-proliferator-activated receptor alpha activates transcription of the rat hepatic malonyl-CoA decarboxylase gene: a key regulation of malonyl-CoA level. *Biochem J* (2004) **378**: 983-990.
- Lee, J., Padhye, A., Sharma, A., Song, G., Miao, J., Mo, Y. Y., Wang, L., Kemper, J. K. A pathway involving farnesoid X receptor and small heterodimer partner positively regulates hepatic sirtuin 1 levels via microRNA-34a inhibition. *J Biol Chem* (2010) **285**: 12604-12611.
- Lee, Y. H., Giraud, J., Davis, R. J., White, M. F. c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. *J Biol Chem* (2003) **278**: 2896-2902.
- Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., Staels, B. Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev* (2009) **89**: 147-191.

- Leuschner, U. F., Lindenthal, B., Herrmann, G., Arnold, J. C., Rossle, M., Cordes, H. J., Zeuzem, S., Hein, J., Berg, T. High-dose ursodeoxycholic acid therapy for nonalcoholic steatohepatitis: a double-blind, randomized, placebo-controlled trial. *Hepatology* (2010) **52**: 472-479.
- Li, H., Zhu, H., Xu, C. J., Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* (1998) **94**: 491-501.
- Li, J., Yuan, J. Caspases in apoptosis and beyond. *Oncogene* (2008) **27**: 6194-6206.
- Li, N., Fu, H., Tie, Y., Hu, Z., Kong, W., Wu, Y., Zheng, X. miR-34a inhibits migration and invasion by down-regulation of c-Met expression in human hepatocellular carcinoma cells. *Cancer Lett* (2009a) **275**: 44-53.
- Li, N., Muthusamy, S., Liang, R., Sarojini, H., Wang, E. Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1. *Mech Ageing Dev* (2011) **132**: 75-85.
- Li, S., Chen, X., Zhang, H., Liang, X., Xiang, Y., Yu, C., Zen, K., Li, Y., Zhang, C. Y. Differential expression of microRNAs in mouse liver under aberrant energy metabolic status. *J Lipid Res* (2009b) **50**: 1756-1765.
- Li, T., Francl, J. M., Boehme, S., Chiang, J. Y. Regulation of cholesterol and bile acid homeostasis by the CYP7A1/SREBP2/miR-33a axis. *Hepatology* (2013).
- Li, Z. Z., Berk, M., McIntyre, T. M., Feldstein, A. E. Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearyl-CoA desaturase. *J Biol Chem* (2009c) **284**: 5637-5644.
- Liechti, F., Dufour, J. F. Treatment of NASH with ursodeoxycholic acid: cons. *Clin Res Hepatol Gastroenterol* (2012) **36 Suppl 1**: S46-52.
- Lindor, K. D., Kowdley, K. V., Heathcote, E. J., Harrison, M. E., Jorgensen, R., Angulo, P., Lymp, J. F., Burgart, L., Colin, P. Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: results of a randomized trial. *Hepatology* (2004) **39**: 770-778.
- Ljungman, M. Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. *Neoplasia* (2000) **2**: 208-225.
- Lomonaco, R., Ortiz-Lopez, C., Orsak, B., Webb, A., Hardies, J., Darland, C., Finch, J., Gastaldelli, A., Harrison, S., Tio, F., Cusi, K. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. *Hepatology* (2012) **55**: 1389-1397.
- Lopez, J. M., Bennett, M. K., Sanchez, H. B., Rosenfeld, J. M., Osborne, T. F. Sterol regulation of acetyl coenzyme A carboxylase: a mechanism for coordinate control of cellular lipid. *Proc Natl Acad Sci U S A* (1996) **93**: 1049-1053.
- Machado, M. V., Ferreira, D. M., Castro, R. E., Silvestre, A. R., Evangelista, T., Coutinho, J., Carepa, F., Costa, A., Rodrigues, C. M., Cortez-Pinto, H. Liver and muscle in morbid obesity: the interplay of Fatty liver and insulin resistance. *PLoS One* (2012) **7**: e31738.
- Malhi, H., Bronk, S. F., Werneburg, N. W., Gores, G. J. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *J Biol Chem* (2006) **281**: 12093-12101.

References

- Malhi, H., Gores, G. J. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin Liver Dis* (2008) **28**: 360-369.
- Mancini, M., Nicholson, D. W., Roy, S., Thornberry, N. A., Peterson, E. P., Casciola-Rosen, L. A., Rosen, A. The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. *J Cell Biol* (1998) **140**: 1485-1495.
- Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., McCullough, A. J., Natale, S., Forlani, G., Melchionda, N. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* (2001) **50**: 1844-1850.
- Mari, M., Caballero, F., Colell, A., Morales, A., Caballeria, J., Fernandez, A., Enrich, C., Fernandez-Checa, J. C., Garcia-Ruiz, C. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Metab* (2006) **4**: 185-198.
- Mariash, C. N., Seelig, S., Schwartz, H. L., Oppenheimer, J. H. Rapid synergistic interaction between thyroid hormone and carbohydrate on mRNAs14 induction. *J Biol Chem* (1986) **261**: 9583-9586.
- Masure, S., Haefner, B., Wesselink, J. J., Hoefnagel, E., Mortier, E., Verhasselt, P., Tuytelaars, A., Gordon, R., Richardson, A. Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. *Eur J Biochem* (1999) **265**: 353-360.
- Meier, P. J. Molecular mechanisms of hepatic bile salt transport from sinusoidal blood into bile. *Am J Physiol* (1995) **269**: G801-812.
- Micheau, O., Tschopp, J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* (2003) **114**: 181-190.
- Min, H. K., Kapoor, A., Fuchs, M., Mirshahi, F., Zhou, H., Maher, J., Kellum, J., Warnick, R., Contos, M. J., Sanyal, A. J. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metab* (2012) **15**: 665-674.
- Miura, T., Ouchida, R., Yoshikawa, N., Okamoto, K., Makino, Y., Nakamura, T., Morimoto, C., Makino, I., Tanaka, H. Functional modulation of the glucocorticoid receptor and suppression of NF-kappaB-dependent transcription by ursodeoxycholic acid. *J Biol Chem* (2001) **276**: 47371-47378.
- Moustafa, T., Fickert, P., Magnes, C., Guelly, C., Thueringer, A., Frank, S., Kratky, D., Sattler, W., Reicher, H., Sinner, F., Gumhold, J., Silbert, D., Fauler, G., Hofler, G., Lass, A., Zechner, R., Trauner, M. Alterations in lipid metabolism mediate inflammation, fibrosis, and proliferation in a mouse model of chronic cholestatic liver injury. *Gastroenterology* (2012) **142**: 140-151 e112.
- Murakami, Y., Yasuda, T., Saigo, K., Urashima, T., Toyoda, H., Okanoue, T., Shimotohno, K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* (2006) **25**: 2537-2545.
- Musso, G., Gambino, R., Biroli, G., Carello, M., Faga, E., Pacini, G., De Michieli, F., Cassader, M., Durazzo, M., Rizzetto, M., Pagano, G. Hypoadiponectinemia predicts the

