Universidade de Lisboa
Faculdade de Medicina de Lisboa

Programa de Formação Médica Avançada (PFMA), 4ª Edição

What is the role of caspase-2 mediated lipoapoptosis in the pathogenesis of the metabolic syndrome-associated liver disease, nonalcoholic fatty liver disease (NAFLD)?

Mariana Luísa Verdelho Moutinho Machado

Orientador: Prof. Doutora Helena Maria Ramos Marques Cortez-Pinto

Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, especialidade de Gastrenterologia

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PROLOGUE

After finishing medical school in 2002 and before starting to work in the clinical set, there was a gap of 3 months. Meanwhile, I asked Prof. Carmo Fonseca, at the time director of Instituto de Medicina Molecular from Faculdade de Medicina de Lisboa, to be integrated in a short research project. As a result, I collaborated with Prof. Helena Cortez-Pinto in a project regarding genetic susceptibility to Alcoholic Liver Disease. What started as a short collaboration, end up being a long-lasting team. With her I learned how enjoyable and exciting it is to ask relevant clinical questions and study them in depth. Prof. Helena Cortez-Pinto has always challenged me to want to inquire instead of passively approach clinical problems, and always helped me and allowed me to find a way to answer them. Also, she generously has included me in her research. And that is how I came across Nonalcoholic Fatty Liver Disease (NAFLD), which soon after acquaintance became the main focus of my research. NAFLD is also the subject of this thesis, being Professor Helena Cortez-Pinto, one of the world's leading experts in NAFLD, my PhD mentor/supervisor. I have profound gratitude for Prof. Helena Cortez-Pinto for her mentoring since soon after medical school, during Gastroenterology residency and now in the PhD; and most of all, for her friendship.

I started the PhD when I was admitted to the IV edition of "Programa de Formação Médica Avançada, PFMA" (Program for Advanced Medical Education), in October 2011. This is a full-time basis PhD program for MDs supported by the Gulbenkian Foundation (FCG), Champalimaud Foundation (FC), the Ministry of Health and the Fundação para a Ciência e Tecnologia (FCT). Prof. Leonor Parreira was the first director of PFMA, followed by Prof. António Coutinho from 2011 to 2013 and afterward by Prof. Jorge Soares. Prof. João Ferreira, Prof. Thiago Lopes Carvalho and Prof. Francisca Fontes were the program coordinators in the IV edition. In this program, students received six months' graduate courses by an international faculty, followed by doctoral thesis work. Graduate courses took place at leading Portuguese biomedical research institutions: Instituto Gulbenkian de Ciência (IGC); Instituto de Medicina Molecular (IMM)/Faculdade de Medicina da Universidade de Lisboa; IPATIMUP, Porto; Faculdade de Ciência Médicas, Universidade Nova de Lisboa; Faculdade de Medicina da Universidade do Porto.

The work in this doctoral thesis is translational research in the field of NAFLD. It specifically studies the role of an apoptotic protein, caspase-2. It was all performed at Anna Mae Diehl's lab, Duke University, Durham, NC, USA. Prof. Anna Mae Diehl has

been called “the mother of NAFLD”, because of seminal work she has been developing in the clinical and basic research sets, since soon after NAFLD was first described, and that allowed major breakthroughs in the understanding of this complex disease. She generously accepted me in her lab, where I learned not only the laboratorial techniques, but also how to plan research to answer specific questions, how to interpret and integrate results and how to communicate our findings. I am truly thankful to her.

When I arrived to Anna Mae Diehl’s lab, I wanted to study NAFLD. NAFLD is the ectopic accumulation of fat in the liver, which usually occurs in association with obesity, insulin resistance and the metabolic syndrome. It is the liver pandemic of our time, being the number one cause of chronic liver disease in the Western world. Intriguingly, whereas NAFLD is very frequent, only a minority of patients develops progressive disease and is at risk of developing liver cirrhosis and end-stage liver disease. Why only a minority of patients progress is not known. Furthermore, clinicians still cannot accurately predict which patient will progress. Also, there is no available effective treatment for NAFLD. As such, it is pressing to better understand the pathogenesis of NAFLD, and to develop better therapeutic approaches. To study a disease, we must first know what tools we have available to do so. There are many animal models of NAFLD, and the confusion in the field is enormous. As such, and because two of the most used dietary mouse models were being used in the lab, I performed a head-to-head comparison of both diets, which helped us better planning future experiments. We started to study caspase-2 because Prof. Sally Kornbluth asked Prof. Anna Mae Diehl for collaboration after exciting discoveries of the role of caspase-2 in apoptosis induced by lipotoxicity in the *Xenopus laevis* oocyte model. Being the liver the metabolic maestro by excellence, it would be a good system to study caspase-2. Prof. Anna Mae Diehl immediately linked it to NAFLD, the lipotoxic induced liver disease, and she invited me to lead the project. I further expanded the study of caspase-2 to its role in obesity and the metabolic syndrome, the lipotoxic induced adipose tissue disease. After demonstrating a role of caspase-2 in the development and progression of NAFLD, we tested a potential therapeutic strategy to modulate caspase-2 activation and hence liver disease. In the lab, under close orientation and guidance by Prof. Anna Mae Diehl, I planned the experiments, executed them and interpreted them, for all the projects I was assigned.

This thesis is divided in 4 chapters and a unifying conclusion. The first chapter is a literature review of the pathogenesis of NAFLD, focusing in the topics that are relevant to a better understanding of the rational of this work. This chapter is an adaptation of a book

chapter I wrote by invitation of Prof. Anna Mae Diehl and under her orientation, entitled *Pathogenesis of NAFLD,* for the textbook *Zakim and Boyer's Hepatology, 7th edition*. The following three chapters regard to different questions that were studied: comparison of two animal models of NAFLD, the role of caspase-2 in the pathogenesis of the metabolic syndrome and NAFLD, and a therapeutic approach to modulate Coenzyme A metabolism and caspase-2 activation in a mouse model of NAFLD. Those chapters start with an extended introduction composed by a literature review followed by the rational and objectives of the experimental work. The organization follows as per protocol, with the sub-chapters methods, results and discussion. They also include adaptations of the original articles that describe the work, and that were published or are still in submission. The chapter on animal models includes an adaptation of the original article: *Machado MV, et al. Mouse models of diet-induced nonalcoholic steatohepatitis reproduce the heterogeneity of the human disease. Plos One 2015;10(5):0127991*. The chapters on the role of caspase-2 in the pathogenesis of the metabolic syndrome and NAFLD include the adaptation of two original articles, one published and the other one submitted, respectively: *Machado MV, et al. Reduced lipoapoptosis, hedgehog pathway activation and fibrosis in caspase-2 deficient mice with non-alcoholic steatohepatitis. Gut 2015;64(7):1148-57* and *Machado MV, et al. Caspase-2 promotes obesity, the metabolic syndrome and nonalcoholic fatty liver disease. Cell Death & Disease 2016;7:e2096*. The chapter on the pre-clinical trial to modulate Coenzyme A metabolism includes the adaptation of one original article in submission: *Machado MV, et al. Vitamin B5 and N-acetylcysteine in nonalcoholic steatohepatitis: a pre-clinical study in a dietary mouse model. Digestive Disease & Sciences 2016;61(1):137-48.*

During the PhD, I also had the opportunity to write reviews on NAFLD, with Prof. Helena Cortez-Pinto, that helped me expand my knowledge on the topic and to better plan my doctoral thesis work. Those include:

Book chapters:

- **Machado MV**, Cortez-Pinto H. Fígado Gordo Não Alcoólico. In: *Gastrenterologia Essencial*. Editors: Pedro Narra Figueiredo, Leopoldo Matos. Lidel Publications, 2012.
- **Machado MV**, Cortez-Pinto H, Evaluating the NAFLD patient: putting in perspective non-invasive and invasive methods. p. 63-70. In: *Therapy in Liver*

Diseases. Editors: Ginés P, Forns X, Fernandez J, Crespo G, Rodes J, Arroyo V. Elsevier Publications, Barcelona, 2013.

Journal articles:

- Carvalhana S, **Machado MV**, Cortez-Pinto H. Improving dietary patterns in patients with nonalcoholic fatty liver disease. *Curr Opin Clin Nutr Metab Care* 2012 Sep;15(5):468-73.
- **Machado MV**, Cortez-Pinto H. Non-invasive diagnosis of non-alcoholic fatty liver disease – a critical appraisal. *J Hepatol* 2013;58(5):1007-19.
- **Machado MV**, Cortez-Pinto H. Leptin in the treatment of lipodystrophy-associated nonalcoholic fatty liver disease: are we there already? *Expert Rev Gastroenterol Hepatol* 2013;7(6):513-5.
- **Machado MV**, Cortez-Pinto H. Management of fatty liver disease with the metabolic syndrome. *Expert Rev Gastroenterol Hepatol* 2014;8(5):487-500.
- **Machado MV**, Cortez-Pinto H. Nuclear receptors: how do they position in non-alcoholic fatty liver disease treatment? *Liver Int* 2014;34(9):1291-4.
- **Machado MV**, Cortez-Pinto H. Non-alcoholic fatty liver disease – what the clinician needs to know. *World J Gastroenterol* 2014;20(36):12956-80.

Furthermore, I had the opportunity to write reviews on NAFLD and mechanisms of fibrogenesis, with Prof. Anna Mae Diehl, that also helped me expand my knowledge on the topic and to better plan my doctoral thesis work. Those include:

Book chapters:

- **Machado MV**, Diehl AM. Hedgehog signaling in the liver. In: *Signaling Pathways in Liver Disease; 3rd edition*. Editors: Jean-François Dufour and Pierre-Alain Clavier. Wiley-Blackwell Publisher, 2015. ISBN: 978-1-118-66339-4.
- **Machado MV**, Diehl AM. The role of developmental morphogens in liver regeneration. In: *Liver regeneration: basic mechanisms, relevant models and clinical application; 1st edition*. Editor: Udayan Apte. Elsevier Publisher, 2015. ISBN: 978-0-12-420128-6.
- **Machado MV**, Diehl AM. Animal Models of Nonalcoholic Fatty Liver Disease. In: *Alcoholic and non-alcoholic fatty liver disease: bench to bedside*. Editors: Naga Chalasani, Szabo Gyongyi. Springer International Publishing, 2015. ISBN: 978-3-319-20537-3.

- **Machado MV**, Diehl AM. Pathogenesis of Nonalcoholic Fatty Liver Disease. In: *Zakim and Boyer's Hepatology, 7th edition*. Editor: Arun Sanyal. Elsevier Publisher, 2016.

Journal articles:

- **Machado MV**, Yang Y, Diehl AM. The benefits of restraint: a pivotal role for IL-13 in hepatic glucose homeostasis. *J Clin Invest* 2013;123(1):115-7.
- Michelotti GA, **Machado MV**, Diehl AM. NAFLD, NASH and liver cancer. *Nat Rev Gastroenterol Hepatol* 2013;10(11):656-65.
- **Machado MV**, Diehl AM. Liver renewal: detecting misrepair and optimizing regeneration. *Mayo Clin Proc* 2014;89(1):120-30.
- Angulo P, **Machado MV**, Diehl AM. Fibrosis in nonalcoholic fatty liver disease: mechanisms and clinical complications. *Semin Liver Dis* 2015;35(2):132-145.

SUMMARY

Nonalcoholic fatty liver disease can be considered the hepatic manifestation of obesity and the metabolic syndrome. It is the number one cause of chronic liver disease in the Western world. Lipotoxicity in the liver induces epithelial lesion that triggers a wound healing response. In susceptible subjects the wound healing response is ineffective in repairing and regenerating the injured liver, leading to scarring, fibrogenesis and eventually hepatic cirrhosis. Though important advances in the knowledge of the pathogenesis of NAFLD have occurred since its firsts descriptions in 1980, gaps in knowledge still precludes Hepatologists to find an effective treatment for this pandemic.

We first extensively characterized and compared two dietary mouse models of NAFLD, methionine-choline deficient (MCD) and Western diets. We found that MCD diet induces severe disease with significant fibrosis, whereas Western diet induces mild disease, but associates with obesity, insulin resistance and the metabolic syndrome.

Afterwards, we demonstrated a pivotal role of caspase-2 in the development of the metabolic syndrome, NAFLD, progression to severe liver disease and hepatic fibrogenesis. Caspase-2 was up-regulated in human NAFLD and in in several different mouse models of NAFLD, correlating with the degree of fibrosis. Also, caspase-2 deficient mice were protected from the metabolic syndrome and liver injury/fibrosis in both MCD and Western diet mouse models.

Finally, we found that in different mouse models of NAFLD, hepatic free coenzyme A content is decreased, which could potentiate caspase-2 activation. We conducted a preclinical trial in mice submitted to MCD diet, treating them with coenzyme A precursors. This approach failed to correct hepatic free coenzyme A levels and had no impact in liver histology or caspase-2 expression/activation.

Our work places caspase-2 as a potential therapeutic target for obesity-associated diseases, such as type 2 diabetes mellitus and NAFLD.

Keywords: NAFLD, fibrosis, metabolic syndrome, caspase-2, coenzyme A

SUMÁRIO

O Fígado Gordo Não Alcoólico (FGNA) pode ser considerado a manifestação hepática da obesidade e do síndroma metabólico (Loria et al., 2005; Machado and Cortez-Pinto, 2014a). É a primeira causa de doença hepática crónica no Ocidente (Bellentani et al., 2010; Clark et al., 2003). Desde as primeiras descrições de FGNA, em 1980 (Ludwig et al., 1980), conheceram-se avanços importantes no entendimento da patogénese do FGNA, no entanto, muito está ainda por desvendar, o que se traduz na inexistência de um tratamento eficaz para esta pandemia.

O FGNA, classicamente, tem sido dicotomizado em esteatose simples, não progressiva, e esteato-hepatite não alcoólica (EHNA), potencialmente progressiva (Matteoni et al., 1999), evoluindo para cirrose hepática em 20% dos casos (Angulo et al., 1999). O que distingue EHNA de esteatose simples é a presença de lesão celular, lipotoxicidade, que se manifesta morfologicamente com a balonização dos hepatócitos, e morte celular, predominantemente sob a forma de apoptose. A lipotoxicidade no fígado induz lesão epitelial que desencadeia uma resposta reparadora/de cicatrização. Os hepatócitos em sofrimento, prestes a morrer, enviam sinais de alarme que vão actuar em células inflamatórias para eliminar os agentes agressores e as células agonizantes, mas que quando ocorre de forma continuada tem o potencial de agravar a lesão epitelial. As alarminas, libertadas pelos hepatócitos que iniciaram processos de apoptose, também actuam nas células estreladas, produtoras de matriz, por forma a conter a lesão e manter a arquitectura hepática, no entanto, quando ocorre de forma continuada promove deposição excessiva de colagénio e processos de fibrogénese. Em indivíduos susceptíveis, a resposta reparadora não permite regeneração eficaz do fígado lesado, levando a excessiva cicatrização, fibrogénese e eventual cirrose hepática (Machado and Diehl, 2014).

A caspase-2 é uma proteína pró-apoptótica da família das caspases que, apesar de ser uma das primeiras caspases descritas e a mais conservada filogeneticamente, é também a mais enigmática das caspases (Bouchier-Hayes and Green, 2012). Sabe-se que a caspase-2 é um indutor da apoptose, ainda que menos potente que outras caspases como a caspase-8 ou -9 (Bergeron et al., 1998). A sua posição na cascata da apoptose é ainda controversa, uma vez que partilha características de caspases iniciadoras e efectoras. Admite-se, contudo, que seja uma caspase iniciadora actuando acima da mitocôndria, em resposta a estímulos intracelulares como stress oxidativo, lesão do DNA e alguns estímulos metabólicos (Andersen and Kornbluth, 2009). De facto, recentemente, foi descrita a indução de

apoptose dependente de caspase-2 em resposta a acumulação de ácidos gordos saturados, no contexto de privação de nutrientes, no modelo de oócitos de *Xenopus laevis* (Johnson et al., 2013). Por outras palavras, a caspase-2 surge assim como potencial mediador de lipotoxicidade, fazendo a ponte entre lesão por ácidos gordos saturados e morte celular.

Nesta tese de doutoramento, propusemo-nos a avaliar o papel da caspase-2 no desenvolvimento e progressão do FGNA, a doença hepática secundária a lipotoxicidade nos hepatócitos. Propusemo-nos também, de forma mais genérica, a avaliar o papel da caspase-2 no desenvolvimento da obesidade e síndroma metabólico, condições secundárias a lipotoxicidade nos adipócitos.

Como estudo preparatório, avaliámos os principais modelos animais dietéticos disponíveis para estudar o FGNA. Assim, efectuámos um estudo comparativo entre duas dietas indutoras de EHNA em ratinho: dieta deficiente em metionina e colina e dieta ocidental (isto é, rica em gorduras predominantemente saturadas, suplementada com colesterol e glicose/frutose). Apesar destas duas dietas serem muito utilizadas e de haver apoiantes para cada uma delas, um estudo comparativo entre as duas dietas, aplicadas ao mesmo tempo, no mesmo laboratório, em animais com o mesmo pedigree genético, ainda não tinha sido feito. Verificámos que os dois modelos são complementares: o modelo de dieta deficiente em metionina e colina desenvolve doença hepática mais grave com maior lesão celular, inflamação e fibrose, mas não se associa a síndroma metabólico e suas complicações, pelo contrário, os ratinhos desenvolvem caquexia. Por outro lado, o modelo de dieta ocidental induz EHNA ligeira, com fibrose incipiente, mas associa-se a ganho ponderal, síndroma metabólico e resistência à insulina/diabetes mellitus tipo 2.

Numa primeira fase, avaliámos se havia diferenças na expressão hepática da caspase-2 no FGNA, tanto em biopsias de doentes, como em vários modelos animais de ratinho, dietéticos e genéticos. Estudámos a expressão de caspase-2 nos seguintes modelos de ratinho: dieta deficiente em metionina e colina, dieta ocidental, ratinhos geneticamente obesos e diabéticos, ob/ob e db/db sob dieta standard ou deficiente em metionina e colina. Verificámos, tanto em humanos como nos modelos animais, que o FGNA se associa a um aumento da expressão de caspase-2 nos hepatócitos, com predilecção para os hepatócitos balonizados. De notar que, a expressão da caspase-2 se associa positivamente à gravidade de fibrose hepática. Este dado é muito relevante, uma vez que, a presença e gravidade de fibrose hepática é o melhor predictor de mau prognóstico no FGNA (Angulo et al., 2015a; Ekstedt et al., 2015).

De seguida, aplicámos as duas dietas indutoras de EHNA a ratinhos geneticamente deficientes em caspase-2, bem como a controlos geneticamente normais. No modelo da dieta ocidental, os ratinhos deficientes em caspase-2 estavam marcadamente protegidos do desenvolvimento de obesidade central, dislipidémia, resistência à insulina e esteatose hepática, em suma, estavam protegidos do desenvolvimento de síndroma metabólico. O fenótipo metabólico dos ratinhos deficientes em caspase-2 parece ser consequência de diferenças estruturais no tecido adiposo abdominal. De facto, estes ratinhos, sob dieta standard ou sob dieta ocidental, apresentam um tecido adiposo com maior capacidade proliferativa, o que se traduz num maior número de pequenas células e resistência a hipertrofia celular induzida por excesso calórico. O tecido adiposo dos ratinhos deficientes em caspase-2 também está melhor preparado para lidar com excesso calórico, com maior consumo energético graças a aumento da expressão de proteínas desacoplantes mitocondriais. Finalmente, estes ratinhos são menos sensíveis a morte celular por apoptose de adipócitos no contexto do excesso calórico. A agregação destas propriedades do tecido adiposo, conduzem a uma menor libertação de lípidos para a circulação, impedindo a sua acumulação ectópica em órgãos distantes, nomeadamente no fígado (isto é, esteatose hepática). Curiosamente, a deficiência em caspase-2 não conferiu protecção para esteatose hepática induzida pela dieta deficiente em metionina e colina. Este achado pode ser explicado pelo diferente mecanismo de indução de esteatose hepática neste modelo, que se deve a perturbação na exportação de lípidos pelo fígado sob a forma de lipoproteínas, e não a um aumento de aporte de lípidos circulantes com origem no tecido adiposo como ocorre com a aplicação de uma dieta ocidental.

Em ambos os modelos dietéticos de EHNA, a deficiência de caspase-2 conferiu protecção para lesão hepática, com menor elevação dos enzimas de citólise hepática, menor morte celular por apoptose, menor reacção proliferativa ductular e, de maior importância, menor fibrose hepática.

Finalmente, verificámos que, em diferentes modelos de ratinho de FGNA, há diminuição do conteúdo hepático de coenzima A livre. Redução de coenzima A livre pode potenciar activação da caspase-2 (McCoy et al., 2013a; McCoy et al., 2013b). Assim, efectuámos um ensaio pré-clínico em ratinhos submetidos a dieta deficiente em metionina e colina, que tratámos com precursores de coenzima A, ácido pantoténico ou vitamina B5 e N-acetilcisteína. No entanto, esta estratégia não foi eficaz a normalizar os níveis de coenzima A livre no fígado, bem como não teve impacto na histologia hepática ou expressão/activação da caspase-2.

Este trabalho posiciona a caspase-2 como um potencial alvo terapêutico na abordagem do FGNA/EHNA, e, de forma mais abrangente, do síndroma metabólico, resistência à insulina e suas complicações.

Palavras-chave: FGNA, fibrose, síndroma metabólico, caspase-2, coenzima A.

ABBREVIATIONS

AIF, apoptosis-inducing factor
ALIOS, American Lifestyle-Induced Syndrome
ALT, alanine aminotransferase
Apaf-1, apoptotic protease activating factor 1
AMPK, AMP-activated protein kinase
ApoB, apolipoprotein B
AST, aspartate aminotransferase
BDNF, brain-derived neurotrophic factor
CAMKII, calcium/calmodulin regulated protein kinase II
CARD, caspase-recruitment domain
ChREBP, carbohydrate-responsive element-binding protein
CK-1, caseine kinase 1
CoA, coenzyme A
CoA-GSS, CoA-glutathione disulfide
CPT-1, carnitine palmitoyl transferase I
CTGF, connective tissue growth factor
CYLD, cylindromatosis
DAMP, damage associated molecular pattern
DD, death effector domain
DGAT, acyl-CoA:diacylglycerol acyltransferase
Dhh, Desert hedgehog
DIABLO, direct inhibitor of apoptosis-binding protein with low pI
DISC, death inducing signaling complex
ER, endoplasmic reticulum
ERK, extracellular-signal related kinases
FA, fatty acids
FADD, Fas associated DD
FFA, free fatty acids
FGF, fibroblast growth factor
FLIP, FLICE-like inhibitory protein
GADD-153, DNA damage-inducible gene-153
GBM, gli-binding motif
Gli, glioma-associated oncogene homolog
GPR-78, glucose regulated protein-78
GPx, glutathione peroxidase
GSH, glutathione
GSK, glycogen synthase kinase
H_2O_2, hydrogen peroxyde

HFD, high fat diet

HIF, hypoxia inducible factor

HNE, hydroxynonenal

HNF, hepatocyte nuclear factor

HSC, hepatic stellate cells

IAP, inhibitor of apoptosis proteins

ICH-1, interleukin-1β converting enzyme homolog-1

Ihh, Indian hedgehog

IKK, IkB kinase

IL, interleukin

IR, insulin resistance

IRS, insulin receptor substrate

JAK, Janus kinase

JNK, c-Jun N-terminal kinases

KO, knockout

LALD, lysosomal acid lipase deficiency

LBW, liver-to-body weight

LPC, lysophosphatidyl choline

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MAT, methionine adenosyltransferase

MCD, methionine choline deficient

MCP-1, monocyte chemoattractant protein-1

MLKL, mixed lineage kinase domain-like

MOMP, mitochondrial outer membrane permeabilization

MTP, microsomal triglyceride transfer protein

NAC, N-acetylcysteine

NAFLD, nonalcoholic fatty liver disease

NASH, nonalcoholic steatohepatitis

NEFA, non-esterified fatty acids

NEMO, NFkB-essential modulator

NFkB, nuclear factor kappa B

$O_2^{\cdot-}$, superoxide oxide

ObR, leptin receptor

$^{\cdot}OH$, hydroxyl radical

OPN, osteopontin

oxLDL, oxidized low-density lipoproteins

P, pantothenate

PANK, pantothenate kinase

PAF 16:0, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine

PDGF, platelet-derived growth factor

PEMT, phosphatidylethanolamine N-methyltransferase

PI3K, phosphoinositide 3-kinase

PI3P, phosphatidylinositol-3,4,5-triphosphate

PIDD, p53-induced protein with a death domain

PKA, protein kinase A

PKB, protein kinase B

PKC, protein kinase C

PPAR, peroxisome proliferator-activated receptor

Ptc, patch

PTEN, Phosphatase and tensin homologue deleted on chromosome 10

RAIDD, RIP associated protein with a DD

RIP, receptor interacting protein kinase

ROS, reactive oxygen species

SAMe, S-adenosyl-L-methionine

SCD-1, stearoyl-CoA desaturase-1

Shh, Sonic hedgehog

Smac, second mitochondria-derived activator of caspase

SMA, smooth muscle actin

Smo, smoothened

SOD, superoxide dismutase

SREBP, sterol regulatory element-binding protein

STAM, stelic animal model

STAT, signal transducer and activator of transcription

T2DM, type 2 diabetes mellitus

TGF, transforming growth factor

TIMP, tissue inhibitor of metalloproteinases

TNF-α, tumor necrosis factor-α

TRADD, TNF receptor associated DD

TRAF, TNF-α receptor associated factor

TRAIL, TNF-related apoptosis-inducing ligand

TrPc, transducin repeat-containing protein

TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

UCP, uncoupling protein

UPS, unfolded protein response

VEGF, vascular endothelial growth factor

VLDL, very low-density lipoproteins

WT, wild type

XBP-1, factor X-box binding protein-1

INDEX

PATHOGENESIS OF NAFLD

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the pandemic liver disease of our times (Bellentani et al., 2010; Clark et al., 2003), afflicting over one billion persons worldwide (Loomba and Sanyal, 2013). It is the number one cause of elevation of aminotransferases in the Western world. With a strong association with the growing epidemic of obesity, NAFLD is already the second etiology of liver disease in patients awaiting liver transplantation in the US (Wong et al., 2015). It is also the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma (Wong et al., 2014). With the advent of a curable therapy for chronic hepatitis C, it is expected that NAFLD will rise in the ranking of liver-related morbi-mortality.

The prevalence of NAFLD is roughly one third of the Western population (Vernon et al., 2011). Similar numbers were described in Asian populations (Farrell et al., 2013). Being considered a benign disease, with the majority of patients having a non-progressive phenotype, a patient with NAFLD imposes a 26% increase in health care costs (Baumeister et al., 2008) and, more importantly, associates with excessive mortality (Musso et al., 2011). Up to 5% of patients with NAFLD will progress to liver cirrhosis and end-stage liver disease (Angulo et al., 1999). This is a highly relevant minority that translates in a huge burden of liver disease, given the NAFLD pandemic. Furthermore, having NAFLD increases non-liver morbi-mortality. For example, it increases the risk of developing type 2 diabetes mellitus (T2DM) and has a negative impact in the treatment and complications of T2DM (Anstee et al., 2013; Ekstedt et al., 2006; Ryysy et al., 2000; Targher et al., 2008). NAFLD also increases the cardiovascular risk. In fact, the main cause of death in patients with NAFLD is cardiovascular disease, which is two times more common than in the general population (Adams et al., 2005; Ekstedt et al., 2015).