- severity of hepatic fibrosis and pancreatic Beta-cell dysfunction in nondiabetic nonobese patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* (2005) **100**: 2438-2446.
- Nair, S., Diehl, A. M., Wiseman, M., Farr, G. H., Jr., Perrillo, R. P. Metformin in the treatment of non-alcoholic steatohepatitis: a pilot open label trial. *Aliment Pharmacol Ther* (2004) **20**: 23-28.
- Najafi-Shoushtari, S. H., Kristo, F., Li, Y., Shioda, T., Cohen, D. E., Gerszten, R. E., Naar, A. M. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. *Science* (2010) **328**: 1566-1569.
- Neuschwander-Tetri, B. A., Caldwell, S. H. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* (2003) **37**: 1202-1219.
- Nguyen, A., Bouscarel, B. Bile acids and signal transduction: role in glucose homeostasis. *Cell Signal* (2008) **20**: 2180-2197.
- Nissen, S. E., Wolski, K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med* (2007) **356**: 2457-2471.
- North, B. J., Verdin, E. Interphase nucleo-cytoplasmic shuttling and localization of SIRT2 during mitosis. *PLoS One* (2007) **2**: e784.
- Oberst, A., Bender, C., Green, D. R. Living with death: the evolution of the mitochondrial pathway of apoptosis in animals. *Cell Death Differ* (2008) **15**: 1139-1146.
- Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., Taya, Y. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* (2000) **102**: 849-862.
- Ofei, F., Hurel, S., Newkirk, J., Sopwith, M., Taylor, R. Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* (1996) **45**: 881-885.
- Ogawa, W., Kasuga, M. Cell signaling. Fat stress and liver resistance. *Science* (2008) **322**: 1483-1484.
- Okamura, K., Liu, N., Lai, E. C. Distinct mechanisms for microRNA strand selection by *Drosophila* Argonautes. *Mol Cell* (2009) **36**: 431-444.
- Oliver, T. G., Meylan, E., Chang, G. P., Xue, W., Burke, J. R., Humpton, T. J., Hubbard, D., Bhutkar, A., Jacks, T. Caspase-2-mediated cleavage of Mdm2 creates a p53-induced positive feedback loop. *Mol Cell* (2011) **43**: 57-71.
- Omary, M. B., Ku, N. O., Strnad, P., Hanada, S. Toward unraveling the complexity of simple epithelial keratins in human disease. *J Clin Invest* (2009) **119**: 1794-1805.
- Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L. H., Hotamisligil, G. S. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* (2004) **306**: 457-461.
- Ozcan, U., Yilmaz, E., Ozcan, L., Furuhashi, M., Vaillancourt, E., Smith, R. O., Gorgun, C. Z., Hotamisligil, G. S. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* (2006) **313**: 1137-1140.