A fatty liver associates with a fatty body, as obesity is the main risk factor for NAFLD. Indeed, most patients with NAFLD are either overweight or obese (Marchesini et al., 2003). Also, as compared to lean subjects, overweight have a two-fold increased risk and obese a four-fold increased risk of having NAFLD (Bellentani and Tiribelli, 2001; Fabbrini et al., 2010; Vernon et al., 2011). Not all fat deposits are equal in dictating the susceptibility to develop NAFLD, as was elegantly shown by Rocha et al.: visceral fat positively correlates, whereas subcutaneous fat negatively correlates with hepatic steatosis (Rocha et al., 2011). The apparent protection conferred by the subcutaneous fat can translate more efficient storage capacity of subcutaneous adipose tissue. In opposition,

visceral adipose tissue is metabolically more active, with the potential to release more pro-inflammatory cytokines and to perturb adipokines expression, besides being more prone to insulin resistance (IR), which leads to increased lipolysis and release of fatty acids that can reach the liver (Lonardo et al., 2015).

IR and T2DM are other major risk factors not only for developing NAFLD, but also for developing worse liver disease and for liver-related mortality (Campbell et al., 2012; Porepa et al., 2010). Also, metabolic syndrome as an entity, as well as each of its components (abdominal obesity, hypertension, dyslipidemia and glucose intolerance), is a major risk factor for the development and progression of NAFLD (Machado and Cortez-Pinto, 2014a). This association is so strong; that many authors suggested NAFLD should be called Metabolic Liver Disease (Loria et al., 2005).

NAFLD has become a major health threat because it is a highly prevalent condition, for which effective therapy is still lacking. Treatment is based in life-style changing strategies and programs to lose weight. However, compliance for those strategies is insufficient and the true long-term effect in fibrosis is still not certain (Musso et al., 2012; Promrat et al., 2010). A magic pill to treat NAFLD is yet to be developed. Currently, two drugs showed to be effective in improving histology in NAFLD, vitamin E (Sanyal et al., 2010) and obeticholic acid (Neuschwander-Tetri et al., 2015), however their effects are not robust enough to justify their regular use in clinical practice. The antioxidant vitamin E, although it improved liver injury and inflammation in a set of patients, it had no effect on the most aspired outcome, liver fibrosis (Sanyal et al., 2010). The medical concept of *primum non nocere* advises us to use it carefully, to avoid potential harmful effects such as hemorrhagic strokes (Klein et al., 2011; Schurks et al., 2010). Obeticholic acid did show improvement in fibrosis in an additional 16% of treated patients as compared to placebo. However, of concern, the seminal clinical trial showed that obeticholic acid treatment associated with worsening serum lipid profile and IR (Neuschwander-Tetri et al., 2015).

In order to better treat NAFLD, hepatologists need to better understand its pathology. Although major advances have occurred since its first description in the eighties (Ludwig et al., 1980), there are still huge gaps in knowledge, that makes the pathogenesis of NAFLD an exciting field to study.

PATHOGENESIS OF NAFLD – A 10000-FOOT VIEW

NAFLD refers to the ectopic accumulation of fat in the liver (a.k.a. steatosis) that cannot be attributed to alcohol consumption. Minor amounts of fat deposition in the liver can occur in a healthy subject, but when it occurs in at least 5% of hepatocytes it is considered pathological (Chalasani et al., 2012; Ratziu et al., 2010). Under the umbrella of NAFLD, there is a wide spectrum of liver pathology with very different outcomes. When steatosis is the only abnormal finding, the condition is dubbed "simple steatosis" or, more precisely, "isolated steatosis" (Brunt, 2009; Yeh and Brunt, 2014). Classically, isolated steatosis has been considered to be a benign non-progressive condition. Conversely, when besides steatosis there is evidence of inflammatory activity and cell injury, manifested by hepatocyte ballooning and/or cell death, it is called nonalcoholic steatohepatitis (NASH). NASH has the potential to progress to liver cirrhosis and its complications.

This concept of dichotomy, distinguishing between benign isolated steatosis from potentially progressive NASH, was also classically present in the dogmatic view of the pathogenesis of this disease. For many years, NAFLD was explained by the two-hits hypothesis (Day and James, 1998). According to this model of NAFLD pathogenesis, the liver must be subjected to two insults for the full spectrum of NAFLD to develop. The first insult leads to steatosis, which sensitizes the liver to a second insult that leads to inflammation, cell death, and fibrosis. This interesting concept was supported by seminal papers showing that genetically modified rodents that develop spontaneous steatosis (either in the context of obesity or as a consequence of impaired export of lipids from the liver) are more susceptible to endotoxin exposure and tumor necrosis factor-α (TNF-α)-induced liver injury, quickly developing steatohepatitis (Bjorkegren et al., 2002; Yang et al., 1997). It was postulated that the increased susceptibility to the second hit would be a consequence of oxidative stress and lipid peroxidation inducing inflammation and modulating responses to TNF-α (Berson et al., 1998).

More recently, these concepts have been questioned in light of clinical and experimental data. It has not been rigorously proven that isolated steatosis temporally precedes NASH in patients, although some paired biopsies studies showed progression from isolated steatosis to NASH (McPherson et al., 2015; Pais et al., 2013; Singh et al., 2015; Wong et al., 2010). This issue will be difficult to resolve because histological criteria for defining NASH are

still debated, and show high inter-observer variability. Also, a well performed high-quality liver biopsy corresponds to less than 1:50,000 of the total liver volume, and NAFLD features are not homogeneous across the parenchyma. Thus, potential sampling error confounds interpretations of paired liver biopsies (Machado and Cortez-Pinto, 2013b).

The role of steatosis *per se* in NAFLD pathogenesis has also been questioned. Studies in animal models have revealed that not all fat behaves similarly. Triglycerides (the type of fat that accumulates in hepatocytes in livers with isolated steatosis) are now accepted as being the "good guys" that prevent accumulation of toxic fat. Hence, an alternative theory to explain NAFLD pathogenesis is that toxic fat accumulation in the liver occurs in parallel with neutral triglyceride accumulation (i.e. steatosis), and the toxic fat (but not the triglyceride) leads to NASH (Neuschwander-Tetri, 2010). According to this theory, isolated steatosis and NASH are considered two independent, eventually parallel, conditions (Fielding and Angulo, 2014). The two-hits is also simplistic because we now know that several pathological processes, other than oxidative stress, contribute to liver disease, such as endoplasmic reticulum (ER) stress, perturbed autophagy and deregulated cell death. Adding a layer of complexity is the understanding that inflammation can precede steatosis and itself promote steatosis. Also, some factors (for example IR) are able to promote both steatosis and inflammation. In this context, a unifying proposal is the multiple parallel hits hypothesis (Tilg and Moschen, 2010).

Furthermore, NAFLD is now seen as a systemic disease in the spectrum of metabolic disturbances, in which the adipose tissue plays a central role. More than a liver disease, NAFLD is a manifestation of a sick adipose tissue (Stanton et al., 2011). However, at some point, the pathological processes in the liver of patients with NAFLD/NASH self-perpetuate and continue to progress independently of the physiology of the adipose tissue (Lanthier et al., 2011) and even with improvement in hepatic fat accumulation (Powell et al., 1990).

In the last years, another dogma has been challenged: the idea that NASH, i.e. cell injury/death and inflammation, convey a worse liver prognosis. In fact, it was recently shown in two large epidemiological studies that it is not the presence of NASH, rather the presence and severity of liver fibrosis that dictates liver outcomes (Angulo et al., 2015a; Ekstedt et al., 2015). Hence, it is not liver injury *per se* that dictates the prognosis, but the way an individual responds to liver injury and the ability to mount an adequate repair/regenerative response *versus* an inadequate one leading to scarring and progressive fibrosis (Machado and Diehl, 2014).

A huge progress has occurred since NAFLD was first described 35 years ago (Ludwig et al., 1980), but gaps in knowledge still precludes the development of effective treatment for NAFLD/NASH. As such, to study the pathogenesis of NAFLD is not only exciting, but also a necessity, with foreseeing huge impact in the field of Hepatology.

MECHANISMS OF STEATOGENESIS

Hepatic steatosis has been used indistinctly from liver accumulation of triglycerides. However, even though triglycerides are the main store of lipids in the hepatocyte, other forms of lipids can accumulate in the liver, such as free fatty acids (FFA), diacylglycerol, free cholesterol, cholesterol esters, ceramides and phospholipids (Puri et al., 2007).

The availability of FFA will determine the amount of triglycerides synthesized. In NAFLD, FFA's reach the liver from three main sources: 60% from systemic circulation as non-esterified fatty acids (NEFA's), 15% from portal blood as chylomicrons lipoproteins assembled from dietary fat, and 25% from *de novo* synthesis/lipogenesis in the liver (Donnelly et al., 2005) (**Figure 1**).

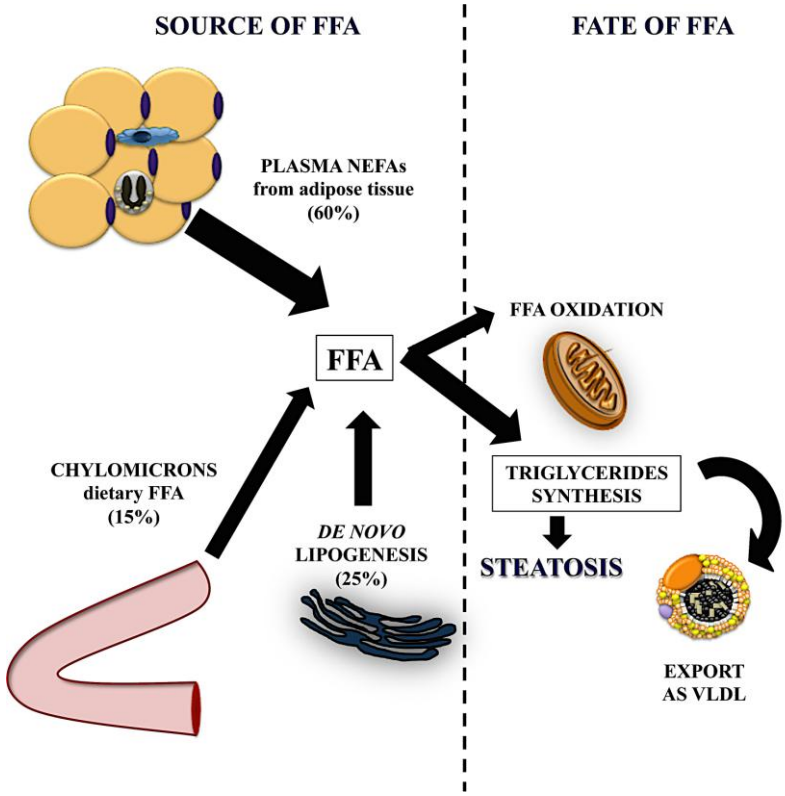


Figure 1. Mechanisms leading to hepatic steatosis

Ectopic fat accumulation in the liver results from an increased influx of lipids, free fatty acids (FFA), to the liver or a decreased lipid disposal. The three main sources of FFA in the liver are: plasmatic nonesterified fatty acids (NEFA's), *de novo* lipogenesis in the liver and dietary FFA. The main source of plasmatic NEFA's is the adipose tissue. Lipolysis in the adipose tissue increases when the adipocyte is overtaxed overwhelming its ability to deal with fat, or in an intrinsically sick adipose tissue (i.e., lipodystrophies). The liver discards fat either by oxidizing it or by exporting it as VLDL. The main place where FA oxidation occurs is the mitochondria, however other organelles, such as peroxysomes, can contribute to fat degradation in conditions of energy surplus. Excessive fat can also accumulate in the hepatocyte, as triglycerides in lipid droplets, leading to steatosis.

The main source of lipids in the liver is, in fact, the adipose tissue. As such, an adipocentric view of the pathogenesis of NAFLD is replacing a hepatocentric one. Circulating NEFA's enter the liver in a non-regulated way, and consequently liver uptake is a function of FFA plasmatic concentrations (Tamura and Shimomura, 2005). Adipose tissue is responsible for 80% of FFA plasmatic pool in the fasting state and 60% in the postprandial state, when fat from diet gains importance (Donnelly et al., 2005). A sick adipose tissue "metastasizes" fat to the liver. Increased NEFA's spill out from the adipose tissue occurs in two opposite situations: either the adipose tissue is intrinsically sick and cannot adequately store energy as occurs in lipodystrophies, or the adipose tissue becomes sick as a consequence of energy surplus that surpasses its natural storage capacity (Machado and Cortez-Pinto, 2014b). IR increases the release of NEFA's from the adipose tissue to systemic circulation. Decreased insulin signaling fails to suppress the hormone-sensitive lipase, increasing release of fatty acids (FA) from triglycerides stored peripherally. Also, by decreasing glucose uptake in the adipose tissue, it decreases glycerol-3-phosphate, necessary for reutilization of FA in triglyceride synthesis (Tamura and Shimomura, 2005).

The second source of hepatic fat in NAFLD is *de novo* lipogenesis. In a healthy lean subject, *de novo* lipogenesis accounts for 5% of hepatic lipids in the fasting state, and increases in the post-prandial period, when availability for lipogenic precursors increases (Donnelly et al., 2005). In a hyperinsulinemic patient with NAFLD, *de novo* lipogenesis increases three-fold in the fasting state (Bays, 2014), but does not further increase after meals, translating an already maximal up-regulation of lipogenic enzymes during fasting (Tamura and Shimomura, 2005). This increased lipogenesis can be explained by hyperinsulinemia and hyperglycemia. Insulin up-regulates expression of sterol regulatory element-binding protein (SREBP-1c) and peroxisome proliferator-activated receptor (PPAR)-γ, which are the major transcriptional factors that regulate lipogenesis by promoting expression of most lipogenic enzymes (Shimomura et al., 1999). Glucose activates carbohydrate-responsive element-binding protein (ChREBP), a transcriptional factor that promotes lipogenesis directly by up-regulation of lipogenic enzymes (Iizuka et al., 2004) and indirectly through increased pyruvate kinase expression, and hence glycolysis, thus providing precursors for lipogenesis (Yamashita et al., 2001). *De novo* lipogenesis not only promotes steatosis by synthesizing more lipids, but also, it inhibits FA oxidation through the accumulation of the intermediate malonyl-CoA (McGarry, 2002).

The third source of fat is the diet. The relevance of this source can increase in hypercaloric high fat diets (Donnelly et al., 2005).

Hepatic steatosis results from an imbalance between the production/uptake of fat and its utilization. The fate of FA in the liver can be divided in 3 routes: oxidation mainly in the mitochondria and to a lesser extent in peroxisomes; synthesis of triglycerides that can be stored as inert lipid droplets; and export from the liver as very low-density lipoproteins (VLDL).

The liver adapts to the overflow of FA increasing mitochondrial β-oxidation and mitochondrial biogenesis (Brady et al., 1985; Ciapaite et al., 2011; Katyare and Howland, 1978). That adaptation occurs not only in response to overflow of FA, but also through the action of adipokines such as leptin, fibroblast growth factor (FGF)-21 and interleukin (IL)-6; as a consequence of hepatic IR; and as a consequence of increased activation of PPAR-α that promotes transcription of lipolytic enzymes (Begriche et al., 2013). Of note, TNF-α represses PPAR-α expression (Beier et al., 1997), and the latter tend to decrease with severe liver injury (Mitsuyoshi et al., 2009), which may limit mitochondrial flexibility as NAFLD progresses.

Mitochondria are organelles highly specialized in extracting efficiently energy from fuel, so we would expect an increased production of ATP in response to increased FA oxidation. However, in patients with NAFLD, liver ATP stores are not increased (Cortez-Pinto et al., 1999), and are even reduced in animal models of NAFLD/NASH (Chavin et al., 1999; Serviddio et al., 2008b). Furthermore, NASH associates with a decreased mitochondrial response to transient energy deficit, which renders the liver more susceptible to ischemic injury (Chavin et al., 1999; Cortez-Pinto et al., 1999; Serviddio et al., 2008b). One mechanism that explains the less efficient ATP production from fuel oxidation is the up-regulation of mitochondrial uncoupling proteins (UCPs). UCP-2 is up-regulated in several animal models of NAFLD/NASH (Chavin et al., 1999; Jiang et al., 2008; Machado et al., 2015c; Serviddio et al., 2008a). It is also up-regulated in human NASH, and correlates with severity of inflammation and fibrosis (Park et al., 2007; Ribeiro et al., 2004). UCPs are proteins localized in the inner membrane of mitochondria that promote proton leak from the outer to the inner mitochondrial membrane, and thus uncoupling oxidative phosphorylation from ATP synthesis (Cortez-Pinto and Machado, 2009).

The aggregate data suggest that steatosis does not occur as a consequence of decreased FA

degradation, though there may be impairments in phosphorylative oxidation. In fact, in NAFLD, an increase in mitochondrial FA oxidation (Bugianesi et al., 2005; Chalasani et al., 2003; Miele et al., 2003; Sanyal et al., 2001) without concomitant up-regulation of mitochondrial respiratory chain (which can even decline as disease progresses) can be in the basis of disturbed redox state (Begriche et al., 2013). Furthermore, the mitochondrial adaptation may not be flexible enough to face the overwhelming FA pressure the steatotic liver is subjected, leading to mitochondrial exhaustion (Knebel et al., 2015). Mitochondrial adaptation is limited by the saturation of rate-limiting enzymes of Krebs citrate cycle and components of the respiratory chain (Knebel et al., 2015). Mitochondrial adaptation is also limited by the FA entry into the mitochondrial matrix through the lipid-level sensitive carnitine shuttle (carnitine palmitoyl transferase I, CPT-1) (Knebel et al., 2015) that is inhibited by malonyl-CoA. To overcome that, FAs are redistributed for degradation in other organelles. In NAFLD, there is a compensatory increase in peroxisomal FA oxidation (Brady et al., 1985). Patients with hepatic steatosis show proliferation and enlargement of hepatic peroxisomes (De Craemer et al., 1995). Peroxisomal lipid degradation has the advantage of allowing unrestricted lipid entry into the organelle. Furthermore, peroxisomes, as compared to mitochondria, are less efficient in extracting energy from the degradation of FA (Knebel et al., 2015). The drawback of peroxisome FA oxidation is the production of hydrogen peroxide, which can perturb redox balance. However, peroxisomes are well equipped with anti-oxidant enzymes, and animal models with impaired peroxisome function develop spontaneous steatohepatitis and hepatocellular carcinoma (Fan et al., 1998). In fact, induction of peroxisomal FA oxidation by PPAR-α agonist protects from NAFLD/NASH in dietary and genetic animal models (Chou et al., 2002; Ip et al., 2003).

The liver can purge excessive fat content by exporting fat as VLDL lipoproteins. VLDL particles contain a core of triglycerides and cholesterol esters, surrounded by phospholipids and the protein apolipoprotein B (apoB) (Fromenty et al., 2004). After translation, apoB is sequentially lipidated in the ER lumen by microsomal triglyceride transfer protein (MTP) and in the Golgi apparatus (Tran et al., 2002). Complete lipidation is necessary for vesicular flow and secretion of apoB into the plasma. Incomplete lipidation targets apoB for ubiquitination and proteasomal degradation (Liao et al., 1998). In IR states and human NAFLD, there is evidence of increased VLDL secretion, probably through up-regulation of MTP and decreased degradation of apoB (Musso et al., 2003;

Taghibiglou et al., 2000). However, some genetic diseases associated with NAFLD/NASH result from deficient VLDL secretion. Abetalipoproteinemia is a rare genetic disease with a mutation in the MTP gene that leads to NASH and cirrhosis associated with fat malabsorption, acanthocytosis and hypocholesterolemia in infancy (Zamel et al., 2008). Familial hypobetalipoproteinemia is a genetic disorder that induces the production of a truncated apoB and impairment of VLDL secretion. As a consequence, patients develop severe hepatic steatosis and NASH, independently of obesity and IR (Amaro et al., 2010). It is estimated to occur in 1:500 to 1:1000 of the population (Tarugi et al., 2007), and clinicians should think of this diagnosis in patients with NASH and low cholesterol levels (i.e. lower than 150 mg/dL total cholesterol and 50 mg/dL HDL-cholesterol) (Schonfeld, 2003). In the differential diagnosis, we should think of lysosomal acid lipase deficiency (LALD), which also manifests with NAFLD and low HDL-cholesterol, though with high LDL-cholesterol, as a result of deficient lysosomal degradation of cholesterol esters and triglycerides. Though extremely rare, with estimated prevalence of 1:40,000 to 1:300,000, it is important to diagnose LALD, since there is now new enzymatic replacement therapy that can be life-saving (Moodie, 2015).

Perturbations in the phospholipid phosphatidylcholine also hamper VLDL assembly and secretion. Phosphatidylcholine can be synthesized through 2 pathways: incorporation of choline into phosphatidyl compounds or through three sequential methylations of phosphatidylethanolamine by phosphatidylethanolamine N-methyltransferase (PEMT) (Takahashi et al., 2012). Choline deficiency is frequent in the Western world and can promote hepatic steatosis by inefficient outflow of lipids from the liver. Choline deficiency can occur as a consequence of low dietary intake, which occurs in more than 90% of the US population (Choline, 2010). Also, obesity-associated dysbiota can promote increased choline degradation in the gut (Dumas et al., 2006). Choline deficiency in association with loss of function PEMT polymorphisms synergically increases the risk for NAFLD (Takahashi et al., 2012).

In conclusion, different roads lead to fat accumulation in the liver. More than the mechanism of fat accumulation *per se*, probably it is the type of fat accumulated and the individual response to lipotoxicity that determines liver outcome.

NAFLD AS THE HEPATIC MANIFESTATION OF ADIPOSOPATHY

The idea that the adipose tissue is a simple inert store of fat is obsolete. We know now that the adipose tissue is a complex and plastic organ that has to adapt to very different conditions such as fuel insufficiency and fuel surplus. In that way, adipose tissue controls whole-body energy status. Also, adipose tissue has important endocrine functions, secreting a wide range of adipokines that regulate many physiological processes such as sensitivity to insulin, appetite, immunity and reproduction (Boyer et al., 2015).

Adiposopathy is a new concept claiming that a positive caloric balance and sedentary lifestyle, in susceptible individuals, leads to adipose tissue dysfunction (Bays, 2014). The adipocyte responds to chronic energy surplus with maladaptive hypertrophy, that is pathological increase in cell size (Sun et al., 2011). Hypertrophic adipocytes, compared to small adipocytes, have increased lipolytic capacity (Laurencikiene et al., 2011), which leads to spill out of NEFAs into the circulation. Circulating NEFAs can accumulate ectopically in other organs such as the liver, heart, muscle, pancreas and kidney (Machado and Cortez-Pinto, 2014b). This response renders an increased metabolic risk. Accordingly, the adipocyte size has a strong positive correlation with risk for developing T2DM, cardiovascular disease and NAFLD (Kloting and Bluher, 2014; Petaja et al., 2013; Wree et al., 2014). The overwhelming increase in the cell size stresses the adipocyte and turns it resistant to insulin and less able to store fat. Adipocytes enlargement results in decreased concentration of cholesterol in the cellular membrane, which contributes to the development of IR (Le Lay et al., 2001). Also, it promotes cytoskeleton disassemble, which is known to impair insulin signaling (Tsakiridis et al., 1997). Furthermore, large adipocytes up-regulate the expression of hormone-sensitive lipase, lipoprotein lipase, leptin, and β-adrenergic receptors (Farnier et al., 2003). This change in gene expression was explained by an increased crosstalk with the extracellular matrix by an enlarged adipocyte, through integrin-β1 signaling, leading to extracellular-signal related kinases (ERK_{1-2}) pathway activation. ERKs are important regulators of adipocyte metabolism (Farnier et al., 2003). Of note, the up-regulation of β-adrenergic receptors is relevant since its stimulation by catecholamines promotes tissue lipolysis during fasting (Koo, 2013).

Other pathological processes occur in the overtaxed adipocyte. For example, increased FA content in the adipocyte promotes oxidative stress (Xu et al., 2012) via mitochondrial production of reactive oxygen species (Chattopadhyay et al., 2015; Paglialunga et al.,

2015), NADPH oxidase activation, and down-regulation of antioxidative enzymes (Furukawa et al., 2004; Han et al., 2012). Oxidative stress in turn, further decreases insulin sensitivity (Paglialunga et al., 2015; Tirosh et al., 1999), deregulates production of adipokines (Furukawa et al., 2004) and induces inflammatory responses (Frohnert et al., 2014; Han et al., 2012). Inflammation further increases oxidative stress establishing a vicious circle (Chandel et al., 2001; Wang and Trayhurn, 2006). Obesity also associates with increased ER stress in the adipose tissue (Boden et al., 2008; Gregor et al., 2009; Li et al., 2015; Ozcan et al., 2004; Sharma et al., 2008). Activation of ER stress further worsens IR through c-Jun N-terminal kinases (JNK) pathway dependent-insulin receptor substrate (IRS)-1 serine phosphorylation (Ozcan et al., 2004). Another important player in the pathology of adiposopathy is hypoxia. In fact, angiogenesis cannot keep up with adipocyte expansion. Enlarged adipocytes compress surrounding vessels decreasing blood flow, and oxidative stress decreases the vasodilator nitric oxide availability, all of the above hampering adipose tissue oxygen supply and hence leading to hypoxia (Bagi et al., 2012; Goossens and Blaak, 2015). Furthermore, adipose tissue expansion induces extracellular matrix remodeling that fails to accommodate the enlarged adipocytes. In fact, adipose tissue becomes enriched with collagen VI, which decreases flexibility of extracellular matrix and further limits its ability to expand. This perpetuates the stress inflicted in the adipose tissue resulting in exacerbated metabolic deregulation (Khan et al., 2009). The mechanisms leading to fibrosis in the adipose tissue are not fully understood, but may be associated with hypoxia-dependent induction of hypoxia inducible factor (HIF)-1α that regulates transcription of several profibrogenic factors such as connective tissue growth factor (CTGF) and tissue inhibitor of metalloproteinases (TIMP)-1 (Finger et al., 2014; Tang et al., 2012). Also, the pro-fibrogenic factor transforming growth factor (TGF)-β is up-regulated by mechanical stress (Liton et al., 2005; Tschumperlin et al., 2003), which can occur in the membrane of adipocytes triggered by the expanded lipid droplet (Khan et al., 2009). In fact, TGF-β expression in the adipose tissue increases in obesity (Samad et al., 1997).

The cellular stress the adipocyte is subjected to may end in cell death. This is a non-intuitive concept, since we think of obesity as an expansion of the adipose tissue. However, cell death is an important and frequent phenomenon in obesity. In fact, in humans and animal models of obesity, adipocyte death increases more than 30 fold (Cinti et al., 2005). After feeding mice 16 weeks with high-fat diet, 80% of adipocytes in visceral

adipose tissue die (Strissel et al., 2007). Hypoxia (Yin et al., 2009), ER stress (Sharma et al., 2008), and mitochondrial dysfunction from oxidative stress or direct damage from cathepsin released from lysosomal permeabilization by FA (Eguchi and Feldstein, 2013; Feng et al., 2011; Gornicka et al., 2012; Masson et al., 2011; Yeung et al., 2006), all can mediate cell death. Cell death decreases the uptake of NEFA's from circulation and induces a boost of release of NEFA's and other toxic products. Also, dying adipocytes initiate an inflammatory response. Obesity associates with recruitment of macrophages (Weisberg et al., 2003; Xu et al., 2003), which remove cellular debris and the free fat released from damaged cells. Of note, more than 90% of macrophages in the adipose tissue surround dead adipocytes, which morphologically produces "crown-like structures" (Cinti et al., 2005). However, the accumulation of macrophages and the induction of a pro-inflammatory M1 phenotype, potentiates an inflammatory state that further worsens IR and metabolic deregulation. In fact, macrophage accumulation and crown-like structures correlate with IR, systemic vascular endothelial dysfunction, hepatic steatosis and NASH (Apovian et al., 2008; Cancello et al., 2005; Cancello et al., 2006; Kolak et al., 2007; Strissel et al., 2007).