References

- Pajunen, P., Kotronen, A., Korpi-Hyovalti, E., Keinänen-Kiukaanniemi, S., Oksa, H., Niskanen, L., Saaristo, T., Saltevo, J. T., Sundvall, J., Vanhala, M., Uusitupa, M., Peltonen, M. Metabolically healthy and unhealthy obesity phenotypes in the general population: the FIN-D2D Survey. *BMC Public Health* (2011) **11**: 754.
- Pan, C., Chen, H., Wang, L., Yang, S., Fu, H., Zheng, Y., Miao, M., Jiao, B. Down-regulation of MiR-127 facilitates hepatocyte proliferation during rat liver regeneration. *PLoS One* (2012) **7**: e39151.
- Panasiuk, A., Dzieciol, J., Panasiuk, B., Prokopowicz, D. Expression of p53, Bax and Bcl-2 proteins in hepatocytes in non-alcoholic fatty liver disease. *World J Gastroenterol* (2006) **12**: 6198-6202.
- Park, H. H., Logette, E., Raunser, S., Cuenin, S., Walz, T., Tschopp, J., Wu, H. Death domain assembly mechanism revealed by crystal structure of the oligomeric PIDDosome core complex. *Cell* (2007) **128**: 533-546.
- Park, J. S., Surendran, S., Kamendulis, L. M., Morral, N. Comparative nucleic acid transfection efficacy in primary hepatocytes for gene silencing and functional studies. *BMC Res Notes* (2011) **4**: 8.
- Patel, S., Flyvbjerg, A., Kozakova, M., Frystyk, J., Ibrahim, I. M., Petrie, J. R., Avery, P. J., Ferrannini, E., Walker, M. Variation in the ADIPOQ gene promoter is associated with carotid intima media thickness independent of plasma adiponectin levels in healthy subjects. *Eur Heart J* (2008) **29**: 386-393.
- Patel, T., Gores, G. J. Apoptosis and hepatobiliary disease. *Hepatology* (1995) **21**: 1725-1741.
- Pearce, L. R., Komander, D., Alessi, D. R. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* (2010) **11**: 9-22.
- Perez-Carreras, M., Del Hoyo, P., Martin, M. A., Rubio, J. C., Martin, A., Castellano, G., Colina, F., Arenas, J., Solis-Herruzo, J. A. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* (2003) **38**: 999-1007.
- Pineau, P., Volinia, S., McJunkin, K., Marchio, A., Battiston, C., Terris, B., Mazzaferro, V., Lowe, S. W., Croce, C. M., Dejean, A. miR-221 overexpression contributes to liver tumorigenesis. *Proc Natl Acad Sci U S A* (2010) **107**: 264-269.
- Piro, S., Spadaro, L., Russello, M., Spampinato, D., Oliveri, C. E., Vasquez, E., Benigno, R., Brancato, F., Purrello, F., Rabuazzo, A. M. Molecular determinants of insulin resistance, cell apoptosis and lipid accumulation in non-alcoholic steatohepatitis. *Nutr Metab Cardiovasc Dis* (2008) **18**: 545-552.
- Pogribny, I. P., Starlard-Davenport, A., Tryndyak, V. P., Han, T., Ross, S. A., Rusyn, I., Beland, F. A. Difference in expression of hepatic microRNAs miR-29c, miR-34a, miR-155, and miR-200b is associated with strain-specific susceptibility to dietary nonalcoholic steatohepatitis in mice. *Lab Invest* (2010) **90**: 1437-1446.

- Ponugoti, B., Kim, D. H., Xiao, Z., Smith, Z., Miao, J., Zang, M., Wu, S. Y., Chiang, C. M., Veenstra, T. D., Kemper, J. K. SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism. *J Biol Chem* (2010) **285**: 33959-33970.
- Previs, S. F., Withers, D. J., Ren, J. M., White, M. F., Shulman, G. I. Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. *J Biol Chem* (2000) **275**: 38990-38994.
- Promrat, K., Kleiner, D. E., Niemeier, H. M., Jackvony, E., Kearns, M., Wands, J. R., Fava, J. L., Wing, R. R. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology* (2010) **51**: 121-129.
- Puri, P., Mirshahi, F., Cheung, O., Natarajan, R., Maher, J. W., Kellum, J. M., Sanyal, A. J. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology* (2008) **134**: 568-576.
- Purushotham, A., Schug, T. T., Xu, Q., Surapureddi, S., Guo, X., Li, X. Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab* (2009) **9**: 327-338.
- Qiao, L., Han, S. I., Fang, Y., Park, J. S., Gupta, S., Gilfor, D., Amorino, G., Valerie, K., Sealy, L., Engelhardt, J. F., Grant, S., Hylemon, P. B., Dent, P. Bile acid regulation of C/EBP β , CREB, and c-Jun function, via the extracellular signal-regulated kinase and c-Jun NH₂-terminal kinase pathways, modulates the apoptotic response of hepatocytes. *Mol Cell Biol* (2003) **23**: 3052-3066.
- Qiao, L., McKinstry, R., Gupta, S., Gilfor, D., Windle, J. J., Hylemon, P. B., Grant, S., Fisher, P. B., Dent, P. Cyclin kinase inhibitor p21 potentiates bile acid-induced apoptosis in hepatocytes that is dependent on p53. *Hepatology* (2002) **36**: 39-48.
- Qiao, L., Studer, E., Leach, K., McKinstry, R., Gupta, S., Decker, R., Kukreja, R., Valerie, K., Nagarkatti, P., El Deiry, W., Molkentin, J., Schmidt-Ullrich, R., Fisher, P. B., Grant, S., Hylemon, P. B., Dent, P. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. *Mol Biol Cell* (2001) **12**: 2629-2645.
- Radu, C., Grigorescu, M., Crisan, D., Lupsor, M., Constantin, D., Dina, L. Prevalence and associated risk factors of non-alcoholic fatty liver disease in hospitalized patients. *J Gastrointest Liver Dis* (2008) **17**: 255-260.
- Raimondi, C., Falasca, M. Targeting PDK1 in cancer. *Curr Med Chem* (2011) **18**: 2763-2769.
- Ramalho, R. M., Cortez-Pinto, H., Castro, R. E., Sola, S., Costa, A., Moura, M. C., Camilo, M. E., Rodrigues, C. M. Apoptosis and Bcl-2 expression in the livers of patients with steatohepatitis. *Eur J Gastroenterol Hepatol* (2006) **18**: 21-29.
- Rao, L., Perez, D., White, E. Lamin proteolysis facilitates nuclear events during apoptosis. *J Cell Biol* (1996) **135**: 1441-1455.
- Ratzliff, V. Treatment of NASH with ursodeoxycholic acid: pro. *Clin Res Hepatol Gastroenterol* (2012) **36 Suppl 1**: S41-45.