Lastly, the sick adipose tissue changes its endocrine properties, decreasing the secretion of adiponectin (Berg et al., 2002; Tsao et al., 2002) and increasing the secretion of IR-inducers, pro-inflammatory, and pro-atherogenic cytokines such as leptin (Friedman and Halaas, 1998), plasminogen-activator inhibitor (PAI)-1 (Shimomura et al., 1996), TNF-α (Hotamisligil et al., 1993; Kirchgessner et al., 1997; Uysal et al., 1997), IL-6 (Fried et al., 1998), MCP-1 (Sartipy and Loskutoff, 2003) and angiotensinogen (Hainault et al., 2002).

Adiponectin is a cytokine only produced in the adipose tissue, but paradoxically its expression decreases with obesity/expansion of adipose tissue (Arita et al., 1999; Bidulescu et al., 2013; Hu et al., 1996; Yatagai et al., 2003). Lower adiponectin levels also associate with T2DM (Spranger et al., 2003; Statnick et al., 2000; Weyer et al., 2001). Importantly, adiponectin inversely correlates with the presence of NAFLD, NASH and severity of fibrosis (Lemoine et al., 2009; Ma et al., 2009; Machado et al., 2012; Musso et al., 2005a; Nannipieri et al., 2009; Polyzos et al., 2011). Adiponectin protected from steatosis and steatohepatitis in animal models of NAFLD (Asano et al., 2009; Fukushima et al., 2009; Matsunami et al., 2010; Tomita et al., 2008). In fact, adiponectin has insulin-sensitizing, anti-inflammatory and anti-fibrotic functions (Finelli and Tarantino, 2013). It protects from steatosis through its effects in whole-body metabolism, through

improvement of mitochondrial function, and through decreased production of reactive oxygen species (Handa et al., 2014; Lin et al., 2014; Yamauchi et al., 2007). Adiponectin is a PPAR-α agonist, ultimately increasing FA β-oxidation (Ghoshal and Bhattacharyya, 2015). It directly sensitizes to insulin favoring tyrosine phosphorylation of members of the downstream pathway of insulin IRS-1 and Akt, while antagonizing the inhibitory serine phosphorylation (Wang et al., 2007). Adiponectin increases anti-inflammatory IL-10 expression and suppresses TNF-α signaling by decreasing its expression and antagonizing its effects (Huang et al., 2008ax); modulates cytokine expression from Kupffer cells by decreasing NFkB activation (Wulster-Radcliffe et al., 2004); and antagonizes IL-1 through increased expression of IL-1 receptor antagonist (Wolf et al., 2004). It inhibits fibrogenesis through decreased production of TGF-β (Tomita et al., 2008) and through direct effects in hepatic stellate cells (HSC) blocking transdifferentiation into myofibroblasts (Adachi and Brenner, 2008; Handy et al., 2011).

Leptin is another important adipokine in the pathogenesis of NAFLD. It is the master regulator of appetite, being a potent anorexigen by its action in the hypothalamus (Takahashi et al., 2012). Many genetic models of obesity/NAFLD take advantage of inherited impairment on leptin signaling. Mice genetically deficient in leptin are hyperphagic, develop morbid obesity, severe IR and severe hepatic steatosis. Leptin, besides regulating food intake, has many anti-steatogenic actions, decreasing gluconeogenesis and *de novo* lipogenesis, while increasing hepatic FA oxidation (Machado and Cortez-Pinto, 2013a). However, leptin-deficient mice develop mild NASH and are protected from fibrosis, even when submitted to other insults to the liver (Diehl, 2005). In fact, leptin has many other functions, namely it modulates body fat composition, insulin activity [decreasing insulin production and secretion (Seufert et al., 1999), while increasing sensitivity to insulin (German et al., 2009; Howard and Flier, 2006)], thermogenesis, immune responses and fibrogenesis (Stojsavljevic et al., 2014). Leptin promotes hepatic fibrogenesis through direct effect in HSC preventing apoptosis (Ikejima et al., 2007), promoting its proliferation (Choi et al., 2010) and transdifferentiation into myofibroblasts (Aleffi et al., 2005; Cao et al., 2007; Choi et al., 2010). Leptin production is a direct function of adipose tissue mass, hence it is increased in patients with obesity. However, hyperleptinemia induces partial leptin resistance, with resistance to the anorexigenic effects, but maintaining peripheral effects, namely pro-fibrogenic ones (El-Haschimi et al., 2000; Knight et al., 2010; Munzberg et al., 2004; Scarpace and Zhang,

2009). In human NAFLD, leptin is believed to be increased, and some studies (though not all) showed a positive association with severity of steatosis, inflammation and fibrosis (Aller et al., 2008; Angulo et al., 2004; Argentou et al., 2009; Canbakan et al., 2008; Chitturi et al., 2002; Huang et al., 2008b; Hui et al., 2004; Kashyap et al., 2009; Le et al., 2007; Lemoine et al., 2009; Lydatakis et al., 2006; Machado et al., 2012; Medici et al., 2010; Munoz et al., 2009; Musso et al., 2005b; Polyzos et al., 2015; Uygun et al., 2000; Wong et al., 2006; Yalniz et al., 2006).

Several other adipokines may be important in the development and progression of NAFLD, such as resistin (Pagano et al., 2006; Senates et al., 2012; Shen et al., 2014; Tan et al., 2015; Tsochatzis et al., 2008; Wong et al., 2006; Zhou et al., 2013), visfatin (Aller et al., 2009; Auguet et al., 2013; Genc et al., 2013; Jarrar et al., 2008) and retinol-binding protein 4 (Alkhouri et al., 2009b; Cengiz et al., 2010; Chang et al., 2015; Schina et al., 2009; Terra et al., 2013). However, their functions and role in the pathogenesis of NAFLD are still controversial.

In summary, energy surplus induces pressure in the adipose tissue to expand. Because adipocytes have a limited capacity to expand, they become stressed and undergo programmed cell death, which initiates an important inflammatory response. This leads to IR in the adipose tissue, deregulation of adipokines production and spill out of FA into the circulation. FA can then accumulate ectopically in other organs, which together with the increase in pro-inflammatory cytokines induces IR in the muscle and in the liver, as well as NAFLD, cardiovascular and renal disease (**Figure 2**).

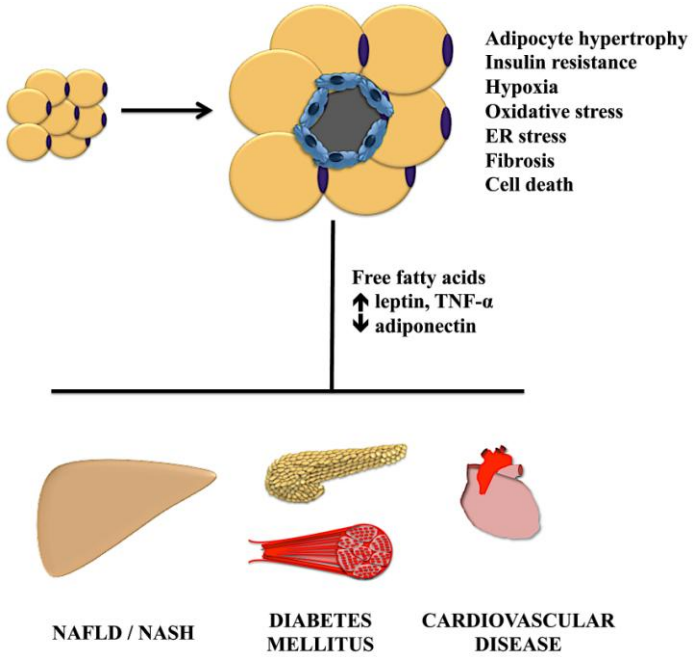


Figure 2. Adiposopathy, the common source of the metabolic syndrome, cardiovascular disease and NAFLD

The overtaxed adipocyte responds with hypertrophy, which is a maladaptive response. Since adipocytes have a limited capacity to expand, adipocyte hypertrophy subjects the cell to several stresses such as oxidative stress, hypoxia, ER stress. The adipose tissue becomes insulin resistant and elicits matrix remodeling that further worsens its physiology. Eventually, adipocytes in the visceral pads undergo programmed cell death, which initiates an important inflammatory response. This further worsens IR, leading to deregulation of adipokines production and spill out of FA into the circulation. FA can then accumulate ectopically in other organs, which together with the increase in pro-inflammatory cytokines induces IR in the muscle and in the liver, as well as NAFLD, cardiovascular and renal disease.

MECHANISMS OF LIPOTOXICITY: TRANSITION FROM STEATOSIS TO STEATOHEPATITIS

Lipid accumulation in the liver induces cell injury through oxidative stress, ER stress and impaired autophagy, resulting in a condition known as lipotoxicity (Machado and Cortez-Pinto, 2014b). Furthermore, NAFLD occurs in a context of obesity and the metabolic syndrome, which, in association with specific dietary patterns, modify the gut microbiota (Backhed et al., 2004; Ley et al., 2005; Mouzaki et al., 2013; Murphy et al., 2010; Raman et al., 2013; Shanab et al., 2011; Turnbaugh et al., 2008; Turnbaugh et al., 2009; Zhu et al., 2013). The obesity/NAFLD-associated dysbiota further promotes obesity and metabolic deregulation by direct actions in the gut and whole-body metabolism (Backhed et al., 2007; Cani et al., 2007; Le Roy et al., 2013; Turnbaugh et al., 2006; Vrieze et al., 2012). Obesity and gut dysbiota also associates with impairment of intestinal barrier and increased gut permeability (Brun et al., 2007; Farhadi et al., 2008; Miele et al., 2009; Wigg et al., 2001). The net result may be an abnormal over-stimulation of the immune system, the liver being a main target as a consequence of its close proximity through portal system. In the liver, injured epithelial cells also release alarm signals that further activate the immune system (Kubes and Mehal, 2012). Moreover, lipid accumulation in immune cells, such as Kupffer cells, contributes for its activation (Ioannou et al., 2013; Leroux et al., 2012). Inflammation is a double-edge sword and may itself aggravate epithelial cells injury, establishing a vicious circle and chronic liver injury.

In a steatotic liver the main lipids that accumulate are triglycerides. Steatosis severity is assessed by the hepatic content of triglycerides, which has been associated with worse metabolic profile and cardiovascular risk (Bian et al., 2011; Ducluzeau et al., 2013; Graner et al., 2015; Jin et al., 2015; Rijzewijk et al., 2010). However, triglycerides *per se* do not seem to be toxic to the liver. On the contrary, triglycerides accumulation may, in fact, be a compensatory defense mechanism, acting as a buffer for truly toxic fat accumulation. Data from animal models beautifully illustrate this concept. Genetically modified mice deficient in MTP cannot export triglycerides and develop hepatic steatosis with accumulation of neutral triglycerides, but do not develop NASH (Raabe et al., 1999). Inhibition of the last step on triglycerides biosynthesis (at the level of acyl-CoA:diacylglycerol acyltransferase, DGAT), in dietary mouse models of NASH, decreased hepatic triglyceride content at expense of an increase in FFA (Yamaguchi et al., 2007; Yu et al., 2005). Consequently, despite a decrease in hepatic steatosis, those mice developed worse liver injury with

increased evidence of lipoperoxidation, apoptosis and fibrosis (Yamaguchi et al., 2007). If not triglycerides, which lipids can be toxic to the liver (**Table 1**)?

Table 1 – Lipids that can promote lipotoxicity

Lipid Type	Role in NAFLD/NASH	Mechanisms of Action
Triglycerides	• Inhibiting triglyceride synthesis in animal models of NASH worsens liver disease. • No difference in triglycerides content in patients with simple steatosis or NASH.	• Neutral lipid acts as a buffer preventing accumulation of toxic lipids. • Role in IR controversial.
Fatty Acids (FA)	• No evidence of increase in FA content in NASH compared with simple steatosis. • Patients with NAFLD (and even more if NASH) have increased dietary consumption of saturated *versus* polyunsaturated FA. • Dietary animal models with severe NASH have higher FFA hepatic content than models with mild NASH.	• Saturated FA induce IR via production of diacylglycerol. • Saturated FA promote apoptosis through increased oxidative stress and production of LPC and ceramides.
Lysophosphatidyl choline (LPC)	• Patients with NASH, as compared with patients with simple steatosis, have increased plasmatic levels of LPC.	• LPC promotes apoptosis inhibiting the synthesis of mitochondrial cardiolipin and recruiting pro-apoptotic Bid.
Ceramides	• Patients with T2DM and morbid obesity have higher plasma levels of ceramides. • Patients with NASH *versus* simple steatosis have higher plasma levels. • In an animal model of NASH, inhibiting ceramide synthesis improved hepatic steatosis, apoptosis and fibrosis.	• Ceramides promote IR. • Ceramides promote apoptosis.
Diacylglycerol	• Patients with NASH have lower hepatic diacylglycerol levels as compared with patients with simple steatosis. • In a mouse model of NASH, despite having decreased hepatic diacylglycerol content, Kupffer cells showed increased diacylglycerol content, correlating with a proinflammatory profile.	• Diacylglycerol promotes IR. • Diacylglycerol may modulate inflammation through protein kinase C activation.
Cholesterol	• In human studies, NASH patients have increased cholesterol content as compared with patients with simple steatosis. • Patients with NASH *versus* simple steatosis have higher intake of cholesterol in the diet. • Dietary animal models with supplementation of cholesterol induce worse liver disease.	• In mouse models, cholesterol accumulation in Kupffer and HSC associates with more pro-inflammatory and pro-fibrogenic phenotype, respectively. • Cholesterol crystals can activate the inflammasome.

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; IR, insulin resistance; FA, fatty acids; LPC, lysophosphatidyl choline; HSC, hepatic stellate cells.

Fatty acids are considered the major hepatotoxic, particularly saturated FA. In fact, *in vitro* incubation of hepatocytes with monosaturated FA induces lipid accumulation, having no effect on cell viability; whereas incubation with saturated FA tremendously decreases viability through increase in apoptosis, despite inducing minor lipid droplets accumulation (Li et al., 2009). Of note, triglycerides biosynthesis preferentially esterify monosaturated FA; and saturated FA must first be desaturated by stearoyl-CoA desaturase-1 (SCD-1) to be incorporated into triglycerides (Bass, 2010). Rodents deficient in SCD-1 submitted to diets to induce NASH develop less steatosis but show higher hepatic content of saturated FA and worse liver injury and fibrosis (Li et al., 2009). Furthermore, the methionine-choline deficient diet animal model of NASH, is the dietary model that induces the worse liver injury, and associates with tremendous down-regulation of SCD-1 expression (Machado et al., 2015c). Saturated FA can induce apoptosis through several mechanisms: increasing production of reactive oxygen species, generating toxic lipids namely ceramides and lysophosphatidyl choline (LPC).

Ceramides can be synthesized in the ER through the condensation of sphingosine and a FA, usually palmitoyl-CoA (Alkhouri et al., 2009a). Ceramides can initiate apoptosis through the generation of channels that increase permeabilization of the mitochondrial outer membrane to proapoptotic intermembrane space proteins (Siskind, 2005; Siskind et al., 2002; Stiban and Perera, 2015). In fact, in human NAFLD, expression of genes in the ceramide metabolism associated with liver fat (Greco et al., 2008); and ceramide plasmatic levels are higher in diabetic and morbidly obese patients, two high-risk populations for developing NAFLD (Haus et al., 2009; Huang et al., 2011), as well as in patients with NASH (Anjani et al., 2015). Moreover, in an animal model of NASH, inhibiting ceramide biosynthesis decreased hepatic steatosis, apoptosis and fibrosis (Kasumov et al., 2015).

Saturated FA palmitate increases LPC content through the action of phospholipase A2 (Han et al., 2008). Subsequently LPC inhibits cardiolipin biosynthesis. Cardiolipin is an important mitochondrial phospholipid that maintains mitochondrial membrane stability and binds to cytochrome c (Ott et al., 2002); as a consequence, decreased cardiolipin content promotes cytochrome c translocation and cell death by apoptosis (Han et al., 2008). Alternatively, LPC can induce apoptosis by recruiting pro-apoptotic Bid (Goonesinghe et al., 2005). In fact, it has been shown recently that patients with NASH, as compared with patients with simple steatosis, have increased plasmatic levels of LPC (Anjani et al., 2015).

Another toxic lipid is diacylglycerol, which is known to induce IR and may have a role in

inflammation through protein kinase C activation (Farrell et al., 2012). In human NASH, hepatic diacylglycerol content is lower as compared with patients with simple steatosis (Puri et al., 2007). However, interestingly, in a mouse model of NASH, it was shown that despite a decrease in hepatic content of diacylglycerol, the Kupffer cells diacylglycerol content was increased compared with control mice, and correlated with a more pro-inflammatory phenotype (Leroux et al., 2012).

Cholesterol has been widely implicated as guilty in the pathogenesis and progression of NASH. Human studies showed increased hepatic free cholesterol content in patients with NASH as compared with simple steatosis (Caballero et al., 2009; Puri et al., 2007). Concordant with this, dietary cholesterol consumption associates with increased risk for NAFLD, NASH and hepatocellular carcinoma (Ioannou et al., 2009; Musso et al., 2003; Yasutake et al., 2009). Also, in animal models of NASH, dietary supplementation with cholesterol worsens liver disease (Shiri-Sverdlov et al., 2006; Van Rooyen et al., 2011; Wouters et al., 2010; Wouters et al., 2008). In those models, accumulation of cholesterol in Kupffer cells associated with a more pro-inflammatory profile (Leroux et al., 2012), whereas accumulation in HSC associated with a more pro-fibrogenic profile (Tomita et al., 2014). Cholesterol modifies membrane permeability and fluidity. Plasma membranes are tolerant to high cholesterol concentrations, however, ER and mitochondrial membranes are susceptible to low increases in cholesterol concentrations, altering those organelles functions, which may lead to ER stress, apoptosis and necrosis of hepatocytes (Musso et al., 2013). More recently, it has been shown that cholesterol can form crystals that accumulate in mice and patients with NASH but not with simple steatosis (Ioannou et al., 2013). Cholesterol in its crystalline form can induce the inflammasome in Kupffer cells, in a process that involves phagolysosomal damage (Duewell et al., 2010).

Lastly, oxidized lipoproteins promote liver injury in animal models of NASH (Bieghs et al., 2013; Yimin et al., 2012). Oxidized LDL mimic pathogen-related epitopes and are phagocytized by Kupffer cells, which acquire a foamy appearance. Because oxidized-LDL, unlike acetylated-LDL, are poorly degraded, they accumulate in lysosomes, ultimately leading to disruption of its membrane with activation of the inflammasome and promotion of a pro-inflammatory state (Bieghs et al., 2013; Walenbergh et al., 2013).

In summary, in regards to hepatic steatosis there is more than the eye can see. Clinically we can easily evaluate triglyceride content either by direct assessment with imaging techniques such as ^{1}H-magnetic resonance spectroscopy or evaluating the presence of lipid droplets in liver sections. However, triglycerides by themselves do not promote liver

injury, they rather act as an innocent bystander that draw our attention to lipid metabolism dysfunction in the liver. Triglycerides are even considered protective, since they can shunt lipids to an inert storage status, as opposed to accumulation of toxic metabolic active forms of lipids. Possibly, the management of NAFLD will shift from strategies that decrease liver fat, to strategies that do increase inert lipid droplets but eliminate unwanted noxious lipid species.

THE ROLE OF CELL DEATH

A *sine qua non* condition for the diagnosis of NASH is the presence of hepatocellular injury manifested as ballooning, mostly on zone 3, and/or evidence of cell death (Brunt, 2009). Cell injury leading to cell death occurs as a consequence of several toxic processes such as direct lipotoxicity, oxidative stress, ER stress, immune cells attack on hepatocytes, among others. Cell death can occur as apoptosis, necrosis or necroptosis (Luedde et al., 2014).

Apoptosis is a form of programmed cell death, in which an unhealthy cell commits suicide, in a controlled way, in order to avoid massive leak of cellular contents that would cause injury in the surrounding tissue and induce a strong inflammatory response (Brenner et al., 2013; Luedde et al., 2014; Machado and Cortez-Pinto, 2011). Morphologically, apoptosis is characterized by unique features, including cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, intranucleosomal DNA fragmentation, phosphatidylserine exposure and, ultimately, fragmentation into membrane-enclosed apoptotic bodies, which can be sequestered by macrophages or other engulfing cells (Wyllie et al., 1980).

Apoptosis can be initiated by external cues, such as TNF-α, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), which bind to death receptors promoting activation of the initiator caspase-8 (extrinsic pathway); or by internal cues, such as ER stress, oxidative stress, DNA damage, which lead to mitochondrial outer membrane permeabilization (MOMP), release of cytochrome c and activation of the initiator caspase 9 (intrinsic pathway). Both the initiator caspase-8 and caspase-9 are able to cleave, and hence activate, effector caspases 3/7 (Machado and Cortez-Pinto, 2011) (**Figure 3**).

In the extrinsic pathway, binding of the death ligand to its specific receptor (TNFR1 or TNFR2 for TNF-α, CD95 or Fas for Fas ligand, and DR3/Wsl-1/Tramp, DR4/ TRAIL-R1, DR5/TRAIL-R2/TRICK2/Killer and DR6 for TRAIL), induces multimerization of the receptor, recruitment of adaptor molecules [for example, FADD (Fas associated DD), TRADD (TNF receptor associated DD), RIP (receptor interacting protein kinase), RAIDD (RIP associated protein with a DD) and FLIP (FLICE-like inhibitory protein] and assembly of a death inducing signaling complex (DISC). DISC is able to recruit and oligomerize caspase-8, leading to caspase-8 auto-catalytic activation (Juo et al., 1998; Varfolomeev et al., 1998). DISC can also activate caspase-2 and -10 (Degterev et al., 2003).

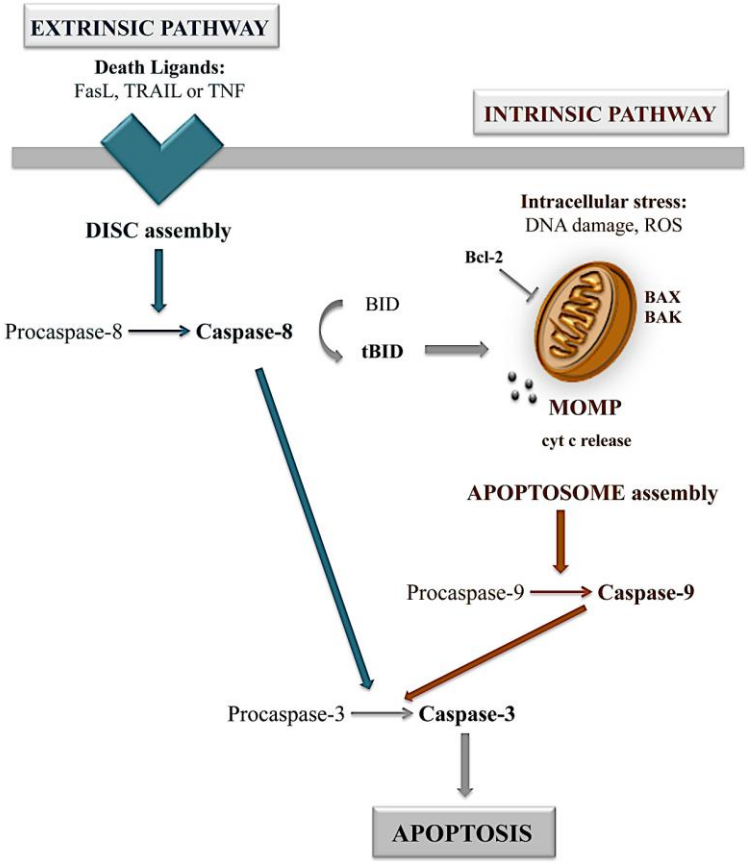


Figure 3. Pathways of apoptosis. The extrinsic pathway is dependent on death receptors that, through a signaling cascade, activate initiator caspases-8. The intrinsic pathway depends on intracellular organelle dysfunction, mostly mitochondria, leading to impaired membrane permeability and cytochrome c release, which ultimately induces caspases-9 activation. Both caspases-8 and -9 activate the effector caspases-3/7, which leads to apoptosis. Cytochrome c release can be prevented by antiapoptotic proteins such as Bcl-2, and can be promoted by proapoptotic proteins such as Bax.

Active caspase-8 can directly activate effector caspases-3 and -7 in type I cells, which are able to produce sufficient amounts of active caspase-8, in order to activate enough downstream caspases to ensue apoptosis (Scaffidi et al., 1999). However, type II cells such as hepatocytes, are not able to activate enough caspase-8, and hence need an additional step of mitochondrial amplification of the death signal, in a signaling cascade shared with the intrinsic pathway. In those cells, caspase-8 cleaves proapoptotic Bcl-2 family member BID (Li et al., 1998; Luo et al., 1998). Truncated BID (tBID) translocates to the mitochondria, induces oligomerization of Bax and Bak in mitochondrial channels (Korsmeyer et al., 2000; Martinez-Caballero et al., 2009), allowing release of cytochrome c. Cytosolic cytochrome c induces the assembly of the apoptosome complex, constituted by seven apoptotic protease activating factor 1 (Apaf-1) adaptor molecules, each one bound to one molecule of cytochrome c and a dimer of pro-caspase-9 (Acehan et al., 2002; Cain et al., 2000; Li et al., 1997; Liu et al., 1996; Zou et al., 1999). This elicits a conformational change and activation of caspase-9.

MOMP allows not only the release of cytochrome c from the mitochondria, but also other proteins such as apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) and

endonuclease G (Degterev et al., 2003). AIF and endonuclease G translocate to the nucleus where they induce chromatin condensation and DNA fragmentation (Daugas et al., 2000; Li et al., 2001). In its turn, Smac/DIABLO inhibits cytosolic apoptosis inhibitors (Martinez-Ruiz et al., 2008).

Animal models of NASH show activation of both intrinsic and extrinsic apoptosis pathways (Farrell et al., 2009). In fact, mice deficient in TRAIL receptor or caspase-3 are protected from diet-induced NASH (Idrissova et al., 2015; Thapaliya et al., 2014). Regarding caspase-8 deficiency, the effect depends on the cell type targeted: deletion in mature, terminally differentiated hepatocytes protects from NASH (Hatting et al., 2013), whereas deletion in more immature cells worsens it (Gautheron et al., 2014). Human studies also corroborate the role of apoptosis in NASH (Feldstein et al., 2003; Ramalho et al., 2006; Ribeiro et al., 2004).

Apoptosis is a strong driver force to hepatic fibrogenesis in NASH, as supported by studies in animal models with increased or inhibited apoptosis that developed worsen or abrogated fibrosis respectively (Hatting et al., 2013; Idrissova et al., 2015; Locatelli et al., 2013; Luedde et al., 2007; Machado et al., 2015b; Takehara et al., 2004; Thapaliya et al., 2014; Vick et al., 2009), and by studies treating animal models of NASH with inhibitors of apoptosis showing prevention/improvement of fibrosis (Anstee et al., 2010; Barreyro et al., 2015; Canbay et al., 2004; Witek et al., 2009). It can promote fibrogenesis through several mechanisms: HSC can engulf apoptotic bodies, increasing their fibrogenic potential (Canbay et al., 2003b; Jiang et al., 2009; Zhan et al., 2006); Kupffer cells can also phagocyte apoptotic bodies increasing secretion of pro-inflammatory and pro-fibrogenic mediators that paracrinally act on HSC (Canbay et al., 2003a); also, cells committed to apoptosis release passively damage associated molecular patterns (DAMPs) and actively substances such as Hedgehog ligand, PDGF and IL-33 that can elicit a repair response which includes activation of HSC (Arshad et al., 2012; Machado et al., 2015b; Rangwala et al., 2011).