-
- Ratzliff, V., de Ledinghen, V., Oberti, F., Mathurin, P., Wartelle-Bladou, C., Renou, C., Sogni, P., Maynard, M., Larrey, D., Serfaty, L., Bonnefont-Rousselot, D., Bastard, J. P., Riviere, M., Spenard, J. A randomized controlled trial of high-dose ursodesoxycholic acid for nonalcoholic steatohepatitis. *J Hepatol* (2011) **54**: 1011-1019.
- Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z., Oren, M. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* (2007) **26**: 731-743.
- Rayner, K. J., Sheedy, F. J., Esau, C. C., Hussain, F. N., Temel, R. E., Parathath, S., van Gils, J. M., Rayner, A. J., Chang, A. N., Suarez, Y., Fernandez-Hernando, C., Fisher, E. A., Moore, K. J. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *J Clin Invest* (2011) **121**: 2921-2931.
- Reinehr, R., Becker, S., Hongen, A., Haussinger, D. The Src family kinase Yes triggers hyperosmotic activation of the epidermal growth factor receptor and CD95. *J Biol Chem* (2004) **279**: 23977-23987.
- Ribeiro, P. S., Cortez-Pinto, H., Sola, S., Castro, R. E., Ramalho, R. M., Baptista, A., Moura, M. C., Camilo, M. E., Rodrigues, C. M. Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *Am J Gastroenterol* (2004) **99**: 1708-1717.
- Richieri, G. V., Kleinfeld, A. M. Unbound free fatty acid levels in human serum. *J Lipid Res* (1995) **36**: 229-240.
- Ridlon, J. M., Kang, D. J., Hylemon, P. B. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* (2006) **47**: 241-259.
- Riedl, S. J., Shi, Y. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* (2004) **5**: 897-907.
- Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M., Puigserver, P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* (2005) **434**: 113-118.
- Rodrigues, C. M., Fan, G., Ma, X., Kren, B. T., Steer, C. J. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. *J Clin Invest* (1998a) **101**: 2790-2799.
- Rodrigues, C. M., Fan, G., Wong, P. Y., Kren, B. T., Steer, C. J. Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. *Mol Med* (1998b) **4**: 165-178.
- Rodrigues, C. M., Ma, X., Linehan-Stieers, C., Fan, G., Kren, B. T., Steer, C. J. Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. *Cell Death Differ* (1999) **6**: 842-854.
- Rodrigues, C. M., Sola, S., Sharpe, J. C., Moura, J. J., Steer, C. J. Tauroursodeoxycholic acid prevents Bax-induced membrane perturbation and cytochrome C release in isolated mitochondria. *Biochemistry* (2003) **42**: 3070-3080.

- Rolo, A. P., Teodoro, J. S., Palmeira, C. M. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radic Biol Med* (2012) **52**: 59-69.
- Romashkova, J. A., Makarov, S. S. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* (1999) **401**: 86-90.
- Rong, M., Chen, G., Dang, Y. Increased miR-221 expression in hepatocellular carcinoma tissues and its role in enhancing cell growth and inhibiting apoptosis in vitro. *BMC Cancer* (2013) **13**: 21.
- Rupinder, S. K., Gurpreet, A. K., Manjeet, S. Cell suicide and caspases. *Vascul Pharmacol* (2007) **46**: 383-393.
- Rutkowski, D. T., Wu, J., Back, S. H., Callaghan, M. U., Ferris, S. P., Iqbal, J., Clark, R., Miao, H., Hassler, J. R., Fornek, J., Katze, M. G., Hussain, M. M., Song, B., Swathirajan, J., Wang, J., Yau, G. D., Kaufman, R. J. UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. *Dev Cell* (2008) **15**: 829-840.
- Sabio, G., Cavanagh-Kyros, J., Ko, H. J., Jung, D. Y., Gray, S., Jun, J. Y., Barrett, T., Mora, A., Kim, J. K., Davis, R. J. Prevention of steatosis by hepatic JNK1. *Cell Metab* (2009) **10**: 491-498.
- Sabio, G., Das, M., Mora, A., Zhang, Z., Jun, J. Y., Ko, H. J., Barrett, T., Kim, J. K., Davis, R. J. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. *Science* (2008) **322**: 1539-1543.
- Saha, M. N., Jiang, H., Yang, Y., Zhu, X., Wang, X., Schimmer, A. D., Qiu, L., Chang, H. Targeting p53 via JNK pathway: a novel role of RITA for apoptotic signaling in multiple myeloma. *PLoS One* (2012) **7**: e30215.
- Sale, E. M., Sale, G. J. Protein kinase B: signalling roles and therapeutic targeting. *Cell Mol Life Sci* (2008) **65**: 113-127.
- Saltiel, A. R., Kahn, C. R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* (2001) **414**: 799-806.
- Salunga, T. L., Cui, Z. G., Shimoda, S., Zheng, H. C., Nomoto, K., Kondo, T., Takano, Y., Selmi, C., Alpini, G., Gershwin, M. E., Tsuneyama, K. Oxidative stress-induced apoptosis of bile duct cells in primary biliary cirrhosis. *J Autoimmun* (2007) **29**: 78-86.
- Sanchez-Alcazar, J. A., Schneider, E., Hernandez-Munoz, I., Ruiz-Cabello, J., Siles-Rivas, E., de la Torre, P., Bornstein, B., Brea, G., Arenas, J., Garesse, R., Solis-Herruzo, J. A., Knox, A. J., Navas, P. Reactive oxygen species mediate the down-regulation of mitochondrial transcripts and proteins by tumour necrosis factor-alpha in L929 cells. *Biochem J* (2003) **370**: 609-619.
- Sanyal, A. J. AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology* (2002) **123**: 1705-1725.
- Sanyal, A. J., Campbell-Sargent, C., Mirshahi, F., Rizzo, W. B., Contos, M. J., Sterling, R. K., Luketic, V. A., Shiffman, M. L., Clore, J. N. Nonalcoholic steatohepatitis: association of

References

- insulin resistance and mitochondrial abnormalities. *Gastroenterology* (2001) **120**: 1183-1192.
- Sanyal, A. J., Chalasani, N., Kowdley, K. V., McCullough, A., Diehl, A. M., Bass, N. M., Neuschwander-Tetri, B. A., Lavine, J. E., Tonascia, J., Unalp, A., Van Natta, M., Clark, J., Brunt, E. M., Kleiner, D. E., Hoofnagle, J. H., Robuck, P. R. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* (2010) **362**: 1675-1685.
- Sauve, A. A., Celic, I., Avalos, J., Deng, H., Boeke, J. D., Schramm, V. L. Chemistry of gene silencing: the mechanism of NAD⁺-dependent deacetylation reactions. *Biochemistry* (2001) **40**: 15456-15463.
- Sayed, D., Abdellatif, M. MicroRNAs in development and disease. *Physiol Rev* (2011) **91**: 827-887.
- Schattenberg, J. M., Schuchmann, M. Diabetes and apoptosis: liver. *Apoptosis* (2009) **14**: 1459-1471.
- Schattenberg, J. M., Singh, R., Wang, Y., Lefkowitz, J. H., Rigoli, R. M., Scherer, P. E., Czaja, M. J. JNK1 but not JNK2 promotes the development of steatohepatitis in mice. *Hepatology* (2006) **43**: 163-172.
- Schenk, S., Saberi, M., Olefsky, J. M. Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest* (2008) **118**: 2992-3002.
- Schmucker, D. L., Ohta, M., Kanai, S., Sato, Y., Kitani, K. Hepatic injury induced by bile salts: correlation between biochemical and morphological events. *Hepatology* (1990) **12**: 1216-1221.
- Schungel, S., Buitrago-Molina, L. E., Nalapareddy, P., Lebofsky, M., Manns, M. P., Jaeschke, H., Gross, A., Vogel, A. The strength of the Fas ligand signal determines whether hepatocytes act as type 1 or type 2 cells in murine livers. *Hepatology* (2009) **50**: 1558-1566.
- Seely, B. L., Staubs, P. A., Reichart, D. R., Berhanu, P., Milarski, K. L., Saltiel, A. R., Kusari, J., Olefsky, J. M. Protein tyrosine phosphatase 1B interacts with the activated insulin receptor. *Diabetes* (1996) **45**: 1379-1385.
- Sesti, G. Pathophysiology of insulin resistance. *Best Pract Res Clin Endocrinol Metab* (2006) **20**: 665-679.
- Shalini, S., Dorstyn, L., Wilson, C., Puccini, J., Ho, L., Kumar, S. Impaired antioxidant defence and accumulation of oxidative stress in caspase-2-deficient mice. *Cell Death Differ* (2012) **19**: 1370-1380.
- Shang, J., Chen, L. L., Xiao, F. X., Sun, H., Ding, H. C., Xiao, H. Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase. *Acta Pharmacol Sin* (2008) **29**: 698-706.
- Shaulian, E., Karin, M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* (2002) **4**: E131-136.
- Shin, S., Lee, Y., Kim, W., Ko, H., Choi, H., Kim, K. Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. *Embo J* (2005) **24**: 3532-3542.

- Sieghart, W., Losert, D., Strommer, S., Cejka, D., Schmid, K., Rasoul-Rockenschaub, S., Bodingbauer, M., Crevenna, R., Monia, B. P., Peck-Radosavljevic, M., Wacheck, V. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. *J Hepatol* (2006) **44**: 151-157.
- Singh, R., Wang, Y., Xiang, Y., Tanaka, K. E., Gaarde, W. A., Czaja, M. J. Differential effects of JNK1 and JNK2 inhibition on murine steatohepatitis and insulin resistance. *Hepatology* (2009) **49**: 87-96.
- Sola, S., Amaral, J. D., Castro, R. E., Ramalho, R. M., Borralho, P. M., Kren, B. T., Tanaka, H., Steer, C. J., Rodrigues, C. M. Nuclear translocation of UDCA by the glucocorticoid receptor is required to reduce TGF-beta1-induced apoptosis in rat hepatocytes. *Hepatology* (2005) **42**: 925-934.
- Sola, S., Castro, R. E., Kren, B. T., Steer, C. J., Rodrigues, C. M. Modulation of nuclear steroid receptors by ursodeoxycholic acid inhibits TGF-beta1-induced E2F-1/p53-mediated apoptosis of rat hepatocytes. *Biochemistry* (2004) **43**: 8429-8438.
- Sola, S., Ma, X., Castro, R. E., Kren, B. T., Steer, C. J., Rodrigues, C. M. Ursodeoxycholic acid modulates E2F-1 and p53 expression through a caspase-independent mechanism in transforming growth factor beta1-induced apoptosis of rat hepatocytes. *J Biol Chem* (2003) **278**: 48831-48838.
- Solinas, G., Naugler, W., Galimi, F., Lee, M. S., Karin, M. Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. *Proc Natl Acad Sci U S A* (2006) **103**: 16454-16459.
- Sotillo, E., Laver, T., Mellert, H., Schelter, J. M., Cleary, M. A., McMahon, S., Thomas-Tikhonenko, A. Myc overexpression brings out unexpected antiapoptotic effects of miR-34a. *Oncogene* (2011) **30**: 2587-2594.
- Srivastava, S., Chan, C. Application of metabolic flux analysis to identify the mechanisms of free fatty acid toxicity to human hepatoma cell line. *Biotechnol Bioeng* (2008) **99**: 399-410.
- St George, A., Bauman, A., Johnston, A., Farrell, G., Chey, T., George, J. Effect of a lifestyle intervention in patients with abnormal liver enzymes and metabolic risk factors. *J Gastroenterol Hepatol* (2009) **24**: 399-407.
- Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., Mak, T. W. Regulation of PTEN transcription by p53. *Mol Cell* (2001) **8**: 317-325.
- Strazzullo, P., Barbato, A., Galletti, F., Barba, G., Siani, A., Iacone, R., D'Elia, L., Russo, O., Versiero, M., Farinero, E., Cappuccio, F. P. Abnormalities of renal sodium handling in the metabolic syndrome. Results of the Olivetti Heart Study. *J Hypertens* (2006) **24**: 1633-1639.
- Sun, C., Zhang, F., Ge, X., Yan, T., Chen, X., Shi, X., Zhai, Q. SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B. *Cell Metab* (2007) **6**: 307-319.