Necrosis refers to non-programmed cell death that occurs after overwhelming cell injury with ATP depletion, and results in rapid cellular swelling, disruption of organelles and plasma membrane, with massive release of cellular contents (Yang et al., 2015). Hence, necrosis induces a strong inflammatory response (Brenner et al., 2013; Luedde et al., 2014).

More recently, a different form of cell death has been described, necroptosis. Necroptosis is a programmed necrosis, which uses the machinery of apoptosis but the final outcome is

cell swelling and content leak (Gautheron et al., 2014; Luedde et al., 2014). This type of cell death can be inhibited by necrostatin-1 (Galluzzi et al., 2012). After activation of death receptors, cell decides between apoptosis and necroptosis according to the action of 2 kinases: receptor-interacting protein (RIP)-1 and 3. Ligand binding to the death receptors TNFR1 or TNFR2, recruits RIP-1 and several E3 ubiquitin ligases such as TNF-α receptor associated factor 2/5 (TRAF2/5) and inhibitor of apoptosis proteins cIAP1 and cIAP2, assembling complex I (Wertz and Dixit, 2008). This complex allows ubiquitination of RIP1, which promotes NFkB pathway activation and cell survival (O'Donnell et al., 2007). When TNFR1 is activated in the absence of cIAP or in the presence of the deubiquitinating enzyme cylindromatosis (CYLD), RIP1 translocates to a secondary cytoplasmatic complex, complex II or ripoptosome (Bertrand et al., 2008; Feoktistova et al., 2011; Moquin et al., 2013). Complex II is composed by FADD, caspase-8 and cFLIP. RIP3 binds to RIP1 in complex II. Activation of caspase-8 determines the choice between cell death by apoptosis or necrosis. If caspase-8 is active, it cleaves RIP1 from RIP3 and apoptosis ensues. On the other hand, if caspase-8 is inactive, RIP1 and RIP3 form a complex, necrosome, initiating necroptosis (**Figure 4**). In this process, RIP3 induces oligomerization of pseudokinase mixed lineage kinase domain-like (MLKL). MLKL can then bind to phosphatidylinositol phosphates (Kaiser et al., 2013; Thapa et al., 2013) and subsequently modify sodium and calcium ion channels flux, increasing osmotic pressure and promoting plasma membrane rupture (Thapa et al., 2013; Vanden Berghe et al., 2010). An interesting study showed evidence of increased necroptosis (up-regulation of RIP-3) in human NASH and in methionine-choline deficient diet murine model. Also, abrogation of necroptosis through RIP-3 ablation protected mice from NASH and fibrosis (Gautheron et al., 2014).

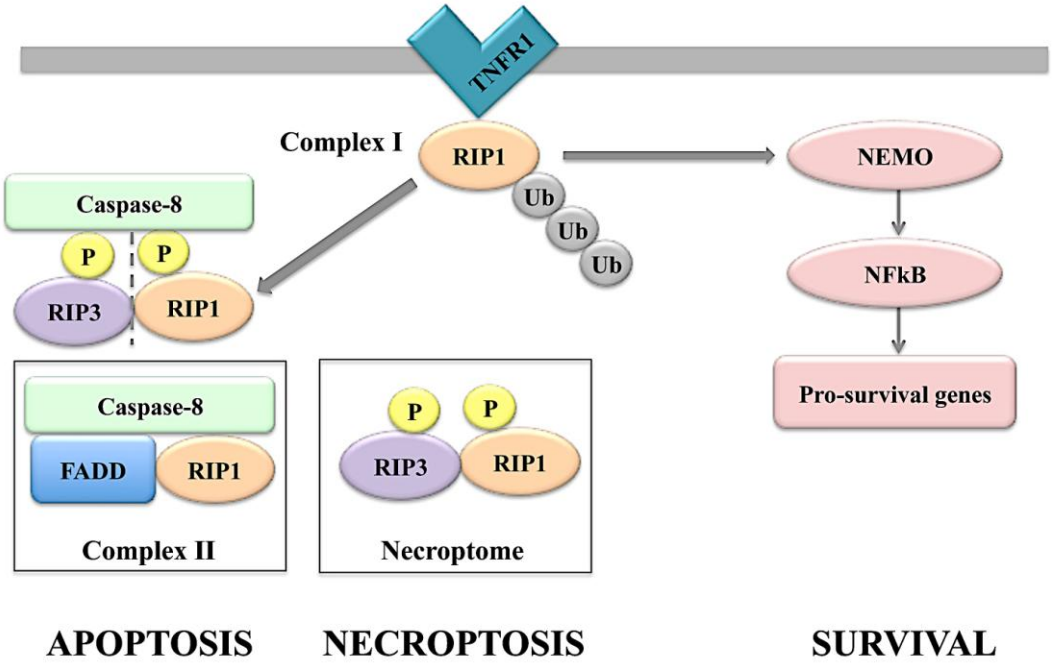


Figure 4. Deciding cell fate: to survive, to die from apoptosis *or* necroptosis.

Upon the binding of death receptor to its ligand, RIP1 is recruited and subsequently ubiquitinated. The polyubiquitinated RIP1, in turn, binds to NEMO, the regulatory subunit of NFkB, to promote NFkB activation, which leads to the induction of pro-survival genes to counter the death signals. The polyubiquitinated RIP1 can also migrate to the cytoplasm, where RIP1 is de-ubiquitinated by A20, the de-ubiquitylating enzyme. RIP1 and RIP3 can then form a pro-necrotic complex followed by phosphorylation on both kinases and induction of necroptosis. In circumstances in which caspase-8 is activated, RIP1 and RIP3 can be cleaved by caspase-8, and the pro-necrotic complex is blunted, which stimulates the cell to undergo apoptosis (adapted from Yang et al., 2015).

In summary, lipotoxic injury in the liver leads to epithelial cell death that can manifest in different forms, the more controlled apoptosis, uncontrolled necrosis, or a necrosis-like controlled form of death, necroptosis. Chronic cell death is a turning point in the pathogenesis of NASH, even though it does not seem to occur in a massive form to justify failure of liver metabolic functions. However, it rings the alarm to initiate a wound-healing response. Dying cells send messages to HSC promoting matrix remodeling in order to confine the insult, however if sustained promotes fibrogenesis. The immune system is also activated, in order to remove the dead cells, but can become overly active and worsen inflammation/tissue injury, as well as fibrogenesis through paracrine signaling in HSC. Lastly, progenitor cells are recruited to replace dead cells, but can, *per se* induce worse injury through the release of pro-inflammatory and pro-fibrogenic signals. In NASH, although there is progenitor cells activation, full maturation into differentiated epithelial

cells may be inhibited, perpetuating expansion of the progenitor pool (histologically illustrated by ductular reaction) (Machado et al., 2015a), further maintaining liver injury (Richardson et al., 2007) and increasing the risk for development of hepatocellular carcinoma (Gandhi et al., 2015; Xu et al., 2014; Ye et al., 2014) (**Figure 5**).

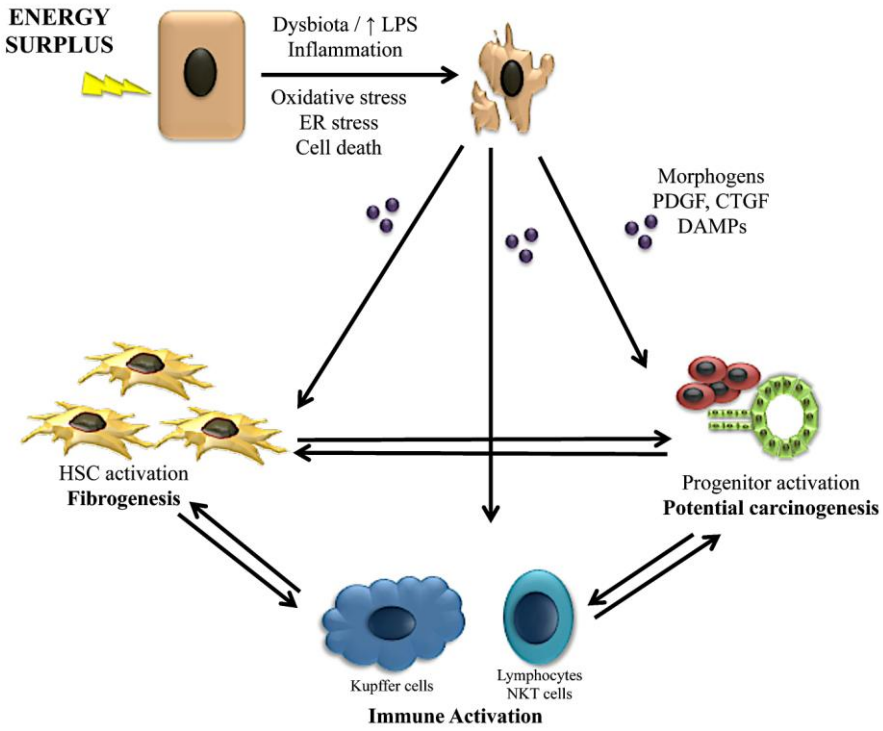


Figure 5. The liver responds to hepatocyte death with a wound healing response

Energy surplus leads to fat accumulation in the hepatocytes, which promote oxidative stress, endoplasmic reticulum (ER) stress and cell death. The injury of hepatocytes is promoted by an inflammatory state, among other factors, favored by a deregulated gut microbiota and increase in lipopolysaccharide (LPS). Injured and dying hepatocytes release damage associated molecular patterns (DAMPs) and morphogens (e.g. hedgehog and Wnt) that act on the immune system increasing inflammation, in stellate cells and progenitors cells activating them and inducing fibrogenesis and pathways of hepatocarcinogenesis. Once started, the regenerative/repair response perpetuates through crosstalk between the different cell types involved.

MECHANISMS OF FIBROGENESIS

Fibrosis progression and cirrhosis are a consequence of defective regeneration/repair. Chronic injury in the liver elicits a wound healing response that cannot compensate cell death and cannot be turned off due to sustained noxious stimulus (Angulo et al., 2015b). Fibrogenesis consists on the deposition of excessive abnormal extracellular matrix enriched in collagen, fibronectin and diverse glycosaminoglycans, that induces scarring and changes the normal tissue architecture (Odena and Bataller, 2012), ultimately leading to cirrhosis. The main cell type that produces extracellular matrix is the myofibroblast. Myofibroblasts are scarce in the normal liver, but accumulate in chronic liver disease. Its main origin upon lipotoxic injury in NASH is the transdifferentiation from quiescent HSC, the liver pericytes that line the perisinusoidal Disse space (Friedman, 2013). Other sources of myofibroblasts are portal fibroblasts, circulating fibrocytes and bone-marrow derived cells.

Several factors contribute to HSC activation, proliferation and transdifferentiation into myofibroblasts, such as the profibrogenic cytokines TGF-β (Dooley et al., 2000), platelet-derived growth factor (PDGF) (Kinnman et al., 2001; Kinnman et al., 2000), CTGF (Huang and Brigstock, 2012; Paradis et al., 2002), IL-13 (Liu et al., 2011; Liu et al., 2012), and osteopontin (Lee et al., 2004; Syn et al., 2011); morphogens such as hedgehog (Choi et al., 2009; Lin et al., 2008; Sicklick et al., 2005; Yang et al., 2008) and Wnt (Cheng et al., 2008; Myung et al., 2007), adipokines such as leptin (Choi et al., 2009; Saxena et al., 2004) and resistin (Dong et al., 2013), and vasoactive substances such as thrombin (Duplantier et al., 2004; Gaca et al., 2002), angiotensin II (Bataller et al., 2000; Yokohama et al., 2006) and endothelin-1 (Kinnman et al., 2000; Tangkijvanich et al., 2001) (**Table 2**).

The fibrogenic factor most studied in NAFLD is hedgehog. Hedgehog is a morphogen that regulates tissue patterning during development. In mammals, there are three hedgehog ligand counterparts: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Dhh expression is restricted to the nervous tissue and testis, whereas Shh and Ihh are widely expressed. Shh is the predominant ligand expressed in the proximal gut and Ihh in the hindgut (Merchant and Saqui-Salces, 2014).

Table 2 – Fibrogenic pathways in NAFLD

Factor	Cellular sources	Pre-clinical and clinical evidence in NAFLD
TGF-β	• Kupffer cells • Hepatocytes • Cholangiocytes • HSC	• Increased mRNA expression in livers of human NASH. • Increased serum levels in patients with NASH, correlate with fibrosis in some, but not all studies. • Pharmacological inhibition decreases myofibroblast activation in mouse models of NASH.
Hedgehog (Hh)	• Injured hepatocytes • Progenitors • Cholangiocytes • HSC • Kupffer cells • NKT cells	• Hh ligands and Hh-responsive cells increase in NASH and correlate with fibrosis stage in mice and humans. • Genetic over-activation of Hh pathway promotes fibrosis in mouse NASH models. • Pharmacological inhibition of Hh improves fibrosis in mouse NASH models.
Wnt	• Hepatocytes • Endothelial cells • Kupffer cells • Cholangiocytes • HSC	• Increased liver mRNA expression of components of Wnt pathway in patients with severe *versus* mild NASH.
Leptin	Receptor: • Hepatocytes • HSC • Kupffer cells • Endothelial cells	• Leptin deficiency promotes hepatic steatosis, but confers resistance to fibrosis in animal models of NASH. • Leptin serum levels correlate with liver fibrosis in most, but not all human studies.
Resistin	• Adipocytes • Kupffer cells	• Increased serum levels in patients with NASH, correlate with fibrosis in some, but not all studies.
PDGF	• Cholangiocytes • Platelets • Kupffer cells	• Increased liver mRNA expression in patients with severe *versus* mild NASH.
CTGF	• Cholangiocytes • HSC	• Increased liver mRNA expression in human NASH. • Increased serum levels in human NAFLD, correlates with fibrosis stage.
IL-13	• NKT cells • Kupffer cells	• NASH patients, but not patients with simple steatosis, have increased HSC expression of high-affinity IL-13 receptor (IL13Rα2). • Treatment with IL13R-directed cytotoxin decreases fibrosis and injury in a rat model of NASH.
Osteopontin	• Cholangiocytes • Kupffer cells • NKT cells • HSC	• Mice deficient in osteopontin are protected from liver injury in dietary models of NASH. • Increased serum levels in patients with NASH, correlate with fibrosis in some, but not all studies.
FXR	• Hepatocytes • Cholangiocytes • HSC	• Liver FXR expression decreases in human NASH and inversely correlates with NAS score. • Treatment with FXR agonist improves histology in a mouse model of NASH. • Treatment of NASH patients with FXR agonist improves liver histology, including fibrosis.
Angiotensin II	Receptor: • Endothelial cells • Kupffer cells • HSC	• Treatment with angiotensin II antagonists consistently associated with decreased fibrosis progression in different animal models of NASH.

In hedgehog-responsive target cells, hedgehog ligand binds to its receptor, Patch (Ptc) (Briscoe and Therond, 2013). Ptc is a transmembrane protein that constitutively represses Smoothened (Smo). When hedgehog binds to Ptc, it eliminates Ptc repressor activity on Smo (Briscoe and Therond, 2013). Activation of Smo regulates the activity of zinc-finger transcriptional factors, glioma-associated oncogene homologs (Gli) (**Figure 6**).

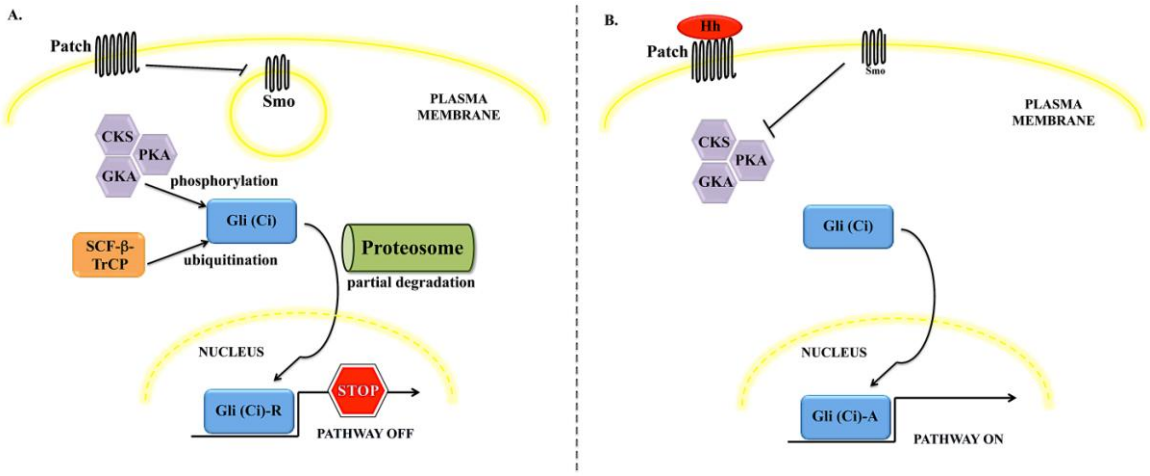


Figure 6. The hedgehog signaling pathway.

A. In the absence of ligand, Patch represses Smo, which is confined to vesicles, being inactive. That allows the sequential phosphorylation by several kinases: protein kinase A (PKA), glycogen synthase-3β (GSK3β) and casein kinase-1 (CK1). Thereafter, ubiquitination by Skip-Cullin-F-box (SCF) protein/β-Transducing repeat Containing Protein (TrCP) primes the phosphorylated Gli to limited proteosomic degradation, exposing the N-terminal repressor domain (GliR), which translocates to the nucleus and represses transcription of target genes.

B. When hedgehog binds to Patch, it releases the inhibitory effect of Patch in Smo contained in cytoplasmic vesicles. That abrogates phosphorylation and subsequent degradation of Gli. Full-length protein translocates to the nucleus promoting gene transcription.

In the absence of hedgehog ligands, Gli undergoes sequential serine phosphorylations by protein kinase A (PKA), glycogen synthase kinase (GSK)-3β and different members of caseine kinase 1 (CKI) family. Phosphorylation promotes binding of Gli to β-transducin repeat-containing protein (βTrCp) that primes it for ubiquitination and targeting to the proteasome for partial degradation. This generates a Gli N-terminal, which translocate to the nucleus, repressing transcription (Gli-R). When Smo is activated by Hedgehog ligand-Ptc interaction, it blocks Gli phosphorylation and subsequent proteosomal degradation. This allows the full-length Gli protein to enter the nucleus and act as a transcriptional activator through the binding of Gli-binding motif (GBM) (Falkenstein and Vokes, 2014) (**Figure 6**). Important target genes are vascular endothelial growth factor (VEGF),

angiopoietin 1 and 2 (endothelial cells); snail, twist-2, FoxF1, α-smooth muscle actin (α-SMA), vimentin, IL-6 (fibroblasts/myofibroblasts); and sox-2, sox-9 and nanog (stem/progenitor cells) (Merchant and Saqui-Salces, 2014).

In mammals, there are 3 Gli proteins: Gli-1, Gli-2 and Gli-3. The three Gli proteins behave differently: Gli-1 does not have a repressor domain and it is not proteolytically processed, besides being a direct transcription target for Gli-2; Gli-3 acts mainly as a transcriptional repressor because the majority of full-length Gli-3 protein is proteolytically processed; and Gli-2 acts mainly as a transcriptional activator although it can have a weak repressing activity. Gli-2 proteolytical processing to form transcriptional repressor is extremely inefficient and Gli-2 full-length protein is readily degraded. Hedgehog ligand signaling suppresses both processing and degradation of Gli-2. Thus, hedgehog signaling typically results in accumulation of full-length Gli-2 (Pan et al., 2006).

In a healthy adult liver, hedgehog ligand can barely be detectable, however, it is consistently up-regulated in all forms of liver injury, including NASH (Choi et al., 2011). Several cell types can produce hedgehog ligands in NAFLD. Whereas healthy hepatocytes do not produce hedgehog, injured ballooned hepatocytes do, being a major source in NAFLD (Guy et al., 2012; Machado et al., 2015b; Rangwala et al., 2011). The expression of hedgehog by ballooned hepatocytes positively correlates with the severity of fibrosis (Guy et al., 2012). Other sources of hedgehog ligands are reactive ductular cells, immune cells such as Kupffer cells and NKT cells, and HSC itself (Jung et al., 2008). Hedgehog ligands act autocrinally and paracrinally in hedgehog-responsive cells, promoting ductular/progenitor cells proliferation and secretion of pro-inflammatory and pro-fibrogenic cytokines (Omenetti et al., 2009); immune cells such as NKT cells promoting its proliferation and production of pro-fibrogenic IL-13 and osteopontin (Syn et al., 2012; Syn et al., 2010; Syn et al., 2009), macrophages/Kupffer cells, promoting a shift to M2, pro-fibrogenic and tumor-tolerant phenotype (Pereira et al., 2013); sinusoidal endothelial cells promoting vascular remodeling and sinusoidal capillarization (Francis et al., 2012; Xie et al., 2013); and HSC promoting its viability, proliferation and activation (Lin et al., 2008; Michelotti et al., 2013; Sicklick et al., 2005). Hence, hedgehog acts as a maestro orchestrating the major pathological processes that drive progression to liver cirrhosis and hepatocellular carcinoma. In agreement with the aggregate data, strategies that improve lipotoxicity associate with decrease hedgehog activation, in humans and animal models of NASH (Guy et al., 2015; Machado et al., 2015b). Furthermore, animal studies showed that

strategies abrogating the hedgehog pathway associate with improvement of steatohepatitis and fibrosis progression (Hirsova et al., 2013).

The fibrogenic process perpetuates because the chronic insult to the liver subsides. Also, subjects susceptible to develop worse liver disease with fibrosis progression to cirrhosis, may have an inherently overly-active fibrogenic pathways or a decreased ability to turn off those pathways even when the insult has ceased. In fact, a recent report showed that susceptibility to cirrhosis in rodent models is heritable and epigenetically regulated (Zeybel et al., 2012). In other words, cirrhosis seems to result from defective repair in a chronically injured liver.

ANIMAL MODELS TO STUDY NAFLD

INTRODUCTION

Overview of the available animal models of NAFLD/NASH

Animal models are indispensable tools to better understand the pathogenesis and test new therapeutic approaches for human diseases. It is even more important in human diseases, as NAFLD, that evolve slowly, and thus impose lengthy studies to determine how interventions impact natural history. Furthermore, NAFLD encompasses diverse phenotypes difficult to differentiate non-invasively, and that can only be accurately differentiated by liver biopsy, which prompts ethical concerns when applied solely for research purposes. There are many animal models for NAFLD, none of them perfect. Some are more suitable for studying the metabolic derangements, others for studying inflammation and fibrogenesis. The most used animals are rodents, particularly mice, in whom NAFLD can be induced through specific diets or through genetic manipulation.

Dietary rodent models of NASH

Methionine-choline deficient diet model

Feeding rodents a methionine-choline deficient (MCD) diet is one of the most used models of NASH. The MCD diet is high in sucrose (40%) and fat (10-20%), but lacks methionine and choline. This diet leads to hepatic steatosis because it impairs fat export from the liver as VLDL; compromises hepatic β-oxidation of FA; and increases hepatic uptake of FA (Kanuri and Bergheim, 2013; Takahashi et al., 2012). In addition to inducing hepatic fat accumulation, the MCD diet changes the hepatic lipid profile, increasing the saturated *versus* monosaturated fatty acid ratio, which probably increases lipotoxicity in hepatic steatosis. The altered lipid profile is the result of the down-regulation of SCD-1, which catalyzes the conversion of saturated FA (e.g., palmitate and stearate) into their monosaturated derivatives (e.g., palmitoleate and oleate) (Rizki et al., 2006). The MCD diet also restricts the synthesis of phosphatidylcholine, which is an essential component of VLDL, as well as a major biliary phospholipid that catalyzes biliary micelle formation.

Distinct liver phenotypes are observed when one or the other amino acid is selectively depleted (Caballero et al., 2010). Whereas rodents fed the MCD diet develop NASH (i.e., hepatic steatosis, inflammation and liver cell death), adding back methionine prevents

NASH, although simple steatosis persists (Oz et al., 2008). Methionine-mediated rescue from liver inflammation and injury may reflect the fact that methionine, and S-adenosyl-L-methionine (SAMe)-dependent methylation reactions are important for glutathione (GSH) synthesis, protection from reactive oxygen species (ROS) injury, DNA methylation, membrane fluidity, and equilibrium between proliferation and apoptosis (Wortham et al., 2008). The high sucrose content of the MCD diet accentuates its ability to induce steatosis and liver injury, by providing a lipogenic substrate (Pickens et al., 2009; Rizki et al., 2006).

Mice fed MCD diet reproducibly develop steatosis and steatohepatitis within 2 weeks. Unlike human NASH, steatosis affects mainly portal areas (in opposition for predilection for peri-venular areas in humans), and hepatocellular ballooning is not prominent. Like human NASH, however, liver cell death (caused by necrosis and lipoapoptosis) and inflammation are exuberant in MCD diet-fed mice, which develop many hepatic necroinflammatory foci, containing lymphocytes, neutrophils and activated Kupffer cells (Leclercq et al., 2000). Perisinusoidal and pericellular fibrosis emerges within 4 weeks, is reproducibly robust at week 8-10, and maximal by week 16 (Itagaki et al., 2013; Syn et al., 2009). Interestingly, MCD diet-induced liver fibrosis is reversible if standard chow diets are resumed within 16 weeks of MCD diet initiation, but it cannot be reversed if chow feeding is resumed after that time point (Itagaki et al., 2013; Mu et al., 2010). Also, as noted in NASH patients who develop advanced liver fibrosis, steatohepatitis (particularly steatosis) lessens in mice with advanced liver fibrosis due to prolonged MCD diet feeding (Itagaki et al., 2013).

Recent studies have demonstrated that MCD diet-fed mice develop portal hypertension. This begins before fibrosis is evident and is associated with increased mesenteric arterial and portal venous flow, arterial hypo-responsiveness to vasoconstrictors, increased intrahepatic resistance from mechanical factors (e.g., sinusoidal narrowing by steatotic and swollen hepatocytes), as well as, adipocytokine effects on contractile function of HSC and sinusoidal endothelial cell biology (Francque et al., 2012; Francque et al., 2010).

Despite its many similarities with fibrosing NASH in humans, the MCD diet model has been criticized because it lacks certain metabolic risk factors that seem important in human NAFLD. Mice fed the MCD diet are not obese. Rather, they develop significant weight loss (Anstee and Goldin, 2006). Weight loss in MCD diet-fed rodents is likely to be multifactorial (Oz et al., 2008). Choline deficiency and, to a lesser extent, methionine deficiency, decreases intestinal absorption of dietary fat, leading to steatorrhea and

reduced capture of dietary energy (Tidwell, 1956). MCD diets also induce hypermetabolism as a result, at least in part, of an increase in sympathetic nervous system outflow to adipose tissue during chronic methionine depletion (Jha et al., 2014). Furthermore, MCD diets increase lipase activity in visceral adipose tissue, permitting an increased flux of FA into liver mitochondria. This increases mitochondrial uncoupling, which decreases the efficiency of energy extraction from nutrients and reduces hepatic ATP synthesis (Rizki et al., 2006).

The adipokine profile of MCD diet-fed rodents is also unlike that of patients with NASH. NASH patients generally exhibit hyperleptinemia and reduced circulating levels of adiponectin, while mice with MCD diet-induced NASH have low levels of leptin and no decrease in adiponectin (Takahashi et al., 2012).

The MCD model also lacks other metabolic features associated with human NAFLD, such as dyslipidemia and IR. In fact, MCD diet-fed rodents demonstrate decreased levels of total cholesterol and triglycerides (Weltman et al., 1996), decreased serum levels of insulin and glucose, and increased peripheral insulin sensitivity (Rinella and Green, 2004). However, despite showing peripheral insulin sensitivity, MCD diet fed rodents exhibit features of hepatic IR (Leclercq et al., 2007; Schattenberg et al., 2005).