References

- Sykes, S. M., Mellert, H. S., Holbert, M. A., Li, K., Marmorstein, R., Lane, W. S., McMahon, S. B. Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell* (2006) **24**: 841-851.
- Sykes, S. M., Stanek, T. J., Frank, A., Murphy, M. E., McMahon, S. B. Acetylation of the DNA binding domain regulates transcription-independent apoptosis by p53. *J Biol Chem* (2009) **284**: 20197-20205.
- Sykitis, G. P., Papavassiliou, A. G. Serine phosphorylation of insulin receptor substrate-1: a novel target for the reversal of insulin resistance. *Mol Endocrinol* (2001) **15**: 1864-1869.
- Takehara, T., Tatsumi, T., Suzuki, T., Rucker, E. B., 3rd, Hennighausen, L., Jinushi, M., Miyagi, T., Kanazawa, Y., Hayashi, N. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. *Gastroenterology* (2004) **127**: 1189-1197.
- Tanno, M., Sakamoto, J., Miura, T., Shimamoto, K., Horio, Y. Nucleocytoplasmic shuttling of the NAD⁺-dependent histone deacetylase SIRT1. *J Biol Chem* (2007) **282**: 6823-6832.
- Tanti, J. F., Jager, J. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Curr Opin Pharmacol* (2009) **9**: 753-762.
- Targher, G., Bertolini, L., Padovani, R., Rodella, S., Tessari, R., Zenari, L., Day, C., Arcaro, G. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* (2007) **30**: 1212-1218.
- Tinel, A., Tschopp, J. The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* (2004) **304**: 843-846.
- Toledo, F., Wahl, G. M. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer* (2006) **6**: 909-923.
- Tomita, K., Teratani, T., Suzuki, T., Oshikawa, T., Yokoyama, H., Shimamura, K., Nishiyama, K., Mataka, N., Irie, R., Minamino, T., Okada, Y., Kurihara, C., Ebinuma, H., Saito, H., Shimizu, I., Yoshida, Y., Hokari, R., Sugiyama, K., Hatsuse, K., Yamamoto, J., Kanai, T., Miura, S., Hibi, T. p53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice. *J Hepatol* (2012) **57**: 837-843.
- Trauner, M., Boyer, J. L. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* (2003) **83**: 633-671.
- Troy, C. M., Rabacchi, S. A., Xu, Z., Maroney, A. C., Connors, T. J., Shelanski, M. L., Greene, L. A. beta-Amyloid-induced neuronal apoptosis requires c-Jun N-terminal kinase activation. *J Neurochem* (2001) **77**: 157-164.
- Tsagarakis, N. J., Drygiannakis, I., Batistakis, A. G., Kolios, G., Kouroumalis, E. A. Octreotide induces caspase activation and apoptosis in human hepatoma HepG2 cells. *World J Gastroenterol* (2011) **17**: 313-321.
- Tsai, W. C., Hsu, S. D., Hsu, C. S., Lai, T. C., Chen, S. J., Shen, R., Huang, Y., Chen, H. C., Lee, C. H., Tsai, T. F., Hsu, M. T., Wu, J. C., Huang, H. D., Shiao, M. S., Hsiao, M., Tsou,

- A. P. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J Clin Invest* (2012) **122**: 2884-2897.
- Tsai, Y. C., Weissman, A. M. The Unfolded Protein Response, Degradation from Endoplasmic Reticulum and Cancer. *Genes Cancer* (2010) **1**: 764-778.
- Tsuchida, T., Shiraishi, M., Ohta, T., Sakai, K., Ishii, S. Ursodeoxycholic acid improves insulin sensitivity and hepatic steatosis by inducing the excretion of hepatic lipids in high-fat diet-fed KK-A(y) mice. *Metabolism* (2011).
- Tuncman, G., Hirosumi, J., Solinas, G., Chang, L., Karin, M., Hotamisligil, G. S. Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance. *Proc Natl Acad Sci U S A* (2006) **103**: 10741-10746.
- Upton, J. P., Austgen, K., Nishino, M., Coakley, K. M., Hagen, A., Han, D., Papa, F. R., Oakes, S. A. Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. *Mol Cell Biol* (2008) **28**: 3943-3951.
- Vakifahmetoglu-Norberg, H., Zhivotovsky, B. The unpredictable caspase-2: what can it do? *Trends Cell Biol* (2010) **20**: 150-159.
- Vanni, E., Bugianesi, E., Kotronen, A., De Minicis, S., Yki-Jarvinen, H., Svegliati-Baroni, G. From the metabolic syndrome to NAFLD or vice versa? *Dig Liver Dis* (2010) **42**: 320-330.
- Vazquez, A., Bond, E. E., Levine, A. J., Bond, G. L. The genetics of the p53 pathway, apoptosis and cancer therapy. *Nat Rev Drug Discov* (2008) **7**: 979-987.
- Ventre, J., Doebber, T., Wu, M., MacNaul, K., Stevens, K., Pasparakis, M., Kollias, G., Moller, D. E. Targeted disruption of the tumor necrosis factor- α gene: metabolic consequences in obese and nonobese mice. *Diabetes* (1997) **46**: 1526-1531.
- Viana, R. J., Ramalho, R. M., Nunes, A. F., Steer, C. J., Rodrigues, C. M. Modulation of amyloid-beta peptide-induced toxicity through inhibition of JNK nuclear localization and caspase-2 activation. *J Alzheimers Dis* (2010) **22**: 557-568.
- Vick, B., Weber, A., Urbanik, T., Maass, T., Teufel, A., Krammer, P. H., Opferman, J. T., Schuchmann, M., Galle, P. R., Schulze-Bergkamen, H. Knockout of myeloid cell leukemia-1 induces liver damage and increases apoptosis susceptibility of murine hepatocytes. *Hepatology* (2009) **49**: 627-636.
- Vinciguerra, M., Sgroi, A., Veyrat-Durebex, C., Rubbia-Brandt, L., Buhler, L. H., Foti, M. Unsaturated fatty acids inhibit the expression of tumor suppressor phosphatase and tensin homolog (PTEN) via microRNA-21 up-regulation in hepatocytes. *Hepatology* (2009) **49**: 1176-1184.
- Viollet, B., Foretz, M., Guigas, B., Horman, S., Dentin, R., Bertrand, L., Hue, L., Andreelli, F. Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. *J Physiol* (2006) **574**: 41-53.
- Voinnet, O. Origin, biogenesis, and activity of plant microRNAs. *Cell* (2009) **136**: 669-687.
- Wajant, H., Pfizenmaier, K., Scheurich, P. Tumor necrosis factor signaling. *Cell Death Differ* (2003) **10**: 45-65.

- Wallach, D., Kang, T. B., Kovalenko, A. The extrinsic cell death pathway and the elan mortel. *Cell Death Differ* (2008) **15**: 1533-1541.
- Wang, D., Wei, Y., Pagliassotti, M. J. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* (2006a) **147**: 943-951.
- Wang, X., Eno, C. O., Altman, B. J., Zhu, Y., Zhao, G., Olberding, K. E., Rathmell, J. C., Li, C. ER stress modulates cellular metabolism. *Biochem J* (2011) **435**: 285-296.
- Wang, Y., Ausman, L. M., Russell, R. M., Greenberg, A. S., Wang, X. D. Increased apoptosis in high-fat diet-induced nonalcoholic steatohepatitis in rats is associated with c-Jun NH2-terminal kinase activation and elevated proapoptotic Bax. *J Nutr* (2008) **138**: 1866-1871.
- Wang, Y., Luo, W., Reiser, G. Proteinase-activated receptor-1 and -2 induce the release of chemokine GRO/CINC-1 from rat astrocytes via differential activation of JNK isoforms, evoking multiple protective pathways in brain. *Biochem J* (2007) **401**: 65-78.
- Wang, Y., Singh, R., Lefkowitz, J. H., Rigoli, R. M., Czaja, M. J. Tumor necrosis factor-induced toxic liver injury results from JNK2-dependent activation of caspase-8 and the mitochondrial death pathway. *J Biol Chem* (2006b) **281**: 15258-15267.
- Watanabe, S., Horie, Y., Suzuki, A. Hepatocyte-specific Pten-deficient mice as a novel model for nonalcoholic steatohepatitis and hepatocellular carcinoma. *Hepatol Res* (2005) **33**: 161-166.
- Welch, C., Chen, Y., Stallings, R. L. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* (2007) **26**: 5017-5022.
- Welsh, G. I., Hers, I., Berwick, D. C., Dell, G., Wherlock, M., Birkin, R., Leney, S., Tavaré, J. M. Role of protein kinase B in insulin-regulated glucose uptake. *Biochem Soc Trans* (2005) **33**: 346-349.
- Westerbacka, J., Lammi, K., Hakkinen, A. M., Rissanen, A., Salminen, I., Aro, A., Yki-Jarvinen, H. Dietary fat content modifies liver fat in overweight nondiabetic subjects. *J Clin Endocrinol Metab* (2005) **90**: 2804-2809.
- Williamson, R. M., Price, J. F., Glancy, S., Perry, E., Nee, L. D., Hayes, P. C., Frier, B. M., Van Look, L. A., Johnston, G. I., Reynolds, R. M., Strachan, M. W. Prevalence of and risk factors for hepatic steatosis and nonalcoholic Fatty liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes Care* (2011) **34**: 1139-1144.
- Wolff, S., Erster, S., Palacios, G., Moll, U. M. p53's mitochondrial translocation and MOMP action is independent of Puma and Bax and severely disrupts mitochondrial membrane integrity. *Cell Res* (2008) **18**: 733-744.
- Xiong, S., Salazar, G., Patrushev, N., Alexander, R. W. FoxO1 mediates an autofeedback loop regulating SIRT1 expression. *J Biol Chem* (2011) **286**: 5289-5299.
- Xu, F., Gao, Z., Zhang, J., Rivera, C. A., Yin, J., Weng, J., Ye, J. Lack of SIRT1 (Mammalian Sirtuin 1) activity leads to liver steatosis in the SIRT1^{+/-} mice: a role of lipid mobilization and inflammation. *Endocrinology* (2010) **151**: 2504-2514.