Several refinements to the diet have been made. Increasing cholesterol content of the diet increased liver fibrosis correlating to an increase in free cholesterol in HSC and its sensitization to activation induced by TGF-β (Tomita et al., 2014). Also, administration of repetitive low doses of lipopolysaccharide (LPS) in mice fed MCD diet increased hepatic inflammation and apoptosis, oxidative stress and fibrosis (Kirsch et al., 2006; Kudo et al., 2009). Feeding rats previously loaded with iron with MCD diet during 4 weeks increased necroinflammation, with a trend toward increased perisinusoidal fibrosis (Kirsch et al., 2006). Finally, adding the methionine adenosyl transferase 1 inhibitor, ethionine, to the drinking water, further increases MCD diet-related liver damage (Syn et al., 2009).

Regular high-fat diet

Dietary fat requirements in rodents are different from humans. Human diets are considered to be high fat when fat comprises more than 30% of total energy requirements (Donnelly et al., 2005). However, fat contributes only 5% of total energy requirements in normal rodent chow (Council, 1995). Hence, healthy rodent diets typically contain 6 fold less fat than healthy human diets. That said, dietary fat content has varied widely in published

studies of high fat diet (HFD) in rodents, ranging from 20% to more than 70% of total energy requirements. This variability makes it difficult to compare study outcomes. The issue is further confounded by the fact that the studies often differ with regards to other key variables that may influence liver outcome, such as are species, strain and gender of the rodent used, dietary fat composition, duration of diet exposure, and age of the rodents when HFD's were introduced.

In one seminal study, Sprague-Dawley rats were fed a liquid, Lieber-DeCarli diet, in which fat contributed 71% energy, *ad libitum* for 3 weeks (Lieber et al., 2004). As compared with low-fat isocaloric diet fed rats, the HFD group did not become obese, but developed IR, and liver pathology with panlobular steatosis, mild inflammatory infiltrates, mitochondrial abnormalities, and oxidative stress. There was, however, no increase in the serum aminotransferases levels or evidence of liver fibrosis other than increase in procollagen-1α gene expression (Lieber et al., 2004). To overcome a potential confounder of *ad libitum* diet consumption (i.e., self-restriction of caloric intake), the same strain of rats was fed a HFD emulsion via gavage for 6 weeks (Zou et al., 2006). When administered HFD via gavage, rats became obese and developed the metabolic syndrome, but liver injury was no worse than when these diets were consumed *ad libitum*. Lengthening the period of HFD exposure had little impact on liver fibrosis. Only mild fibrosis developed in less than 40% of rats that were fed HFD for 43 weeks (Omagari et al., 2008).

Strain-dependent differences in HFD outcomes have been reported in rats and in mice (Asai et al., 2014; Farrell et al., 2014; Nishikawa et al., 2007; Romestaing et al., 2007). In general, mice are relatively resistant to HFD induced liver injury. Most strains develop only mild hepatic inflammation and almost no liver fibrosis unless diet exposure is quite prolonged. For example, although C57BL/6J mice that were fed HFD (with 60% calories from fat) developed obesity, IR, and dyslipidemia, after 5 weeks of HFD exposure liver injury was very mild and mice developed only slight liver perivenular fibrosis (Ito et al., 2007). The model improves when HFD is administered via implanted gastrostomy tube (allowing overfeeding) but even then, mice develop only modest liver damage (Deng et al., 2005; Gaemers et al., 2011). Another interesting approach to increase the food consumption by animals was recently described by Ogasawara *et al*. (Ogasawara et al., 2011). The authors injected mice with gold thioglucose (GTG) IP (2 mg/g weight) and fed HFD (82% calories from fat) for 12 weeks, starting immediately after weaning. GTG

induces lesions in the ventromedial hypothalamus leading to hyperphagia and obesity. GTG-treated mice did become hyperphagic, and developed obesity with increased abdominal adiposity, IR and adipokine deregulation. Mice developed all the histological characteristics of NASH, including hepatocellular ballooning, Mallory-Denk bodies, and pericellular fibrosis (Ogasawara et al., 2011).

Stelic Animal Model (STAM^{TM}) mice provide another model of accelerated NASH. In this model, male mice are injected with 200 ug of streptozotocin at 2 days of age, and feed a HFD (32% calories from fat, of which 22.3% are saturated fats) since weaning. At 8 weeks, animals develop NASH (with severe hepatocellular ballooning but mild steatosis and inflammation), significant fibrosis emerges one week later, and hepatocellular carcinomas are evident after 16 weeks of HFD exposure (Kawai et al., 2012). Besides the fact that the formal STAM^{TM} protocol remains unpublished, the model has an important limitation with regards to mimicking the metabolic milieu of human NAFLD, namely it causes type 1 diabetes rather than type 2 diabetes because streptozotocin destroys pancreatic β-cells.

Lastly, it has been suggested that supplementing a regular HFD with IV injections of oxidized low-density lipoproteins (oxLDL) in the last 2 weeks of 23 weeks of HFD feeding might be useful to induce NASH, worsening lipid metabolism, hepatic steatosis, apoptosis, inflammation and fibrosis (Yimin et al., 2012). *In vitro* studies showed that oxLDL could promote HSC activation (Kang and Chen, 2009; Schneiderhan et al., 2001).

Western or fast-food diet

Epidemiological studies in humans suggest that the fat composition of the diet might play a role in NAFLD pathogenesis/progression because the intake of saturated FA and cholesterol positively associate with NAFLD and NASH (Cortez-Pinto et al., 2006; Musso et al., 2003; Yasutake et al., 2009). Furthermore, high consumption of simple carbohydrates, particularly fructose, also associates with the metabolic syndrome, risk for developing NAFLD, NASH and advanced fibrosis (Abdelmalek et al., 2010; Abid et al., 2009). Researchers have exploited these insights to create models that mimic this "western type" HFD (also known as the "fast-food" diet) by feeding higher amounts of saturated fats and trans-fats and increasing dietary cholesterol content. In some circumstances, simple carbohydrates (equivalent to high fructose corn syrup) are also supplemented to the drinking water, in order to reproduce high-sweetened soda beverage consumption.

The American Lifestyle-Induced Syndrome (ALIOS) diet has become a popular Western (fast food) diet model for inducing NAFLD/NASH. In the ALIOS diet model, 45% dietary calories are derived from fat (enriched with trans-fats from partially hydrogenated vegetable oil), and the HFD is supplemented with sucrose and fructose in the drinking water (42g/L) (Tetri et al., 2008). When C57BL/6 mice (aged 5-6 weeks old at the time of diet initiation) were fed ALIOS diets for a total of 16 weeks, they became obese and insulin resistant. This diet also induced progressive hepatic steatosis, hepatocellular ballooning, and necroinflammatory changes. However, despite having NASH, mice failed to develop evidence of liver fibrosis (Tetri et al., 2008).

Modification of the protocol, increasing dietary cholesterol content has been shown to modulate the propensity for liver fibrosis development during the ALIOS protocol. However, fibrosis does not seem to develop universally, nor the severity to be reproducible across the cohort of mice treated, even after prolonged exposure to the diet (12 to 30 weeks exposure depending on the study) (Charlton et al., 2011; Clapper et al., 2013; Dorn et al., 2014; Ginsberg, 2006; Mells et al., 2012).

Atherogenic Diet

The atherogenic diet, or Paigen diet, contains 1.25% cholesterol and 0.5% cholate. When administered to rodents, it induces progressive, time-dependent liver injury, with steatosis, inflammation, important perisinusoidal fibrosis and hepatocellular ballooning after 6 months. If the diet is also high fat, it worsens liver fibrosis and accelerates NASH development, with hepatocellular ballooning becoming apparent as early as 3 months (Matsuzawa et al., 2007). One limitation of atherogenic diets is that they fail to reproduce several of the metabolic abnormalities that are typical in human NAFLD. Even when the Paigen diet is high in fat, it promotes weight loss and decreased visceral adiposity, does not evoke hypertriglyceridemia, and only modestly decreases insulin sensitivity after very long feeding periods (Matsuzawa et al., 2007).

Choline deficient L-amino acids defined (CDAA) diets

The choline deficient L-amino acids defined (CDAA) diet consists of a diet with low choline content, and in which aminoacids are all pure L-enantiomers. Fisher rats fed CDAA diets develop fibrosing NASH by week 10 (Fujita et al., 2010) and some rats even exhibit cirrhosis by week 12 of diet exposure (Nakae et al., 1992). When administered for one year, 100% animals developed hepatocellular carcinoma (Nakae et al., 1992).

However, this diet induced weight loss (though with increased visceral adiposity), increased levels of adiponectin, and less IR (Fujita et al., 2010). The mechanism by which CDAA diets exacerbate fatty liver damage is not well understood. However, it has been suggested that the lack of oligopeptides may decrease the absorption of methyl donor aminoacids and antioxidant minerals (Nakae et al., 1992). The combination of CDAA with HFD (35% calories as fat), enriched with trans fatty acids (54%) and exposure to diethylnitrosamine (DEN) in drinking water accelerates liver pathology, with almost all rats developing frank cirrhosis and hepatocellular carcinoma in 16 weeks (de Lima et al., 2008).

CDAA with or without HFD administered to mice induce similar lesions, though not so severe (Matsumoto et al., 2013; Pizarro et al., 2015; Povero et al., 2014). However, the rate of hepatocellular carcinoma development is much lower in mice than in rats fed CDAA diet, being below 40% after 9 months of diet (De Minicis et al., 2014; Denda et al., 2002).

Genetic rodent models of NAFLD

ob/ob mice

The ob/ob phenotype resulted from a spontaneous mutation in an outbred colony at Roscoe B. Jackson Memorial Laboratory in 1949, and subsequent transfer of the mutation onto a C57BL/6 background (Ingalls et al., 1950). Forty-five years later, Jeff Friedman discovered that the mutated gene responsible for the phenotype was leptin, an adipokine produced mainly in white adipose tissue (Zhang et al., 1994). The main signaling pathways of leptin are Janus Kinase and Signal Transducer and Activator of Transcription (JAK-STAT), Mitogen-Activated Protein Kinase (MAPK), Phosphoinositide 3-Kinase (PI3K) and AMP-Activated Protein Kinase (AMPK) pathways (Lindstrom, 2007). Leptin's receptor has different isoforms generated by alternative splicing of leptin mRNA (Fellmann et al., 2013). The long form of the receptor (ObRb) encoded by the full-length leptin transcript) is necessary for activation of JAK-STAT pathway. ObRb acts both centrally in the nervous system, and in peripheral tissues, such as adipose tissue, muscle, liver and pancreatic islets. The short form of the leptin receptor (ObRa), which lacks the cytosolic domain of ObRb, is localized predominantly in skeletal muscle and activates the PI3K pathway (Lindstrom, 2007). In the hypothalamus, leptin acts in ventral median nucleus as a potent anorexic agent (Takahashi et al., 2012). Besides regulating satiety,

leptin increases energy expenditure, promotes physical activity, enhances thermogenesis, increases sympathetic tone, and regulates immune cells, such as T cells and macrophages, among many other actions. Consequently, ob/ob mice are hyperphagic, inactive, markedly obese (up to 4 times the weight of wild type animals), have all the features of the metabolic syndrome except hypertension, and exhibit impaired immune function (Diehl, 2005).

Ob/ob mice have deregulated lipid and bile acid metabolism and develop severe hepatic steatosis on regular chow diets (Liang and Tall, 2001). NASH occurs with addition of secondary insults, such as HFD (Li et al., 2003; Trak-Smayra et al., 2011; Trevaskis et al., 2012), MCD diet (de Oliveira et al., 2006; Sahai et al., 2004), or low dose of LPS (Yang et al., 1997). However, ob/ob mice are resistant to fibrosis, even when other triggers for liver injury, such as chronic carbon tetrachloride treatment, are applied (Leclercq et al., 2002; Potter et al., 2003), which makes this model unsuitable to study liver fibrosis and NASH-associated cirrhosis.

Although mutations in the leptin gene are very rare in humans (Takahashi et al., 2012), ob/ob mouse is a good model for human NAFLD because obese patients display functional leptin deficiency caused by hyperleptinemia-induced leptin resistance.

db/db mice

Mice with db/db phenotype were first described in 1996 as a model of T2DM (Hummel et al., 1966). They carry a recessive mutation in the leptin receptor gene (ObRb), resulting in leptin resistance despite normal or increased leptin levels (Chen et al., 1996). Db/db mice start to gain weight at 3-4 weeks of age, and shortly thereafter develop hyperglycemia, polyuria and glycosuria. As with ob/ob mice, db/db mice develop all the features of the metabolic syndrome except hypertension.

Db/db exhibit liver steatosis, without appreciable hepatic inflammation or fibrosis, when fed normal chow. However, in opposition to ob/ob mice that are resistant to fibrosis, db/db mice develop worse fibrosis when exposed to MCD or HFD than lean non-diabetic mice (Sahai et al., 2004; Trak-Smayra et al., 2011; Wortham et al., 2008).

Fa/fa Zucker rats

The fa/fa Zucker rat obese phenotype was observed by Zucker *et al*., in 1961, and demonstrated to result from a spontaneous mutation, named fatty or fa (Zucker, 1965). It is an autosomal recessive mutation that affects the extracellular part of the leptin receptor,

leading to weaker affinity for leptin and altered signal transduction (Fellmann et al., 2013). Zucker fa/fa rats exhibit hyperphagia, obesity and hyperlipidemia, with plasma triglycerides and cholesterol increasing with age. They exhibit mild hyperinsulinemia with IR (though without fasting hyperglycemia), and even moderate hypertension when older. Fa/fa rats also develop spontaneous liver steatosis, with liver lipogenesis increasing, but adipose tissue lipogenesis decreasing, with age (Godbole and York, 1978). However, the hepatic steatosis is mild, and does not typically lead to NASH or fibrosis. Feeding fa/fa rats high fat or high sucrose diets induces T2DM and severe NASH with mild periportal fibrosis (Carmiel-Haggai et al., 2005; Fukunishi et al., 2009, 2010).

Genetic models with lipodystrophy-like phenotype

Several mouse genetic models induce disease of the adipose tissue, leading to NAFLD secondary to impaired adipose tissue lipid storage capacity, that is, lipodystrophy. One example is the transgenic strain in which the nSREBP-1c transgene (which encodes a nuclear-targeted form of SREBP-1c) is under control of the adipose tissue-specific promoter aP2. This leads to overexpression of SREBP-1c localized to adipose depots, and consequently a severe lipoatrophic phenotype, massive early hepatic steatosis and spontaneous NASH with pericellular/perivenular fibrosis at 5 months of age (Nakayama et al., 2007; Shimomura et al., 1998). The aP2-diphteria toxin mouse is another model of lipodystrophy-induced NAFLD that results from the expression of an attenuated diphtheria toxin A chain targeted to the adipose tissue (Nagarajan et al., 2012; Ross et al., 1993).

PTEN null mice

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) encodes a lipid phosphatase, with its main substrate being phosphatidylinositol-3,4,5-triphosphate (PI3P). It acts as a tumor suppressor by inhibiting the PIP3 kinase and serine-threonine protein kinase B (PkB or Akt) pathways (Takahashi et al., 2012). Mice that are global knockouts (KO) for PTEN are not viable, dying early during embryonic development (Stiles et al., 2004). Liver-specific KO are viable, but develop NAFLD with increased synthesis of FA and glycogen. Contrary to human NAFLD, these mice exhibit liver hypersensitivity to insulin (Stiles et al., 2004). Liver-specific PTEN KO mice develop progressive liver disease, with macrovesicular steatosis by week 10 of age, NASH with hepatocellular ballooning, Mallory-Denk bodies and sinusoidal fibrosis by week 40 and hepatocellular

carcinoma with a penetrance of 47% at week 44 and 100% at weeks 74-78 (Horie et al., 2004; Sato et al., 2006).

Methionine adenosyltransferase 1A (MAT1A) deficient mice

Methionine adenosyltransferase (MAT) is the enzyme that catalyzes the conversion of methionine and ATP in SAMe. The isoform 1A is liver-specific (Wortham et al., 2008). KO mice have impaired antioxidant defense and perturbations of lipid metabolism (Lu et al., 2001). Those mice develop spontaneous NASH with fibrosis at 8 months age. Although they are hyperglycemic, their insulin levels are normal and they do not develop other features of the metabolic syndrome.

NEMO deficient mice

The IkB kinase (IKK) is essential for activation of the survival factor NFkB, which regulates cellular responses to inflammation. Mice with global KO of the IKK subunit NFkB-essential modulator (NEMO) develop massive hepatocyte apoptosis and die during embryonic development. However, liver-specific conditional KO mice develop spontaneous NASH, with steatosis, inflammation, hepatocellular ballooning, apoptosis and fibrosis, as young as 8 weeks of age. At 12 months they develop hepatocellular carcinoma with 100% penetrance (Luedde et al., 2007).

Non-rodents animal models of NAFLD

Other animals have been used to model human NAFLD. For example, opossums fed long-term HFD (Chan et al., 2010; Chan et al., 2012) or ossabaw minipigs fed atherogenic diet (Dyson et al., 2006; Lee et al., 2009) can develop fibrosing NASH. However those models are more expensive, require specialized facilities and hence have limited role in the NAFLD research field.

Fish models of NAFLD have been increasingly used to study NAFLD. They have several advantages over more widely used small mammal models: fish are less expensive, have shorter generation times, and are highly fertile. Thus, fish are efficient tools for screening studies. Also, fat and specific proteins can be visualized in live fish using fluorescent tags (Asaoka et al., 2013). However, fish liver structure is different from mammals, and its small size makes molecular analysis of tissue more difficult. The most used fish are zebrafish (*Danio rerio*) and the ricefish medaka (*Oryzias latipes*). Several models of diet-

induced obesity, disruption of ER function or deregulated metabolism of methionine, phospholipids and lipids have allowed important advances in the field (Cinaroglu et al., 2011; Her et al., 2011; Her et al., 2013; Kuwashiro et al., 2011; Matsumoto et al., 2010; Matthews et al., 2009; Miyake et al., 2008; Oishi et al., 2012; Oka et al., 2010; Pai et al., 2013; Sadler et al., 2005; Thakur et al., 2011).

In summary, the quest for the ideal animal model for human NAFLD/NASH continues. Nevertheless, a variety of animal models are currently available. Some of these are more ideal for modeling the physiology of liver steatosis in the context of the metabolic syndrome, while others better model hepatic inflammation and fibrosis. Ultimately, model selection should be guided by the purpose of the study.

Rodents are by far the most used animals to model NAFLD, since they provide a good balance between cost, time necessary for breeding and intervention, and amount of tissue samples that can be obtained. Mice are particularly amenable for genetic manipulation, allowing for causality studies. Two strategies have been used to evoke NAFLD/NASH, dietary and genetic. The dietary models have the advantage of facilitating studies in mice with other genetic manipulations, avoiding breeding crossing experiments to induce NAFLD. One intriguing finding with rodent models is the fact that the models (dietary or genetic) that seem to associate with worse and earlier liver injury, do not associate with the metabolic disturbances described to associate with human NAFLD/NASH, which should prompt us to question the role of those metabolic factors in the progression of human liver disease (**Table 3**).

Table 3. Animal models of NAFLD

	Weight	IR	Inflammation	Fibrosis	Time for fibrosis
Dietary animal models					
MCD diet	↓ 40%	No	+++	+++	4-8 weeks
High fat diet	Obesity	Yes	+/-	+/-	52 weeks
Western diet	Obesity	Yes	+	+	12-25 weeks
CDAA ± high fat diet	Obesity	Yes	++	++	22 weeks
Genetic models					
ob/ob	Obesity	Yes	+	+/- (+/- if MCD diet)	12 weeks
db/db	Obesity	Yes	+	+ (+++ if MCD diet)	12 weeks
PTEN KO	Normal	No	++	+++	40 weeks

More recently, non-rodent models have been used to study NASH, such as opossum and minipigs. However, data on these models is still scarce, and they are more expensive than rodent NAFLD/NASH models. Increasingly, fish are being used to model NAFLD/NASH. Fish provide a very useful model to screen for potential genetic defects associated with disease and for therapeutic approaches, but results still need to be confirmed in mammals.

Rational and aims for the experimental study with head-to-head comparison of two dietary mouse models of NASH

Though much has been learned about NAFLD since its first descriptions thirty five years ago (Ludwig et al., 1980), there are still huge gaps in knowledge regarding its pathogenesis, prognosis, prevention, and treatment. Studying human NAFLD is hampered by the fact that it encompasses a spectrum of conditions difficult to differentiate non-invasively. Also, NAFLD is a very slowly progressive disease, which hinders prospective observational studies. Given these challenges, animal models that mimic human pathology are a necessity. The perfect animal model would develop NAFLD in the context of key risk factors for the human condition (i.e., obesity and the metabolic syndrome), eventually manifest all histological features of NASH (i.e. steatosis, lobular inflammation and hepatocellular ballooning with Mallory-Denk bodies), progress to advanced liver fibrosis, and be susceptible to hepatocellular carcinoma. Importantly, the ideal NASH model should require little time and expense to develop/maintain, and be highly reproducible. There are several diet-induced models of NAFLD/NASH in small animals. Mice are generally preferred due to their short lifespan and the ease of genetically manipulating putative pathogenic/protective pathways.

The most widely used diet to induce NAFLD/NASH is the MCD diet. Standard MCD diet also has a high content of sucrose (40% of energy) and is moderately enriched with fat (10-20%). It is a very reproducible model, consistently inducing a phenotype of severe NASH after 8 weeks of administration (Itagaki et al., 2013). The MCD diet has been criticized, however, because it causes weight loss and does not induce features of the metabolic syndrome, an important risk factor for NAFLD (Machado et al., 2015). HFD is another highly studied approach to develop NAFLD. The heterogeneity of such diets makes it difficult to compare studies from different research groups. However, standard

HFD generally do not induce significant NASH (i.e., liver cell death, inflammation, or fibrosis) even when fed for long-term, despite reproducibly provoking obesity, the metabolic syndrome, and hepatic steatosis (Asai et al., 2014). More recently, a modified HFD has been used to model NAFLD/NASH. This diet is enriched in saturated FA and supplemented with cholesterol as well as high-fructose corn syrup equivalents, mimicking the fast food style that characterizes the "Western diet" (Mells et al., 2012). The Western diet has the advantage of inducing obesity and the metabolic syndrome in mice, although requiring long-term administration.

Even though MCD and Western diets are two popular rodent models for studying NAFLD/NASH, with advocates for each diet strongly criticizing the other model, a direct comparison between them has not yet been done.

Aims:

1. To perform a head-to-head comparison of MCD and Western diets after controlling for multiple variables that impact metabolism and NAFLD pathogenesis: mouse genetic background, age, gender, and inter-animal facility-related differences in light-dark cycling and indigenous microbial flora.
2. To highlight the strengths of each model and provide a rational for evidenced-based lines of orientation to help researches chose the appropriate model for their experiments.

Contribution of the PhD candidate:

The candidate had the idea of comparing the most used diets applied to rodents to model NAFLD/NASH, in order to better clarify the strengths and limitations of each diet and, in that way, to help better planning research in the field. Under close orientation and guidance by Prof. Anna Mae Diehl, the candidate planned all the experiments, executed them and interpreted them. The candidate also wrote the published article (which was edited by Prof. Anna Mae Diehl) that presented the data described in this chapter.

MATERIAL AND METHODS

Animal Studies

Male wild-type (WT) mice C57Bl/6 were obtained from Jackson Laboratory and fed either chow diet (Picolab Rodent diet 20, #5053; n=13 mice); MCD diet (MP Biomedicals, #960439; n=15 mice) for 8 weeks, or Western diet (TD.120330 22% HVO + 0.2% cholesterol diet, Teklad Research, supplemented with high-corn fructose syrup-equivalents in the drinking water, that is 42 g/L glucose and fructose, 55% and 45% respectively, w/w; n=8 mice) for 16 weeks. The characteristics of the diets are summarized in **Supplemental Tables 1 and 2**. Interventions were started at 12 weeks of age for MCD diet and 4 weeks for Western diet, in order to complete the protocol at 20 weeks of age in all mice. Animal care and procedures were approved by the Duke University Institutional Animal Care and fulfilled National Institutes for Health and Duke University IACUC requirements for humane animal care.

Human Samples

Liver biopsies from liver transplant donors (n=5) and adult NASH patients with mild fibrosis (F0-1) (n=5) or severe fibrosis (F3-4) (n=5) were randomly selected from Duke University Health System NAFLD Clinical Database and Biorepository. Patients gave informed consent at time of recruitment. Prior to this analysis patient records were anonymized and de-identified. Studies were approved by the Duke IRB and conducted in accordance with National Institutes of Health and institutional guidelines for human subject research.

Histopathological analysis

Formalin-fixed, paraffin-embedded liver sections were stained with H&E and evaluated for severity of NAFLD, by a trained pathologist, according to criteria described by Brunt *et al*. (Kleiner et al., 2005). Immunohistochemistry was done, as previously described (Choi et al., 2006), with the antibodies specified in **Supplemental Table 3**. Liver fibrosis was assessed by Picrosirius red (#365548, Sigma) staining (Choi et al., 2006). TUNEL staining (11684817910, Roche) was performed according to the manufacturer's suggestions.

Serum and tissue analysis

Liver enzymes were assayed by the Veterinary Diagnostic Laboratory, Division of Laboratory Animal Resources, Duke University Medical Center. Insulin was measured with Ultrasensitive Mouse Insulin ELISA kit (Crystal Chem Inc: #90080); lipids were measured in the serum and liver with Triglyceride Colorimetric Assay kit (Cayman Chemical Company: #10010303), Free Fatty Acid Quantification Kit, (Abcam, ab65341) and Cholesterol Quantification kit (Abcam, ab65359); serum leptin and adiponectin were determined with Abcam mouse ELISA kits, ab100718 and ab108785, respectively. Liver hydroxyproline content was quantified as previously described (Syn et al., 2009).

Molecular Studies

mRNA quantification by Real-time Reverse Transcription-PCR (RT-PCR). Total RNA was extracted from livers using TRIzol (Invitrogen). RNA was reverse transcribed to cDNA templates using random primer and Super Script RNAse H-Reverse Transcriptase (Invitrogen) and amplified. Semiquantitative qRT-PCR was performed using iQ-SYBR Green Supermix (Bio-Rad) and StepOne Plus Real-Time PCR Platform (ABI/Life Technologies), as previously described (Michelotti et al., 2013). For primers, see **Supplemental Table 4**.

Western Blotting. Total proteins were extracted from whole liver using RIPA buffer (Sigma). Proteins were separated by electrophoresis on 4%-20% Criterion gels (BioRad), transblotted into polyvinylidene-difluoride membranes, and incubated with primary antibodies listed in **Supplemental Table 5**.

Statistics

Results were expressed as mean±SEM. Significance was established using Kruskal-Wallis and Mann-Whitney tests, with significance $p<0.05$, corrected for multiple comparisons.

RESULTS

Western diet mimics metabolic profile associated with human NASH

Twenty-week old wild type mice that had been fed chow diet, MCD diet for 8 weeks, or Western diet for 16 weeks, were studied. MCD diet-fed animals weighed 40% less, and Western diet-fed animals 14% more, than age- and gender-matched chow-fed mice (**Figure 7A**).

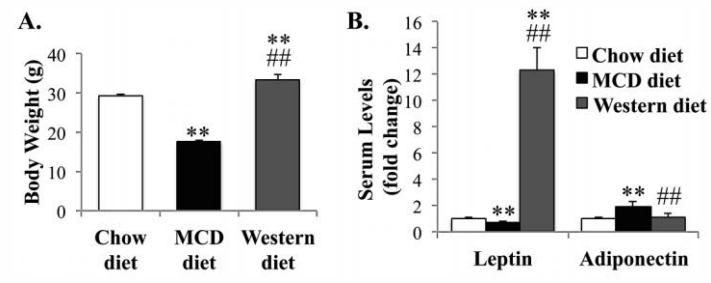


Figure 7. Effects of MCD diet and Western diet on body weight.

WT mice were fed chow diet, MCD diet for 8 weeks, or Western diet for 16 weeks, and sacrificed at 20 weeks of age. A. Body weights. B. Serum adipokines (leptin and adiponectin). Mean±SEM results were graphed. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

Weight loss in the MCD diet-fed group was more pronounced in the beginning of the diet. On average, mice lost 10% body mass per week during the first 2 weeks of MCD diet, 5% in week 3, and 2.5% per week thereafter. MCD diet-related weight loss has been attributed to hypermetabolism (Ntambi et al., 2002) caused by increased sympathetic nervous system outflow to the adipose tissue (Jha et al., 2014), with resultant increased mitochondrial uncoupling leading to less efficient energy extraction from nutrients (Rizki et al., 2006). We observed increased uncoupling protein gene expression in the livers of MCD-fed mice (**Figure 8**).

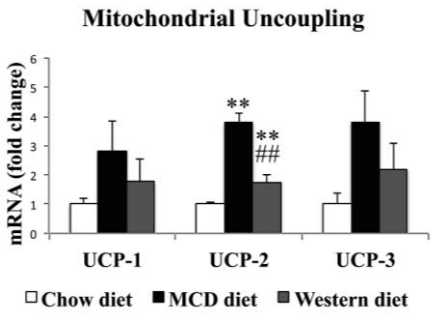


Figure 8. Effects of MCD diet and Western diet on liver mitochondrial uncoupling

qRT-PCR analysis of liver genes encoding mitochondrial uncoupling proteins, in mice fed either chow, MCD or Western diet. Results were normalized to expression in chow-diet fed mice and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

As expected, the Western diet promoted obesity, though with modest weight gain as compared to controls. This excessive weight gain was noteworthy, however, because the Western diet-fed group consumed only half as much solid food as controls fed isocaloric standard chow, as expected given appetite suppressing effect of lipids (Samra, 2010). On the other hand, the Western diet group was permitted *ad libitum* access to drinking water supplemented with high fructose corn syrup, which associates with decreased satiety and obesity (Purnell and Fair, 2013). Concordant with the differences in weight, MCD-diet fed mice were hypoleptinemic and hyperadiponectinemic, whereas Western-diet fed mice were hyperleptinemic relative to controls (**Figure 7B**). This is concordant with the literature, since increase in adiponectin has been described in the MCD diet, however MCD diet also seems to associate with hepatic adiponectin resistance (Larter et al., 2008). Western diet mimicked the metabolic syndrome associated with human NAFLD/NASH, with hyperglycemia/IR as well as dyslipidemia. In contrast, MCD-diet fed mice did not exhibit hyperlipidemia or IR and became severely hypoglycemic with fasting (**Figure 9**).

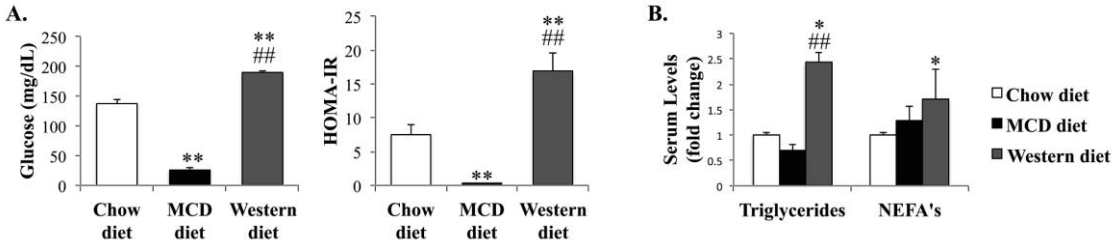


Figure 9. Effects of MCD diet and Western diet on metabolic profile.

A. Fasting serum glucose and HOMA-IR index. B. Serum triglycerides and non-esterified fatty acids (NEFA's). Mean±SEM results were graphed. *p<0.05 and **p<0.005, control *vs*. experimental diet; #p<0.05 and ##p<0.005, MCD *vs*. Western diet.

Of note, although mice fed the MCD diet developed peripheral insulin sensitivity, it is known that MCD diet associates with liver IR, which may mimic the liver effects of metabolic syndrome-phenotype associated with human NAFLD (Leclercq et al., 2007; Schattenberg et al., 2005).

MCD diet induces less hepatic steatosis, but more liver injury, than Western diet

Although both diets induced liver steatosis, only Western diet-fed mice developed hepatomegaly (i.e., increased liver-to-body weight (LBW) ratio) (**Figure 10**).

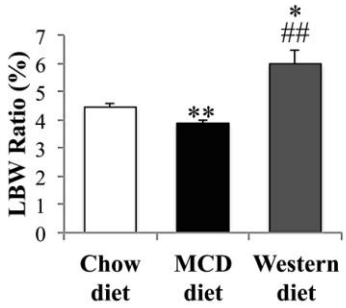


Figure 10. Effects of MCD diet on liver weight.

Liver to body weight (LBW) ratio, in mice fed chow, MCD or Western diet. Mean±SEM results were graphed. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01 MCD *vs*. Western diet.

LBW in MCD-diet fed mice was actually lower than controls, suggesting that MCD diets led to loss of liver mass. Indeed, serum liver enzymes, as well as histologic liver inflammation and the NAS score, were all significantly greater in the MCD diet-fed mice than the Western diet-fed mice, supporting the concept that the MCD diet provoked worse liver damage (**Figure 11**). Although neither diet induced clear pathological findings of hepatocellular ballooning, a hallmark lesion in human-NASH, the MCD diet better mimicked the other pathological findings typical of severe human NASH (**Figure 11**), namely lobular/periportal inflammation and perivenular/perisinusoidal fibrosis.

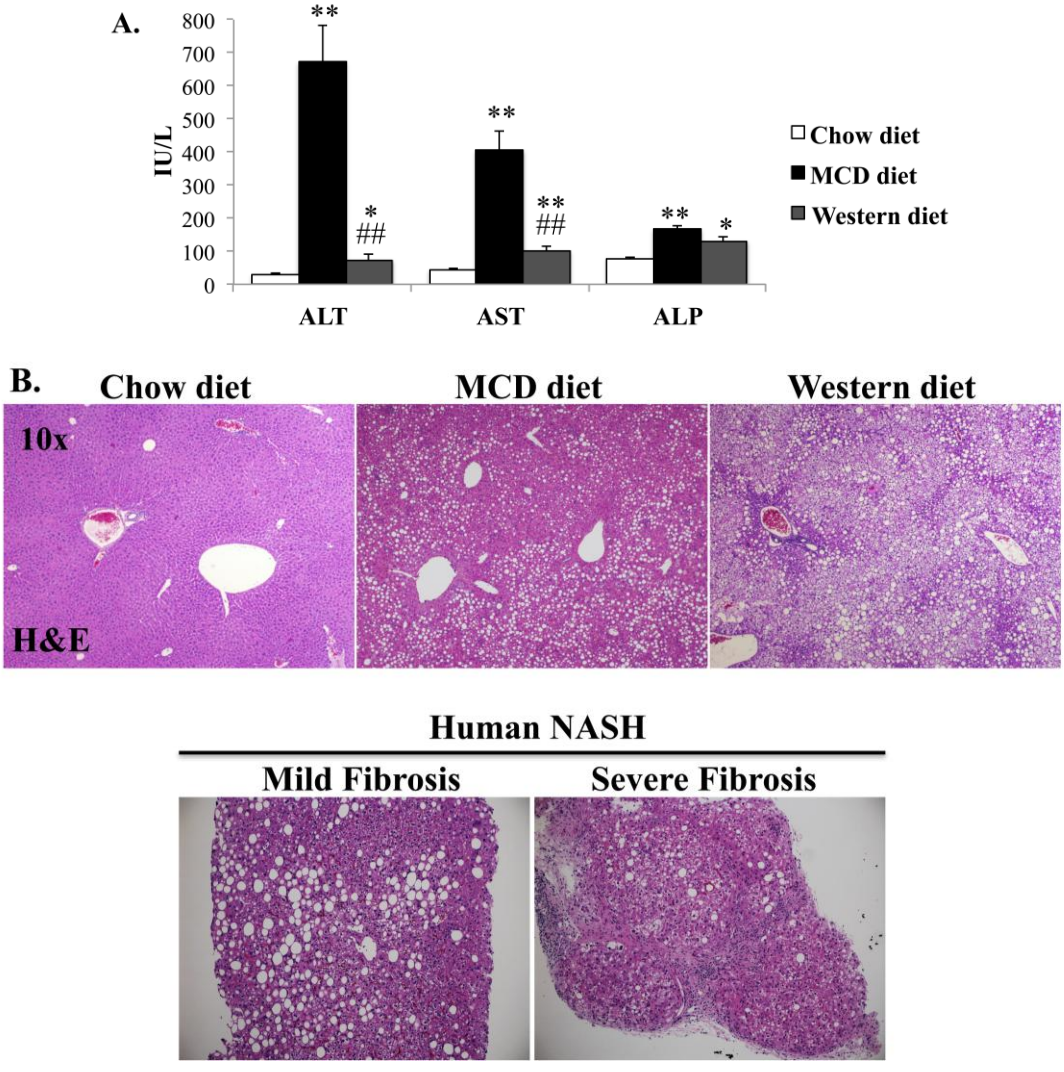
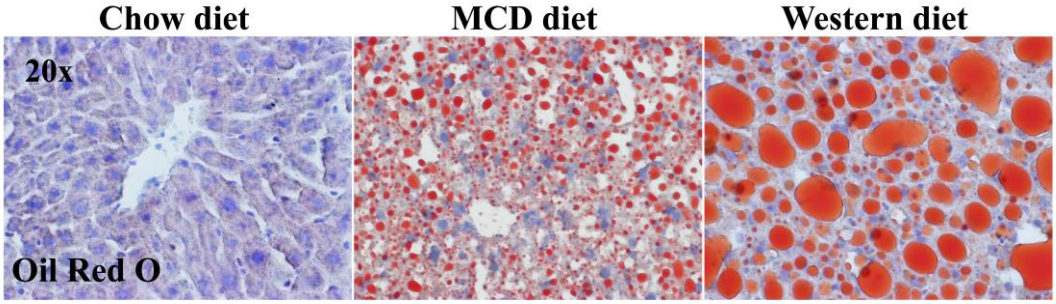


Figure 11. Effects of MCD diet and Western diet on liver injury.

A. Serum aminotransferases (ALT, AST) and alkaline phosphatase (ALP) from mice fed chow, MCD or Western diet. B. H&E staining of representative liver sections from mice (upper panels) and NASH patients with mild or severe fibrosis (lower panels). Results were graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

Hepatic lipid composition and mechanisms of fat accumulation differ in Western diet and MCD diet models

Western diet induced more impressive hepatic steatosis than MCD diet, as assessed by oil-red staining and liver triglyceride concentration. The Western diet supplemented cholesterol and hence increased liver cholesterol content, whereas liver cholesterol content in MCD diet-fed mice was actually lower than chow-fed controls. Interestingly, liver NEFA's, which are believed to be the more active and injury-inducing lipids (Ibrahim et al., 2011), were higher in the MCD-diet group (**Figure 12**).



Liver levels	Chow diet	MCD diet	Western diet
Triglycerides (µg/mg)	5.7±0.6	28.5±5.9**	58.6±5.7**,#
Cholesterol (µg/mg)	2.7±0.3	0.9±0.1**	3.4±0.5*,##
NEFA's (nmol/mg)	0.8±0.1	4.3±1.0*	1.6±0.4

Figure 12. Effects of MCD diet and Western diet on hepatic steatosis.

Oil-red O staining of representative liver sections from mice fed chow, MCD or Western diet (upper panels) and liver lipid levels (lower panel). (D). qRT-PCR analysis of liver genes encoding lipid metabolic enzymes. Results were graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.005, MCD *vs*. Western diet.

The mechanisms driving liver fat accumulation also seemed to differ in the two models. Western diet induced genes involved in *de novo* lipogenesis, whereas MCD diet caused down-regulation of genes for fatty acid esterification and VLDL secretion (**Figure 13**).

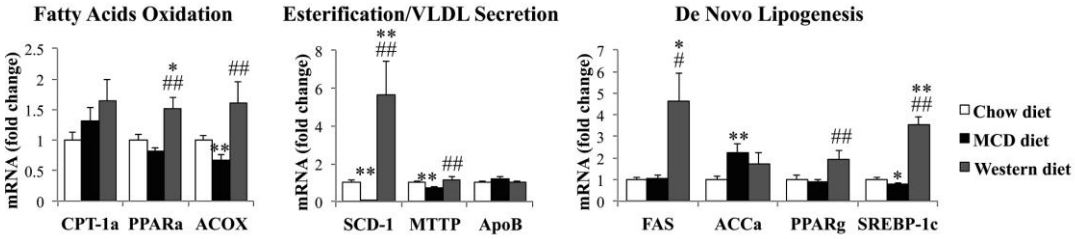


Figure 13. MCD diet and Western diet induce hepatic steatosis through different mechanisms.
qRT-PCR analysis of liver genes encoding lipid metabolic enzymes, mice fed chow, MCD or Western diet. Results were normalized to chow-diet fed mice and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #<0.05 and ##<0.01, MCD *vs*. Western diet.

Worse and more consistent fibrosis and ductular response in MCD diet model

MCD diet was significantly more fibrogenic than Western diet, as assessed by hydroxyproline assay, Sirius red staining, gene expression analysis and immunohistochemistry for HSC activation markers (**Figure 14**). Of note, animal-to-animal variability in the severity of liver fibrosis was greater in the Western diet than in the MCD diet group.

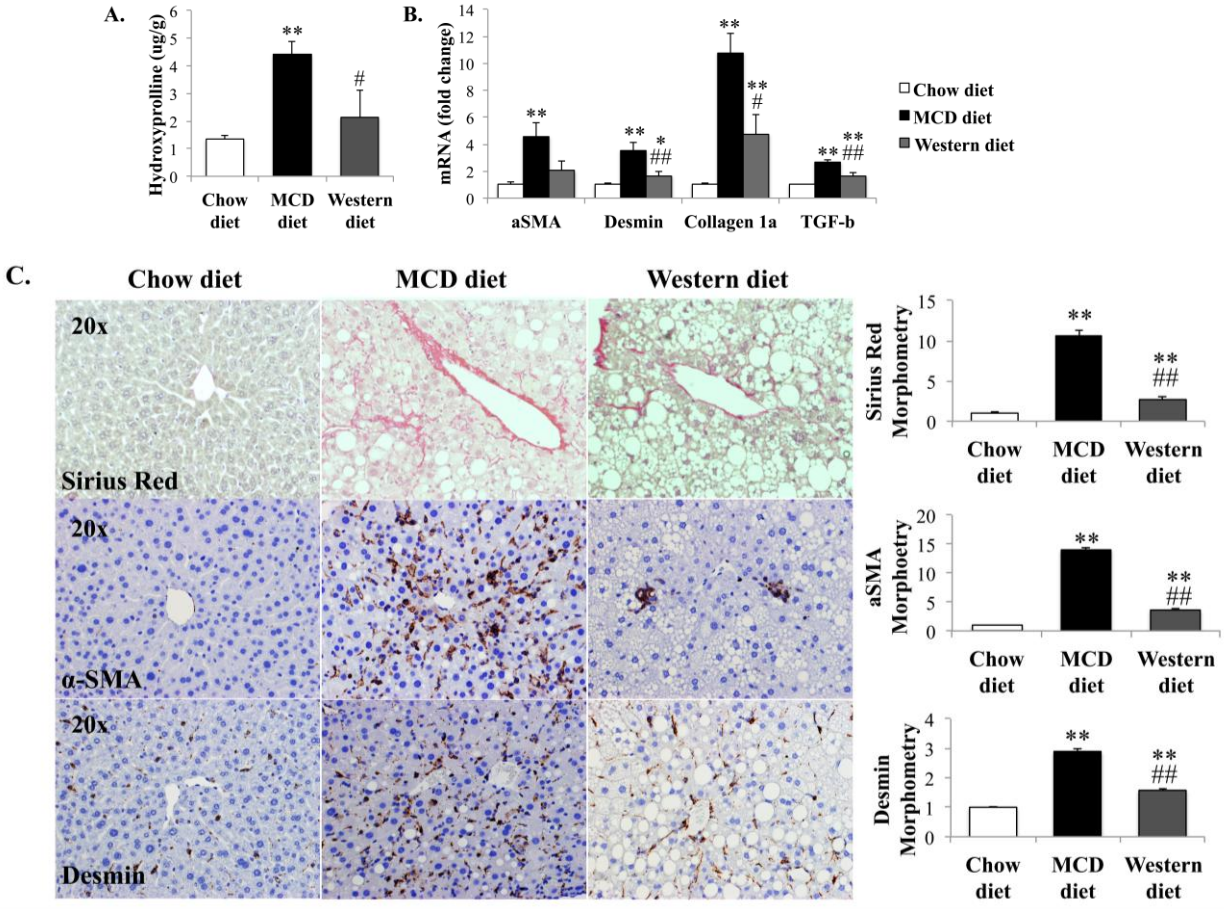


Figure 14. Effects of MCD diet and Western diet on liver fibrosis.

A. Hepatic hydroxyproline content, in mice fed chow, MCD or Western diet. B. qRT-PCR analysis of genes associated with liver fibrosis. C. Liver sections from representative mice stained for markers of fibrosis (Sirius red, α-SMA, and desmin) (left panels) and respective morphometry (right). Results were normalized to chow-fed mice and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

Ductular response intensity (assessed by qRT-PCR analysis and immunohistochemistry for progenitor markers) generally paralleled the severity of the fibrogenic response, both being significantly greater in the MCD diet than the Western diet model (**Figure 15**).

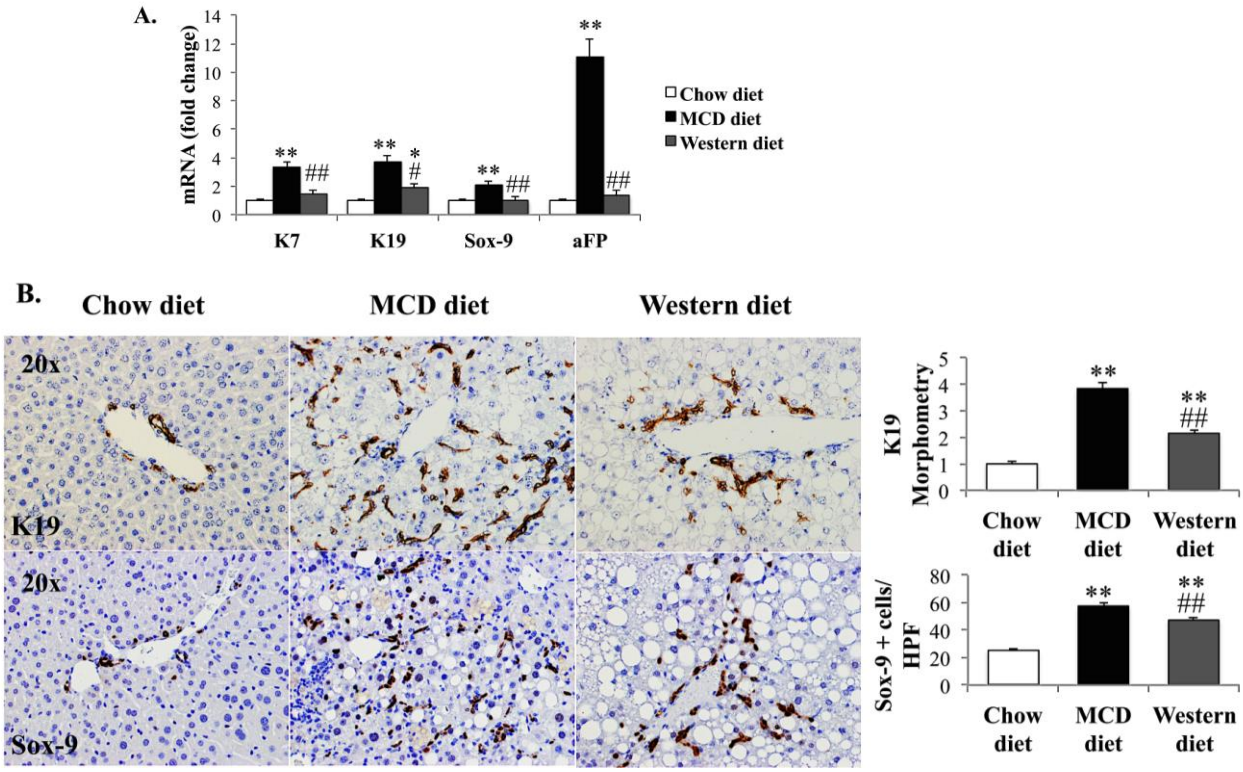
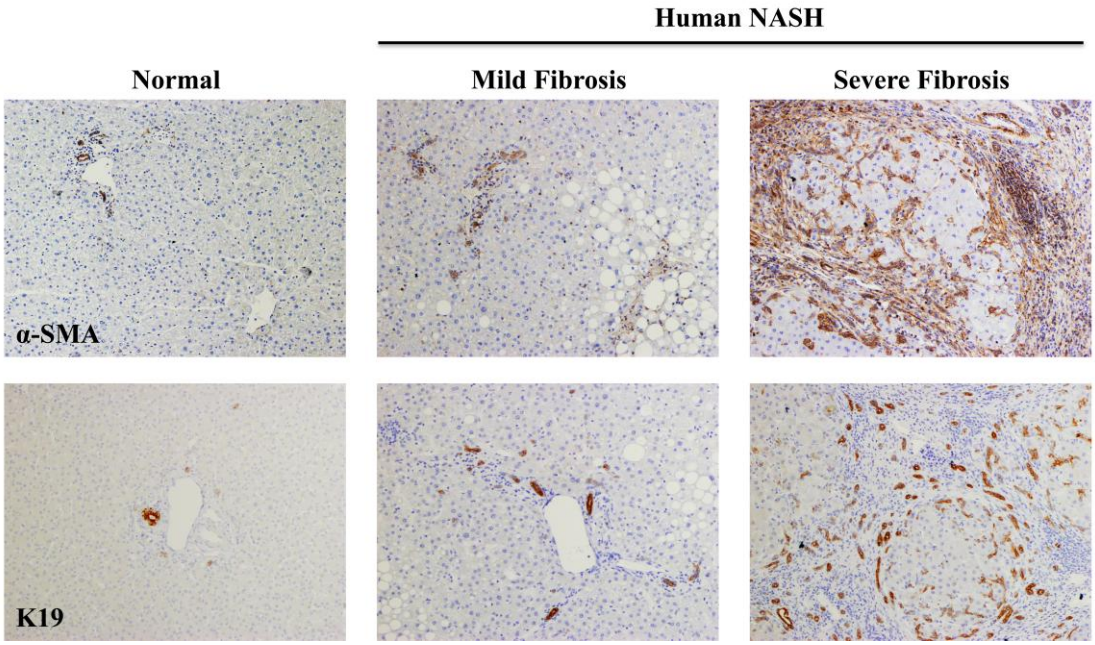


Figure 15. Effects of MCD diet and Western diet on ductular reaction.

A. qRT-PCR analysis of progenitor markers genes. B. Liver sections from representative mice stained for markers of the ductular reaction and progenitor expansion (K19 and Sox-9) (left panels) and respective morphometry (right). Results were normalized to chow-fed mice and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

Most notably, αSMA and K19 immunostaining in the MCD diet-fed mice was quantitatively and qualitatively similar to that of NASH patients with severe fibrosis (**Figure 16**).



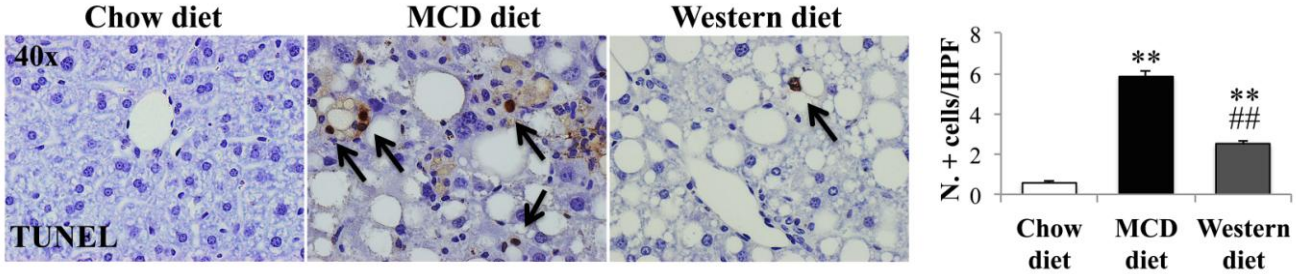
	Mouse Models		Human NASH	
	MCD (8 weeks)	Western diet (16 weeks)	Mild Fibrosis (N=5)	Severe Fibrosis (N=5)
α-SMA	14.0±0.4	3.6±0.2	2.6±0.4	10.8±1.1
K19	3.9±0.2	2.1±0.1	3.7±0.9	9.0±3.8

Figure 16. Fibroductular response in human NASH.

Representative photographs from immunohistochemistry for a-SMA and K19 in liver sections from healthy donors (N=5), adult patients with NASH and mild fibrosis (N=5) or severe fibrosis (N=5). Table comparing morphometry for the above staining in murine models and human samples. Results were expressed in fold-change from chow diet (mouse model) or normal human liver (human NASH samples), with average±SEM. All results were statistically different from respective controls ($p<0.05$).

Worse hepatic apoptosis and inflammation in the MCD diet model

Cleaved PARP, a marker of active apoptosis, and numbers of TUNEL-positive cells (another indicator of cell death) were both significantly greater in MCD diet-fed mice than Western diet-fed mice (**Figure 17**).



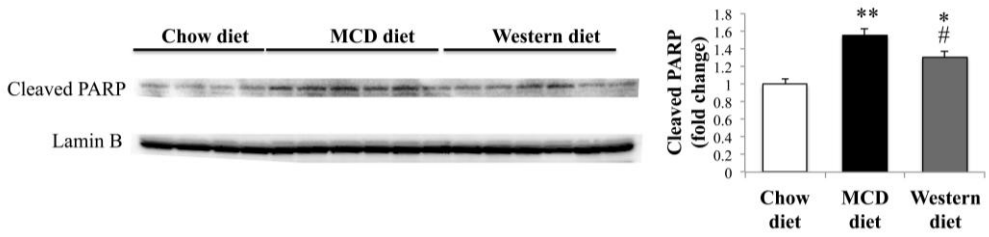


Figure 17. Effects of MCD diet and Western diet on liver cell death.

TUNEL assay in mice fed chow, MCD or Western diet and quantification of number of positive cells per HPF (upper panels). Western blot for cleaved PARP (lower panels). Results were graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

MCD diet induced more liver inflammation than Western diet, as evidenced by higher liver mRNA and protein levels of TNF-α, F4/80 and YM-1, markers of classical and alternative macrophage activation, respectively (**Figure 18**). Infiltration by other immune cells, namely lymphocytes, monocytes and neutrophils, though less marked than macrophage accumulation, was also higher in MCD diet-fed mice compared to Western diet-fed animals, as assessed by qRT-PCR analysis of cell-type specific markers (**Figure 18**).