- Xue, D., Shaham, S., Horvitz, H. R. The Caenorhabditis elegans cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes Dev* (1996) **10**: 1073-1083.
- Yakaryilmaz, F., Guliter, S., Savas, B., Erdem, O., Ersoy, R., Erden, E., Akyol, G., Bozkaya, H., Ozenirler, S. Effects of vitamin E treatment on peroxisome proliferator-activated receptor-alpha expression and insulin resistance in patients with non-alcoholic steatohepatitis: results of a pilot study. *Intern Med J* (2007) **37**: 229-235.
- Yamakuchi, M., Ferlito, M., Lowenstein, C. J. miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci U S A* (2008) **105**: 13421-13426.
- Yamakuchi, M., Lowenstein, C. J. MiR-34, SIRT1 and p53: the feedback loop. *Cell Cycle* (2009) **8**: 712-715.
- Yan, F., Wang, X. M., Liu, Z. C., Pan, C., Yuan, S. B., Ma, Q. M. JNK1, JNK2, and JNK3 are involved in P-glycoprotein-mediated multidrug resistance of hepatocellular carcinoma cells. *Hepatobiliary Pancreat Dis Int* (2010) **9**: 287-295.
- Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., Flavell, R. A. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* (1997) **389**: 865-870.
- Yang, J. I., Yoon, J. H., Myung, S. J., Gwak, G. Y., Kim, W., Chung, G. E., Lee, S. H., Lee, S. M., Kim, C. Y., Lee, H. S. Bile acid-induced TGR5-dependent c-Jun-N terminal kinase activation leads to enhanced caspase 8 activation in hepatocytes. *Biochem Biophys Res Commun* (2007) **361**: 156-161.
- Yang, J. S., Kim, J. T., Jeon, J., Park, H. S., Kang, G. H., Park, K. S., Lee, H. K., Kim, S., Cho, Y. M. Changes in hepatic gene expression upon oral administration of taurine-conjugated ursodeoxycholic acid in ob/ob mice. *PLoS One* (2010) **5**: e13858.
- Yeh, T. C., Chiang, P. C., Li, T. K., Hsu, J. L., Lin, C. J., Wang, S. W., Peng, C. Y., Guh, J. H. Genistein induces apoptosis in human hepatocellular carcinomas via interaction of endoplasmic reticulum stress and mitochondrial insult. *Biochem Pharmacol* (2007) **73**: 782-792.
- Yki-Jarvinen, H. Liver fat in the pathogenesis of insulin resistance and type 2 diabetes. *Dig Dis* (2010) **28**: 203-209.
- Yoshimoto, S., Loo, T. M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., Iwakura, Y., Oshima, K., Morita, H., Hattori, M., Honda, K., Ishikawa, Y., Hara, E., Ohtani, N. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* (2013).
- Yoshizaki, T., Milne, J. C., Imamura, T., Schenk, S., Sonoda, N., Babendure, J. L., Lu, J. C., Smith, J. J., Jirousek, M. R., Olefsky, J. M. SIRT1 exerts anti-inflammatory effects and improves insulin sensitivity in adipocytes. *Mol Cell Biol* (2009) **29**: 1363-1374.
- Youle, R. J., Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* (2008) **9**: 47-59.

References

- Yu, J. W., Shi, Y. FLIP and the death effector domain family. *Oncogene* (2008) **27**: 6216-6227.
- Yuan, Q., Loya, K., Rani, B., Mobus, S., Balakrishnan, A., Lamle, J., Cathomen, T., Vogel, A., Manns, M. P., Ott, M., Cantz, T., Sharma, A. D. MicroRNA-221 overexpression accelerates hepatocyte proliferation during liver regeneration. *Hepatology* (2013) **57**: 299-310.
- Zhang, G., Park, M. A., Mitchell, C., Walker, T., Hamed, H., Studer, E., Graf, M., Rahmani, M., Gupta, S., Hylemon, P. B., Fisher, P. B., Grant, S., Dent, P. Multiple cyclin kinase inhibitors promote bile acid-induced apoptosis and autophagy in primary hepatocytes via p53-CD95-dependent signaling. *J Biol Chem* (2008) **283**: 24343-24358.
- Zhang, J. The direct involvement of SirT1 in insulin-induced insulin receptor substrate-2 tyrosine phosphorylation. *J Biol Chem* (2007) **282**: 34356-34364.
- Zhang, K., Wang, S., Malhotra, J., Hassler, J. R., Back, S. H., Wang, G., Chang, L., Xu, W., Miao, H., Leonardi, R., Chen, Y. E., Jackowski, S., Kaufman, R. J. The unfolded protein response transducer IRE1 α prevents ER stress-induced hepatic steatosis. *Embo J* (2011) **30**: 1357-1375.
- Zhang, Y., Padalecki, S. S., Chaudhuri, A. R., De Waal, E., Goins, B. A., Grubbs, B., Ikeno, Y., Richardson, A., Mundy, G. R., Herman, B. Caspase-2 deficiency enhances aging-related traits in mice. *Mech Ageing Dev* (2007) **128**: 213-221.
- Zhao, T., Li, J., Chen, A. F. MicroRNA-34a induces endothelial progenitor cell senescence and impedes its angiogenesis via suppressing silent information regulator 1. *Am J Physiol Endocrinol Metab* (2010) **299**: E110-116.
- Zhu, H., Yang, Y., Wang, Y., Li, J., Schiller, P. W., Peng, T. MicroRNA-195 promotes palmitate-induced apoptosis in cardiomyocytes by down-regulating Sirt1. *Cardiovasc Res* (2011) **92**: 75-84.
- Zois, C. D., Baltayiannis, G. H., Bekiari, A., Goussia, A., Karayiannis, P., Doukas, M., Demopoulos, D., Mitsellou, A., Vougiouklakis, T., Mitsi, V., Tsianos, E. V. Steatosis and steatohepatitis in postmortem material from Northwestern Greece. *World J Gastroenterol* (2010) **16**: 3944-3949.