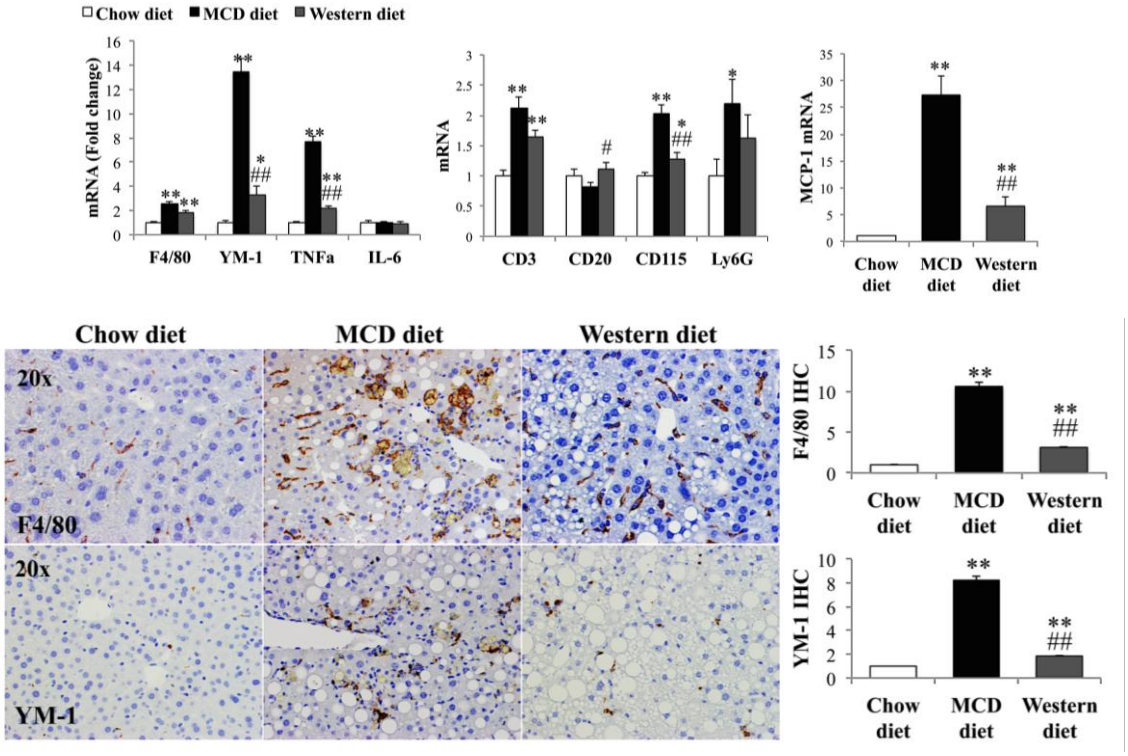


Figure 18. Effects of MCD diet and Western diet on liver inflammation.

qRT-PCR F4/80 and YM-1 (macrophage markers), CD3 (pan-T lymphocyte marker), CD20 (pan-B lymphocyte marker), CD115 (marker of blood monocytes) and Ly6G (the granulocyte differentiation antigen 1) and the chemokine MCP-1 (monocyte chemoattractant protein-1) (upper

panels). Representative photos and morphometry for immuno-histochemistry of macrophage markers (lower panels). Results were normalized to chow-diet fed mice and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.05, MCD *vs*. Western diet.

MCD diet models mechanisms implicated in human NASH pathogenesis/progression

Oxidative stress plays a major role in the pathogenesis of human NASH (Alkhouri et al., 2014; Ucar et al., 2013). To limit liver injury, hydroxyl radical (˙OH), nitric oxide radical (NO˙), and superoxide anion ($O_2^{\cdot-}$) must be neutralized by antioxidant enzymes (superoxide dismutases) which convert $O_2^{\cdot-}$ into hydrogen peroxyde (H_2O_2). The latter is further detoxified by glutathione peroxidase (GPx) or catalase. Similar to humans with severe NASH (Hardwick et al., 2010; Sreekumar et al., 2003), mice fed MCD diet demonstrated decreased expression of antioxidant enzymes (**Figure 19**).

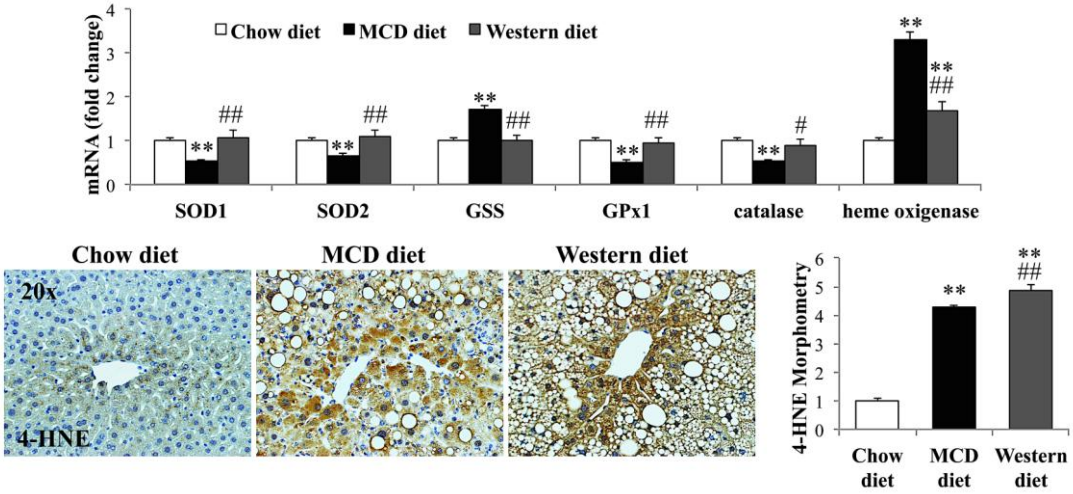


Figure 19. Effects of MCD diet and Western diet on oxidative stress.

qRT-PCR analysis of anti-oxidant enzymes (upper panels) and immunohistochemistry plus morphometry for 4-hydroxynonenal (4-HNE) in representative mice (lower panels). Results were normalized to chow-diet fed mice and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; ##p<0.005, MCD *vs*. Western diet.

MCD diet is also known to deplete SAMe, a methyl donor important for glutathione synthesis and anti-oxidant defense (Wortham et al., 2008). This suggests that MCD diet imposes greater oxidative stress than Western diet. Consistent with this, gene expression of heme oxygenase (a marker of oxidative stress) (Choi and Alam, 1996), was higher in MCD diet-fed mice than Western diet-fed mice. Also, MCD diet- and Western diet-groups had comparable 4-hydroxynonenal accumulation despite the fact that MCD diet-fed

mice had lower hepatic lipid content and thus, less substrate for lipoperoxide production (**Figure 19**).

ER stress and the consequent unfolded protein response (UPR) are other key factors in the pathogenesis of human NAFLD/NASH (Gonzalez-Rodriguez et al., 2014; Henkel and Green, 2013; Lake et al., 2014). MCD-diet provoked greater induction of UPR mediators, transcription factor X-box binding protein-1 (XBP-1) and chaperone glucose regulated protein-78 (GPR-78) than Western diet. It also stimulated greater expression of growth-arrest and DNA damage-inducible gene-153 (GADD153), an important mediator of ER stress-eliciting apoptosis response (**Figure 20**).

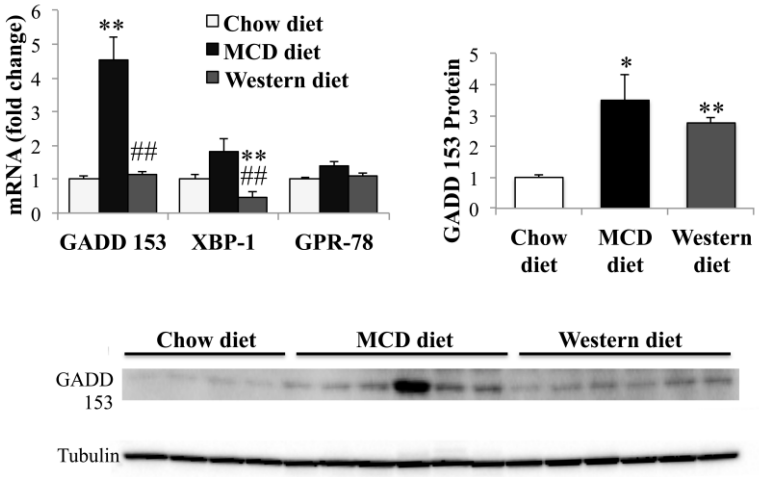


Figure 20. Effects of MCD diet and Western diet on liver ER stress.

qRT-PCR analysis of relevant ER stress-related genes and western blot analysis of GADD-153, in mice fed chow, MCD or Western diet. Results were normalized to chow diet and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

Deregulated autophagy occurs in IR and obesity (Park et al., 2014), and has also been implicated in NASH pathogenesis. Autophagy and steatosis severity are inversely correlated (Kashima et al., 2014). MCD diet-fed mice demonstrated evidence for decreased autophagy based on reduced hepatic accumulation of the active conjugate form of microtubule-associated protein 1 light chain 3, LC3-II (**Figure 21**).

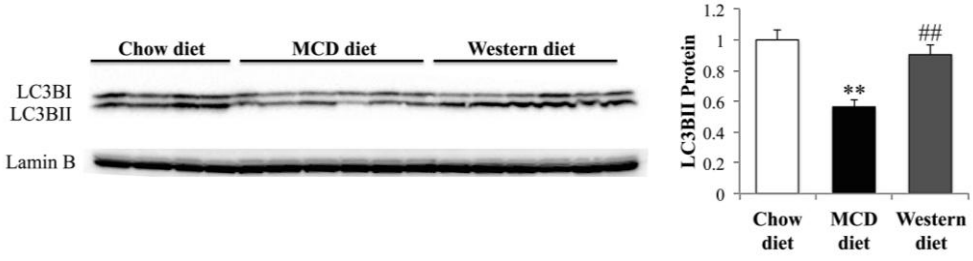


Figure 21. Effects of MCD diet and Western diet on liver autophagy.

Western blot analysis of the autophagy marker, LC3BII, in mice fed chow, MCD or Western diet. Results were normalized to chow diet and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

Thus, three key cell stress-related mechanisms that have been implicated in human NASH pathogenesis (oxidative stress, ER stress, and autophagocytic stress) are significantly more active in the MCD diet model than in the Western diet model.

Finally, hedgehog pathway activation is pivotal in NASH progression, driving profibrogenic responses in animal models and correlating with fibrosis in human NASH (Fleig et al., 2007; Guy et al., 2012; Hirsova and Gores, 2015; Syn et al., 2009). The hedgehog pathway was more activated in MCD diet-fed mice than Western diet-fed mice, with greater induction of Hedgehog ligands and Hedgehog-target genes (**Figure 22**).

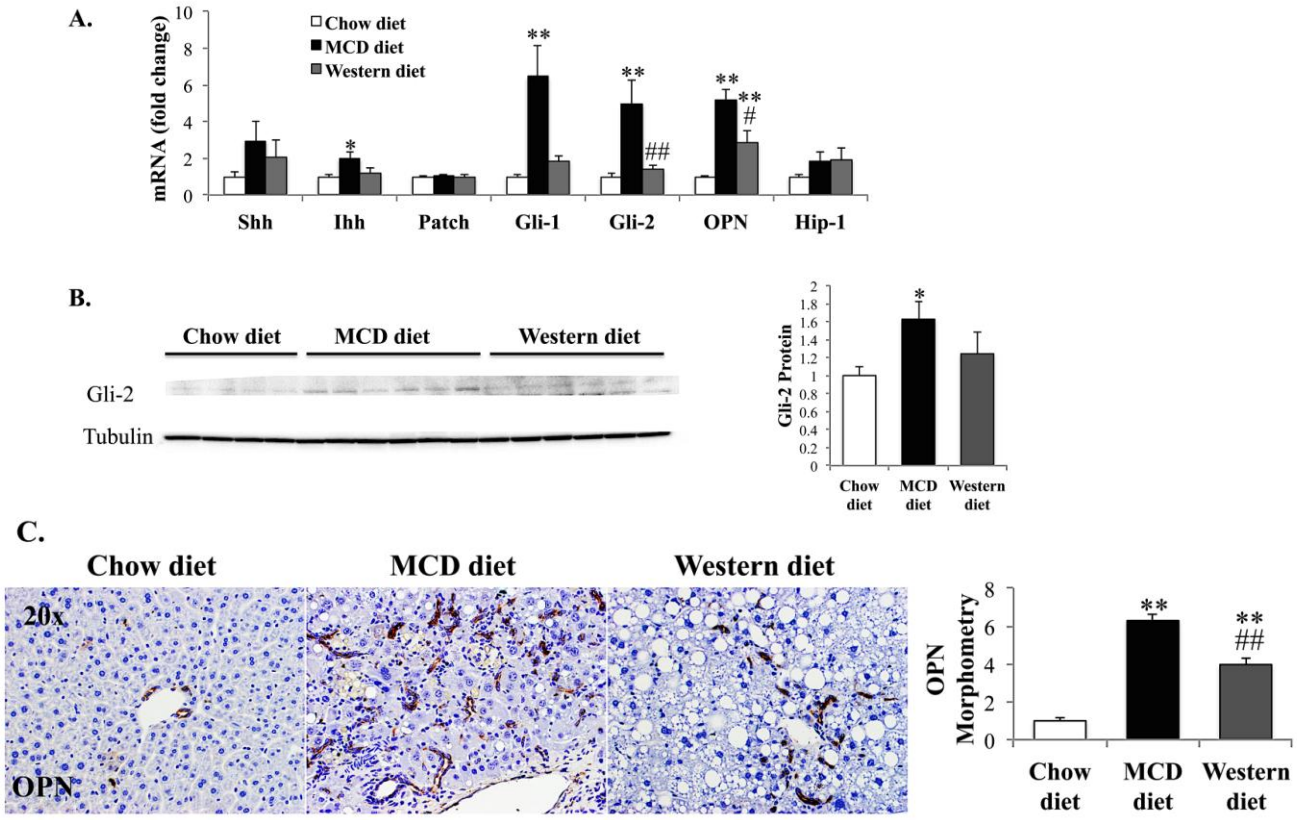


Figure 22. Effects of MCD diet and Western diet on Hedgehog pathway.

A. qRT-PCR analysis of hedgehog ligands (Shh, Ihh), receptor (Patch), hedgehog-regulated transcription factors (Gli1, Gli2), and target genes (OPN, Hip-1) B. Western blot analysis of Gli-2. C. Immunohistochemistry for osteopontin (OPN): sections from representative mice and morphometry. Results were normalized to chow diet and graphed as mean±SEM. *p<0.05 and **p<0.01, control *vs*. experimental diet; #p<0.05 and ##p<0.01, MCD *vs*. Western diet.

DISCUSSION

Efficient, inexpensive, reproducible, and relevant animal models are needed to advance understanding of the mechanisms that drive the pathogenesis and progression of NASH in humans. Two diets, MCD diet and Western diet, have been widely used to model human NASH in rodents. Each approach has its advocates, who often condemn the alternative model for failing to faithfully mimic relevant human pathobiology. The present head-to-head comparison of the two models suggests that such controversy is groundless because each diet beautifully models a different sub-type of human NASH.

Human NAFLD is a heterogeneous disease. Although NAFLD is extremely prevalent, only a minor sub-population of individuals with hepatic steatosis demonstrates NASH at any given point in time (Loomba and Sanyal, 2013). The dichotomy between non-progressive steatosis and progressive NASH (Singh et al., 2015) has been challenged by natural studies that suggested that the presence and severity of fibrosis is the factor that dictates fibrosis, whereas having NASH does not translate in worse liver outcome at long term (Angulo et al., 2015; Ekstedt et al., 2015). Also, it is clear that progression is far from inevitable (Singh et al., 2015). Indeed, "spontaneous" regression of NASH has been documented to occur in some of the placebo group participants in several prospective NASH treatment trials (Neuschwander-Tetri et al., 2015; Sanyal et al., 2010). The variable propensity to improve *versus* progress that exists among individuals with NASH is not well understood. Of note, body mass index/obesity does not seem to be an independent predictor of fibrosis progression or explain the occurrence of rapid fibrosis progression in the 20% of patients that develop progressive fibrosis (Singh et al., 2015).

Clarifying mechanisms that determine whether or not NASH progresses is a major unmet need because such information would provide the basis for risk stratification and more personalized management. Our results show that the Western diet models the more common, relatively non-progressive subtype of NASH, whereas the MCD diet models the less common, more rapidly progressive/aggressive NASH subtype. Because our study was designed to control for genetic and epigenetic factors that are likely to modulate NASH outcomes in humans (i.e., genetic background, gender, age, and living conditions), diet-sensitive factors that modulate NASH were revealed. The results show that Western diet components are relatively weak NASH agonists, while something about the MCD diet strongly enhances NASH progression. Moreover, the MCD diet-associated factor(s) appear to work by stimulating many of the same mechanisms that promote progressive

liver damage in human NASH, including oxidative-, ER-, autophagocytic-stress, and hedgehog pathway activation.

The Western diet-related factors that promote obesity and insulin-resistance are neither sufficient, nor necessary, for NASH progression in mice. Compared to lean, insulin-sensitive MCD diet-fed mice, which developed severe NASH within 8 weeks, obese, insulin-resistant Western diet-fed mice exhibited only mild NASH even after being exposed to the obesogenic and diabetogenic diet for twice that time. This result has important implications for human NASH. Namely, it underscores the primary importance of non-dietary factors (e.g., genetic background, epigenetic exposures) in driving NASH progression, in obese, insulin-resistant humans. While somewhat surprising, other independent lines of emerging evidence in both mice and humans support this concept. Recent trans-generational studies in mice proved that cirrhosis susceptibility is controlled by epigenetic factors that influence gene methylation (Zeybel et al., 2012). Global differences in hepatic gene methylation patterns have been reported in typical patients with mild *versus* severe NASH (Murphy et al., 2013). In humans with advanced NASH, genes that encode key methylation-promoting enzymes are differentially hyper-methylated and under-expressed, and the hepatic genome is generally hypo-methylated relative to humans with mild NASH (Murphy et al., 2013). Given this background, it is noteworthy that MCD diets deplete methyl donors and may promote global hypo-methylation of the hepatic genome (Oz et al., 2008). Thus, the aggregate rodent and human data strongly justify further research to identify key epigenetic mechanisms that control liver health and clarify factors that deregulate these processes to promote tissue damage in individuals with the metabolic syndrome. Contrasting model-dependent differences in various epigenetic profiles (e.g., the hepatic methylome) is likely to identify important diagnostic/therapeutic targets in human NASH because the two mouse models demonstrate significant discrepancies in fibrosis severity, the main prognostic factor in human NASH (Angulo et al., 2015; Ekstedt et al., 2015).

The new data also help to clarify the relative importance of different types of lipids in NASH pathogenesis. Diet-induced obesity and IR were highly efficient tools for generating hepatic triglyceride accumulation. However, triglyceride content *per se* was not predictive of NASH progression, as demonstrated by the fact that liver injury and fibrosis were much more severe in MCD diet-fed mice which had lower hepatic triglyceride content than Western diet-fed mice with non-progressing NASH. High cholesterol diets are known to provoke liver damage in rodents; hypercholesterolemia is certainly

associated with human NASH; and cholesterol-lowering interventions have been correlated with milder forms of NASH in patients (Athyros et al., 2010). Thus, it has been assumed that lowering liver cholesterol content would improve human NASH. However, in the present study, supplementing dietary cholesterol in obese, diabetic mice was not sufficient to trigger NASH progression, although it did significantly increase hepatic cholesterol content. Rather, more severe liver damage occurred in mice that became somewhat depleted of liver cholesterol due to MCD diet administration. In contrast, the hepatic NEFA content and severity of liver damage were strongly correlated. Relative to MCD diets, the obesogenic/diabetogenic Western diet was a relatively weak stimulant for liver NEFA accumulation. This observation has important implications for researchers who wish to investigate the roles of NEFA in NASH pathogenesis.

Our results also emphasize the lack of correlation of metabolic disorders (obesity, IR, T2DM and dyslipidemia) and the severity of liver injury/fibrosis, in dietary animal models. That is also true for genetic models of NASH. For example, severe NASH, cirrhosis and liver cancer have been reported to occur in non-obese mice with targeted genetic defects that enhance insulin sensitivity (Horie et al., 2004), or that disrupt signaling that activates NFkB (a major pro-inflammatory factor) (Luedde et al., 2007). Like MCD diet-fed mice, these genetically altered mice are often dismissed as reagents for studying NASH pathogenesis because they lack factors (e.g., obesity, IR, pro-inflammatory cytokines) that associate with an increased risk for human NASH.

It is worth noting, however, that MCD diet induces hepatic IR despite peripheral insulin hypersensitivity. Moreover, discordance between improvements in IR and improvements in liver histology were recently documented in at least two large human NASH treatment trials (Neuschwander-Tetri et al., 2015; Sanyal et al., 2010), which raises the question whether hepatic IR and not peripheral IR can modulate the severity of liver disease in NAFLD. Also, regarding the difference in the adipokine profile in lean mice and obese mice or patients with NASH, it is true that the MCD diet induces a different profile from obese patients with NAFLD, but this may not be in contradiction with human physiology. In fact, hyperleptinemia in humans is often accompanied by some degree of leptin resistance. Like low leptin levels, leptin resistance reduces leptin signaling in relevant target tissues. Thus, leptin activity is variably inhibited in both human NASH and rodents with MCD diet-induced NASH. On the other hand, humans with NASH appear to be relatively deficient in adiponectin, whereas at least the expression of this adipocytokine is maintained in the MCD diet-fed model. However, several reports described perturbed

hepatic adiponectin signaling, despite normal serum levels, in rodents fed MCD diet (Hsiao et al., 2010; Tsai et al., 2011).

In conclusion, while the ideal animal model for NAFLD/NASH has yet to be discovered, the currently available MCD and Western dietary models provide complementary tools to study this major human disease. The MCD diet model has the advantage of being more efficient and reproducible for inducing severe liver damage and progressive fibrosis. MCD diet-fed rodents activate mechanisms that have been implicated in human NASH progression. Thus, this diet approach models the subgroup of NASH patients with histologically advanced NASH, and it is ideal for studying mechanisms driving NASH-related inflammation/fibrosis, as well as strategies to inhibit these processes. In contrast, the Western diet model mimics the vast majority of obese NAFLD/NASH patients. Such individuals typically have IR and the metabolic syndrome, but relatively mild liver injury. Despite the fact that innumerous diets have been used to induce NAFLD in animal models, Western diet and MCD diet are by far the most used and the better characterized ones. Hence, the Western diet model should be the first choice for studying how NAFLD/NASH impact systemic metabolic and cardiovascular risk for tissue complications related to type 2 diabetes mellitus and atherosclerosis. Because liver injury and fibrosis are relatively mild and slowly progressive in the Western diet model, it also provides an invaluable tool for identifying factors that accelerate NASH progression.

THE ROLE OF CASPASE-2 IN THE PATHOGENESIS OF THE METABOLIC SYNDROME AND ITS LIVER MANIFESTATION, NAFLD

INTRODUCTION

Caspase-2, the odd man among caspases

Caspase-2, formerly known as ICH-1 (interleukin-1β converting enzyme homolog-1) in humans (Wang et al., 1994) or NEDD-2 in mice (Kumar et al., 1994; Kumar et al., 1992), is a member of the family of cysteine-dependent aspartate-specific proteases. It shares with other caspases the catalytic triad residues, consisting of the active site Cys285 (which is a part of the conserved QACXG pentapeptide sequence), His237, and the backbone carbonyl of residue 177 (caspase-1 numbering) (Stennicke and Salvesen, 1999). Caspase-2 was the second caspase being cloned and the evolutionarily most conserved. In fact, caspase-2 is the caspase that shows the highest sequence homology with the archetypical *Caenorhabditis elegans* caspase, Ced-3 (Kumar et al., 1994). Despite being one of the first caspases to be described, the primary function of caspase-2 remains to be determined. Caspase-2 is known to regulate cellular apoptosis (Bouchier-Hayes and Green, 2012). However, it has also been implicated in several other vital processes including regulation of cell cycle checkpoints, the oxidative stress response, autophagy, and senescence (Gitenay et al., 2014; Olsson et al., 2015; Tiwari et al., 2014; Wilson et al., 2015).

The position of caspase-2 in the apoptotic pathway is a matter of debate. Caspases can be divided into initiators (caspases 1, 8, 9 and 10) and executioners (caspases 3, 6 and 7) (Bouchier-Hayes, 2010). Caspase-2 resembles initiator caspases structurally and in the way of activation. However, similarly to executioner caspases, caspase-2 has a strong preference for cleaving in the aspartic acid residue in the P4 position of its substrates, does not directly activate executioner caspases and can be cleaved by caspase-3 (Bouchier-Hayes, 2010). Such characteristics suggest that caspase-2 may act as a proximal responder to auto-activate in apoptosis signaling, and function as an executioner caspase by cleaving cellular targets instead of activating downstream caspases (Degterev et al., 2003). Several reports showed that caspase-2 acts upstream of MOMP, leading to Bid cleavage, Bax translocation to the mitochondria and cytochrome c release (Guo et al., 2002; Lassus et al., 2002; Robertson et al., 2002). Subsequently, cytochrome c release initiates the apoptotic pathway through activation of initiator caspase-9 (Guo et al., 2002) (**Figure 23**).

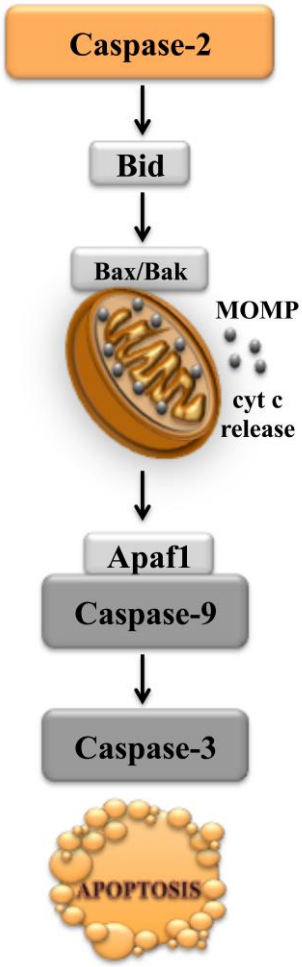


Figure 23. Apoptotic pathway mediated by caspase-2.

Caspase-2 is activated by intracellular cues, and mediates cleavage and activation of Bid, assembly of mitochondrial membrane pores by Bax and Bak, leading to mitochondrial outer membrane permeabilization (MOMP). MOMP has two consequences: it both initiates a proteolytic cascade of pro-apoptotic enzymes and damages mitochondrial function.

Caspase-2 share a similar structure with initiator caspases, and has a long prodomain that contains a caspase-recruitment domain (CARD) or death effector domain (DD) (Tinnikov and Samuels, 2013). Similarly to caspase-8 and -9, caspase-2 activation occurs after recruitment to an activation platform, which allows proximity between molecules of caspase-2 and hence facilitates dimerization (Bouchier-Hayes and Green, 2010). The activation platform, dubbed PIDDosome, is a multiprotein complex composed by PIDD (p53-induced protein with a death domain) and the adaptor molecule RAIDD (receptor-interacting protein-associated ICH-1/CED-3 homologous protein with a death domain) (Tinel and Tschopp, 2004). Binding of caspase-2 to RAIDD occurs through CARD motifs present in both proteins. After dimerization, auto-processing of caspase-2 stabilizes it (Baliga et al., 2004) (**Figure 24**). Cleavage of caspase-2 by caspase-3 also occurs after MOMP (Slee et al., 1999) and does not promote caspase-2 activation (Baliga et al., 2004). Hence, monitoring cleavage alone is not sufficient to identify caspase-2 as the apical caspase (Bouchier-Hayes et al., 2009).

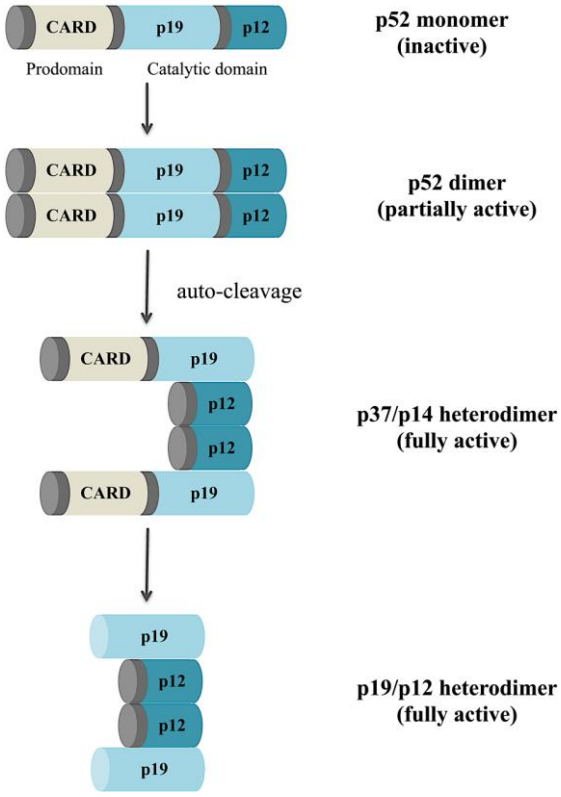


Figure 24. Mechanisms of caspase-2 activation.

In response to proapoptotic signals, monomers of pro-caspase-2 are recruited into close proximity via assembly of PIDDosome. The protease domains of caspase-2 can then interact in a transient dimeric complex and undergo intrinsic auto-catalytic activity, with cleavage between the large and small subunits of the protease domains. That event leads to the formation of an active stable tetrameric complex with maximal catalytic activity. Further processing generates the fully mature $(p19,p12)_2$ caspase-2 enzyme. *Adapted from Baliga BC, Cell Death Differentiation 2004*.

Assembly of PIDDosome and regulation of caspase-2 activation is a complex phenomenon. PIDD can assemble in two complexes, with RAIDD and caspase-2, but also with RIP1 controlling the activity of NEMO and hence NFkB survival pathway (Tinel et al., 2007). The PIDD-RIP1 complex recruits NEMO resulting in the post translational modification sumoylation. NEMO sumoylation facilitates NEMO ubiquitination and phosphorylation leading to the translocation of NEMO from the nucleus to the cytoplasm. Cytoplasmic NEMO releases IkB from NFkB allowing activation of NFkB survival pathway. The aggregate data suggest that PIDD may act as molecular switch between death and survival pathways (Janssens et al., 2005). The recruitment of RIP1 and RAIDD in the PIDDosome seems to be sequential. PIDD can undergo sequential auto-processing in PIDD-C through cleavage at Serine 446 and subsequently PIDD-CC at Serine 588. PIDD-C recruits RIP1 and can translocate to the nucleus, whereas PIDD-CC is exclusively cytoplasmatic and binds to RAIDD (Tinel et al., 2007). If the noxious stimulus is mild, PIDD-C activates NFkB promoting cell survival, whereas if the noxious is overwhelming, PIDD-CC activates caspase-2 leading to cell death (Andersen and Kornbluth, 2009). Caspase-2 can antagonize NFkB activation by competing with PIDD and by cleaving RIP1 (Guha et al., 2010).

Metabolic activity is a master determinant of cell commitment to proliferate or die (Buchakjian and Kornbluth, 2010). Accordingly, caspase-2 activation is highly metabolically regulated (**Figure 25**).

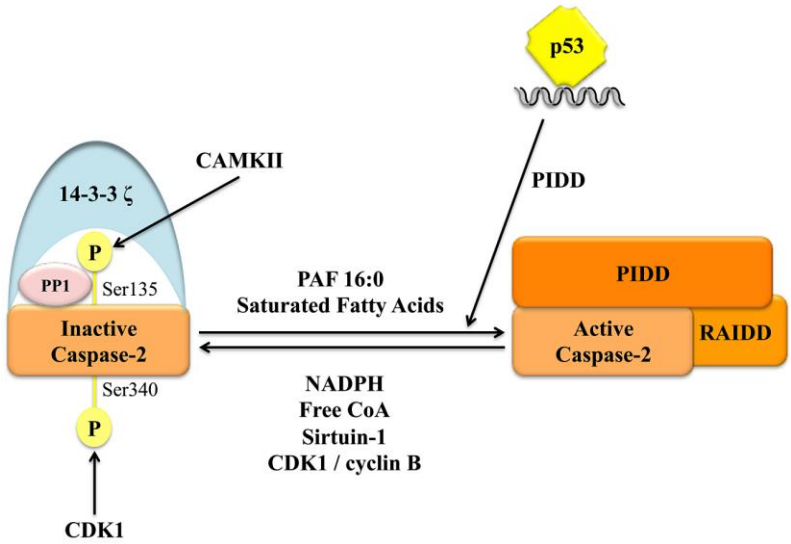


Figure 25. Metabolic regulation of caspase-2.

Caspase-2 is a strong mediator between metabolism and cell death. Caspase-2 is inhibited in response to abundance in NADPH, which is produced by the flux of glucose through the pentose phosphate pathway. NADPH inhibits caspase-2 through activation of calcium/calmodulin regulated protein kinase II (CAMKII) that phosphorylates and inhibits caspase-2 Ser135. Phosphorylated caspase-2 binds to 14-3-3ζ, which prevents its dephosphorylation. Dephosphorylated caspase-2 can assemble in the PIDDosome allowing its activation. Caspase-2 is also sensitive to activation by saturated fatty acids and the metabolite 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF 16:0). During mitosis cdk1 kinase suppresses caspase-2 through phosphorylation of a different serine residue, Ser 340.

Calcium/calmodulin regulated protein kinase II (CAMKII) phosphorylates caspase-2 at Serine-135, which inactivates caspase-2 (Nutt et al., 2005). Phosphorylated caspase-2 binds to small acidic phosphobinding 14-3-3ζ protein, which prevents dephosphorylation by constitutive caspase-2 binding to phosphatase-1 (Nutt et al., 2009). In other words, dephosphorylation of caspase-2 depends on the release from 14-3-3ζ protein. Nutrient availability and hence production of NADPH inhibits caspase-2 activation through activation of CAMKII and by facilitating release of 14-3-3ζ protein from caspase-2 (Buchakjian and Kornbluth, 2010). Free coenzyme A can also directly activate CAMKII and hence inhibit caspase-2 mediated apoptosis (McCoy et al., 2013). The deacetylase

sirtuin-1 enhances binding of 14-3-3ζ protein to caspase-2 and inhibition of sirtuin-1 allows dissociation of 14-3-3ζ protein and activation of caspase-2 apoptosis (Andersen et al., 2011). Sirtuin-1 can be seen as an intracellular energy sensor that detects the availability of NAD^+, in which increased intracellular concentrations of NAD^+ (as occurs with calorie restriction) activates sirtuin-1. Over nutrition associates with inhibition of sirtuin-1 (Kotas et al., 2013) and expectedly over activation of caspase-2. Also, through unknown mechanisms, accumulation of saturated FA or the lipid second messenger 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF 16:0) also induces activation of caspase-2 (Johnson et al., 2013; Ryan et al., 2009).

An intuitive concept is the need to repress apoptosis during cell division. Accordingly, during mitosis caspase-2 activity is suppressed through an evolutionary conserved mechanism involving the kinase cdk1-cyclin B1 phosphorylation of caspase-2 at a serine residue at position 340, which prevents caspase-2 activation (Andersen et al., 2009).

Adding a layer of complexity, Manzl *et al*. showed that PIDD is not necessary for caspase-2 activation (Manzl et al., 2009), and alternative ways of activation have been described, namely activation by caspase-8 through DISC downstream of CD95 (Olsson et al., 2009). Also, caspase-2 has been implicated in apoptosis induced by bacterial pore-forming toxins that mediate potassium efflux and activation of caspase-2 by a high-molecular weight complex different from PIDDosome (Imre et al., 2012). At the transcriptional level, caspase-2 expression is regulated by several transcription factors: SREBP-1c and SREBP-2 increase (Logette et al., 2005a; Logette et al., 2005b) and brain-derived neurotrophic factor (BDNF) decreases caspase-2 expression. Further, p53 and p73 can repress caspase-2 mRNA expression in a p21-dependent manner (Baptiste-Okoh et al., 2008).

Alternative splicing (Han et al., 2013) and regulation of caspase-2 expression by microRNAs modulate its function (Upton et al., 2012). Two caspase-2 isoforms can be expressed through alternative splicing (Wang et al., 1994): long isoform, proapoptotic, caspase-2_L, and short isoform, caspase-2_S, a truncated protein that inhibits apoptosis (Han et al., 2013; Kumar et al., 1994). In the short isoform, exon 9 is introduced. Because exon 9 has a stop codon, its introduction creates a truncated protein (Wang et al., 1994). More recently, it was shown that the two mRNA, from the two isoforms, differ at their 5'-end, upstream of the first exon, being the first exon common to both. This difference is due to the presence of two distinct promoters, being the promoter for the long isoform much

stronger than the one for the short isoform (Logette et al., 2003). Also, the half-life of short isoform mRNA is very short because it can be degraded by nonsense-mediated mRNA decay (Solier et al., 2005). The long isoform is ubiquitously expressed, whereas the short isoform is predominantly expressed in the brain, heart and muscle (Kumar et al., 1997; Wang et al., 1994). Furthermore, post translational modifications, such as N-terminal acetylation (Yi et al., 2011) and phosphorylation, modulate caspase-2 activation (Nutt et al., 2009).

One interesting aspect of caspase-2 is its cellular localization in the nucleus (Colussi et al., 1998) and in the Golgi apparatus (Mancini et al., 2000), apart from the cytosolic localization. Caspase-2 import to the nucleus depends on two nuclear localization signals in the prodomain, however the significance of nuclear localization is unknown, and overexpression of mutants that cannot translocate to the nucleus maintain caspase-2 potential to promote apoptosis (Baliga et al., 2003; Colussi et al., 1998). Also, caspase-2 oligomerization, and hence activation, can only occur in the cytoplasm, and does not occur in the nucleus (Bouchier-Hayes et al., 2009). It has been described that following apoptotic stimuli, caspase-2 translocates from the nucleus to the cytoplasm, where it can initiate the apoptotic cascade (Tinnikov and Samuels, 2013). Nuclear caspase-2 may be implicated in non-apoptotic functions such as regulation of cell-cycle checkpoints (Tinnikov and Samuels, 2013). Recently nuclear caspase-2 has been proposed to regulate the activity of p53-related gene p63. Alternative splicing generates two p63 isoforms: full-length isoform with a transactivation (TA-) domain homologous to full-length p53, and amino-terminally truncated (ΔN-) isoform that lacks the transactivation domain and act as a dominant-negative inhibitor of the full-length p53-related proteins, by dimerization. Caspase-2 cleaves ΔNp63, leading to nuclear export to the cytoplasm, and releasing full-length protein from its inhibitory effect. The net result is TAp63 transcription of relevant target genes such as proapoptotic PUMA and NOXA (Jeon et al., 2012). Caspase-2 also localizes in the Golgi complex, being able to cleave golgin-160, mediating disintegration of the Golgi complex after a pro-apoptotic signal (Mancini et al., 2000).

The function of caspase-2 is enigmatic because, unlike genetically modified mice deficient in caspase-3 or caspase-8 that are embryonically lethal (Degterev et al., 2003), caspase-2 knockout mice are phenotypically normal (Bergeron et al., 1998). A more careful evaluation of caspase-2 deficient mice unravels some phenotypic minor

characteristics: excessive oocyte accumulation, reduced number of facial motor neurons in neonatal stages (Bergeron et al., 1998), and 9% decrease in maximum lifespan (Zhang et al., 2007). Accumulation of oocytes has been attributed to decreased cell death during development. Interestingly, p63 has been shown to mediate DNA-damaged induced oocyte cell death (Suh et al., 2006), and p63 activity can be regulated by caspase-2 (Jeon et al., 2012).

Our work unraveled a new role of caspase-2 in metabolic disease. Although caspase-2 function seems to be dispensable under normal conditions, probably through compensatory action of other proteins, it may be important when adapting to specific stressors, namely metabolic ones, as will be discussed in this chapter.

Rational and aims for the experimental study

Western societies exist in an era of caloric excess that is at odds with evolutionary adaptations to the ancestral low-calorie lifestyle. As Western economics, culture and habits have spread, obesity prevalence has nearly doubled worldwide. In 2014, almost two billion adults were overweight and more than half a billion were obese. It is a fact that obesity kills more than 2.8 million adults per year (C., 2015). The reality in Portugal is not different from the rest of the world, with roughly two thirds of the population being at least overweight and one-fourth obese (C., 2015).

The metabolic syndrome is a cluster of features including abdominal obesity, hypertriglyceridemia, low HDL-cholesterol levels, hypertension, and glucose intolerance/ IR/T2DM (Grundy et al., 2004). Obesity increases the risk for all these aspects of the metabolic syndrome, as well as the risk for cardiovascular disease and multiple cancers. Ectopic fat accumulation in the liver, dubbed NAFLD, is also strongly associated with the metabolic syndrome, having been proposed that NAFLD should be considered a component and a diagnostic criterion for the metabolic syndrome (Loria et al., 2005; Machado and Cortez-Pinto, 2014).

Adipocytes respond maladaptively to chronic energy surplus, resulting in adipocyte hypertrophy (Strissel et al., 2007). Fat-swollen adipocytes become resistant to insulin, decrease expression of adipokines such as the anti-inflammatory insulin sensitizer adiponectin, and increase expression of the satiety factor leptin (Kloting and Bluher,

2014). Stressed adipocytes undergo cell death by apoptosis and paraptosis (Cinti et al., 2005), which has been described in mice with genetic or diet-induced obesity, as well as in humans with morbid obesity (Cinti et al., 2005; Strissel et al., 2007). Adipocyte cell size and cell death correlate with the presence of the metabolic syndrome, IR and NAFLD (Cancello et al., 2005; Kloting and Bluher, 2014; Strissel et al., 2007).

In concordance with the strong association with the MS and the global obesity pandemic, NAFLD is now the number one cause of chronic liver disease in the western world, with more than one third of the population being affected (Bellentani et al., 2010). Although usually benign, in 20-30% of cases, fatty liver may result in serious injury with inflammation and hepatocyte necro-apoptosis, dubbed NASH. NASH may eventuate in progressive fibrosis and cirrhosis (Angulo et al., 1999). Correlative histopathological studies of human liver biopsies, as well as research in cell and animal models (Ribeiro et al. 2004; Machado and Cortez-Pinto, 2011), provide strong evidence of increased hepatocyte apoptosis in NASH. Indeed, apoptosis is believed to be crucial in the pathogenesis of NASH-related cirrhosis. Special attention has been given to effector caspases-3 and -7 (Feldstein et al., 2003; Thapaliya et al., 2014), and more recently, to initiator caspases-8 and -9 (Bechmann et al., 2010; Hatting et al., 2013; Kakisaka et al., 2012; Gautheron et al., 2014). A prevailing concept is that injured hepatocytes behave as undead cells, initiating the apoptotic process but failing to complete it, thereby providing a sustained source of apoptosis-associated molecular signals, including hedgehog, that drive liver inflammation and fibrosis (Hirsova and Gores, 2015; Jung et al., 2010; Kakisaka et al., 2012; Ryoo et al., 2004). Importantly, liver fibrosis is the main factor that dictates liver prognosis (Angulo et al., 2015; Ekstedt et al., 2015). NASH-related cirrhosis is already the second liver disease among adults awaiting liver transplantation in the US (Wong et al., 2015).

The initiator caspase for cellular apoptosis caspase-2 has been implicated in several other vital processes including regulation of cell cycle checkpoints, the oxidative stress response, autophagy, and senescence (Gitenay et al., 2014; Olsson et al., 2015; Tiwari et al., 2014; Wilson et al., 2015; Gitenay et al. 2014; Tiwari et al. 2014; Olsson et al. 2015, Wilson et al. 2015). More recently, caspase-2 was linked to lipoapoptosis, a cell death pathway triggered by excess intracellular accumulation of lipids (Johnson et al., 2013). Extracts prepared from *Xenopus laevis* eggs accumulate long chain FA upon prolonged incubation at room temperature, and undergo the molecular events of apoptosis following caspase-2 activation. Blocking accumulation of long chain FA blunted the caspase-2

response (Johnson et al., 2013). Other lines of evidence also link caspase-2 activation with lipid metabolism. Addition of palmitate to hepatocytes in culture induced apoptotic cell death that was significantly dampened by siRNA-mediated knockdown of caspase-2 (Johnson et al., 2013). These reports raised the intriguing possibility that caspase-2 might play a role in diseases in which lipoapoptosis/lipotoxicity has been implicated in the pathogenic mechanism. A simplified way to understand NAFLD/NASH pathogenesis is to consider adipocyte lipotoxicity as the hallmark of NAFLD and hepatocyte lipotoxicity as the hallmark of NASH. We hypothesized that caspase-2 may be a key link between energy surplus, adipocyte death, and the development of the metabolic syndrome and NAFLD, as well as a link between hepatocyte lipotoxicity and NASH progression. Supporting this hypothesis, rats fed HFD demonstrated increased caspase-2 expression in adipose tissue (Jobgen et al., 2009). Previous work from our group, using immunoblotting of liver samples from morbidly obese NAFLD patients showed increased cleaved caspase-2, though it was not clear if this was a cause or consequence of cell death (Ferreira et al., 2011).

Aims:

1. To evaluate the role of caspase-2 in the development of the metabolic syndrome and its liver manifestation, NAFLD.
2. To evaluate the role of caspase-2 in the progression of NASH.

Contribution of the PhD candidate:

Professor Sally Kornbluth proposed to Professor Anna Mae Diehl to study the role of caspase-2 in liver disease, and the latter immediately linked it to NAFLD. Professor Anna Mae Diehl invited the candidate to lead that project. Under close orientation and guidance by Prof. Anna Mae Diehl, the candidate planned all the experiments, executed them and interpreted them. The candidate also wrote the published article as well as the article still in submission (which were edited by Prof. Anna Mae Diehl) that presented the data described in this chapter.

MATERIAL AND METHODS

Human Samples

Liver biopsies from liver transplant donors (n=4) and adult patients with simple steatosis (n=4), NASH with no or mild fibrosis (n=5), and NASH-cirrhosis (n=5) were randomly selected from Duke University Health System NAFLD Clinical Database and Biorepository. To validate findings in NASH, biopsies from a second independent cohort of 23 NASH patients from Duke University and Liver-Gastroenterology Department, University Hospital, Angers, France were also examined. Patients with histological NASH had no significant alcohol consumption or other chronic liver disease. Studies were conducted in accordance with National Institutes of Health and institutional guidelines for human subject research.

Animal Studies

Male obese, diabetic mice (ob/ob and db/db mice), and wild-type (WT) mice C57Bl/6 were obtained from Jackson Laboratory (Bar Harbor, ME). At least 5 animals per group were fed methionine choline-deficient (MCD) diets (MP Biomedicals, Solon, OH) for 8 weeks. WT mice were also fed MCD diet for 4 weeks. Equal numbers of mice were fed standard rodent food (4.7 kcal/g: 13% calories as fat, 62% carbohydrates and 24% proteins).

In separate experiments male caspase-2 deficient (B6.129SY-casp2$^{tm1Yuan/J}$) and congenic WT C57Bl/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and fed two NASH-inducing diets. In one experiment 5 animals per group were fed high fat (HF)/MCD diets or the same HF diet supplemented with methionine and choline (MP Biomedicals, Solon, OH) for 8 weeks. The HF diets provided 4.5 kcal/g: 20% calories as fat, 58% carbohydrates and 22% proteins. In the other experiment, mice were fed either chow diet (Picolab® Rodent diet 20, #5053; n=4 mice per genotype) or Western diet (TD.120330 22% HVO + 0.2% cholesterol diet, Teklad Research, supplemented with fructose and glucose in the drinking water; n=8 mice per genotype) for 16 weeks, beginning at 4 weeks of age. Diet specifications are in **Supplemental Tables 1 and 2**.

During the last week of the latter diet experiment, glucose and insulin tolerance tests were performed. Glucose tolerance test was performed after 12 hours of fasting, by IP injection of glucose (2g/kg). Glucose levels were measured at 0, 15, 30, 60, 90, 120 and 180 minutes, by tail vein sampling using a glucometer. Insulin tolerance test was performed

after 5 hours of fasting, with IP injection of 0.6 units/kg of human regular insulin at a concentration of 0.2 units/mL. Glucose levels were measured by tail vein sampling at 0, 15, 30, 45, and 60 minutes.

At the end of treatment, mice were fasted for 8 hours and sacrificed.

Animal care and procedures were approved by the Duke University Institutional Animal Care and fulfilled National Institutes for Health and Duke University IACUC requirements for humane animal care.

Histopathological, serum and tissue analysis

Formalin-fixed, paraffin-embedded liver, pancreas and abdominal (epididymal) adipose tissue biopsies were cut into 5 μm serial sections. H&E and Oil red staining were performed.

NAFLD severity was assessed with hematoxylin and eosin staining using criteria described by Brunt *et al*. (Kleiner et al., 2005).

For immunohistochemistry, sections were deparafinized with xylene and rehydrated. Slides were incubated in 3% hydrogen peroxide/methanol. Antigen retrieval was performed by heating in 10 mM sodium citrate (pH 6.0). Sections were incubated overnight with caspase-2 antibody. Horseradish peroxidase-conjugated IgG secondary antibody was used. Antigens were demonstrated by diaminobenzidine (K3466; Dako Envision, Carpinteria, CA). Omitting primary antibodies from reactions eliminated staining, demonstrating specificity. Tissue sections were counterstained with Aqua Hematoxylin-INNOVEX (Innovex Biosciences). Morphometric analysis was done with Metamorph Software (Molecular Devices Corporation, Downingtown, PA). Immunohistochemistry antibodies are specified in **Supplemental Table 3**.

Liver fibrosis was assessed by Picrosirius red (Sigma, ST. Louis, MO) staining. Quantification was done by morphometric analysis in randomly chosen sections (x10 magnification, 20 fields/sample) (Choi et al., 2006).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Boehringer Mannheim, Germany) was performed according to the manufacturer's suggestions.

Serum and tissue analysis

Blood was collected when sacrificing the animals and serum was obtained. A biochemical panel was outsourced to Veterinary Diagnostic Laboratory, Division of Laboratory Animal Resources, Duke University Medical Center.

Insulin was measured with Ultrasensitive Mouse Insulin ELISA kit (Crystal Chem Inc: #90080). Lipids were measured with Triglyceride Colorimetric Assay kit (Cayman Chemical Company: #10010303), Free Fatty Acid Quantification Kit, (Abcam, ab65341) and Cholesterol Quantification kit (Abcam, ab65359); serum leptin and adiponectin were determined with Abcam mouse ELISA kits, ab100718 and ab108785, respectively. Shh was measured by ELISA (Sigma: RAB0431).

Molecular Studies

mRNA quantification by Real-time Reverse Transcription-PCR (RT-PCR). Total RNA was extracted from tissue using TRIzol (Invitrogen). RNA was reverse transcribed into cDNA templates using random primer and Super Script RNAse H-Reverse Transcriptase (Invitrogen). Semiquantitative qRT-PCR was performed using iQ-SYBR Green Supermix (Bio-Rad) on a StepOne Plus Real-Time PCR Platform (ABI/Life Technologies), as previously described (Michelotti et al., 2013). For primers, see **Supplemental Table 4**.

Western Blotting. Total proteins were extracted from liver and abdominal adipose tissue using RIPA buffer (Sigma) supplemented with phosphatase and protease inhibitors (Roche). Equal amounts of protein were separated by electrophoresis on 4%-20% Criterion gels (BioRad), transferred onto polyvinylidene difluoride membranes, and incubated with the primary antibodies specified in **Supplemental Table 5**.

Cell isolation and culture

Mouse adipose tissue stem cells, hepatocytes and HSC were isolated from caspase-2 deficient and WT mice as previously described (Fink and Zachar, 2011; Michelotti et al., 2013; Rangwala et al., 2011). Kupffer cells (Pereira et al., 2013), and sinusoidal endothelial cells (Xie et al., 2013) were also isolated from WT mice. Hepatocytes were seeded onto plastic culture dishes (Sigma, St. Louis, MO) in Dulbelcco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), ITS (1:200; Invitrogen), dexamethasone (1 mM; Sigma, ST. Louis, MO) and antibiotics (Life Technologies). Cells were treated with similar media but with 2% FBS and 2% bovine serum albumin (Sigma) with or without palmitate (1 mM, Sigma)

based on dose and time-curves of apoptosis in WT hepatocytes that defined the optimal dose and duration of palmitate treatment as shown in **Figure 26**.

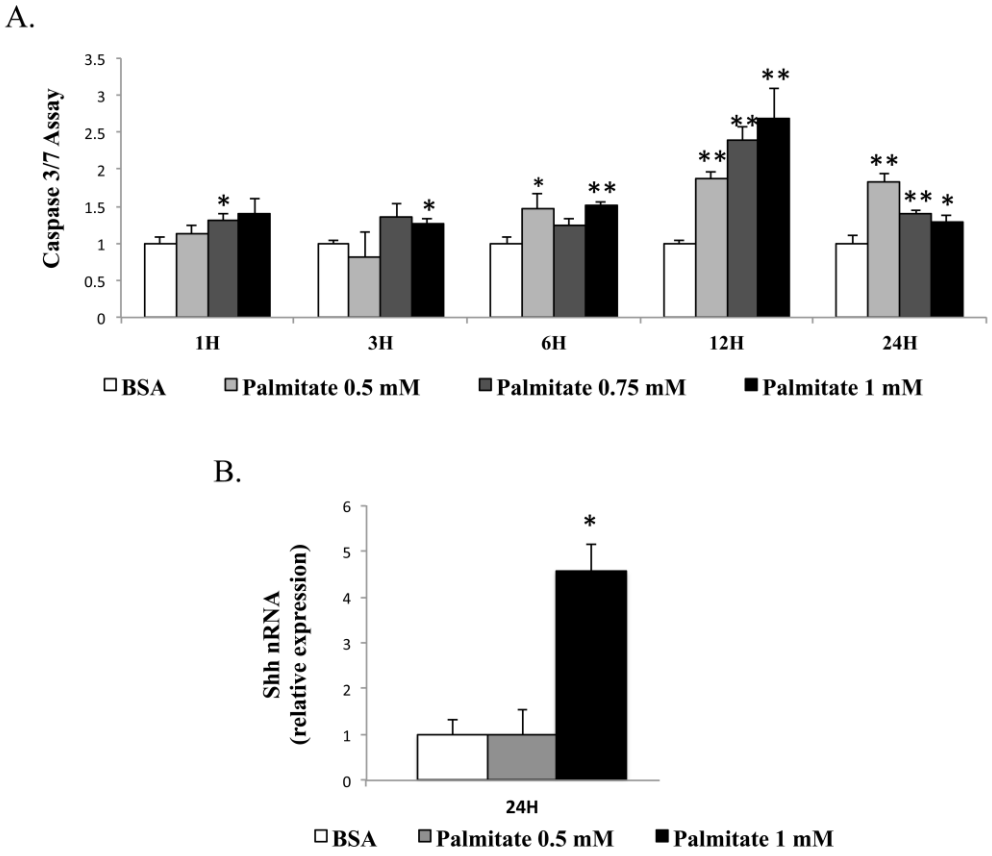


Figure 26: Time and dose-curve for treatment of primary hepatocytes with palmitate – evaluation of apoptosis and production of Sonic hedgehog (Shh) ligand.
A. Primary hepatocytes isolated from WT mice were treated with progressively increasing doses of palmitate (0.5 mM, 0.75 mM and 1 mM) or bovine serum albumin (BSA, 2%), for 1, 3, 6, 12 and 24 hours. Apoptosis was assessed by Caspase-3/7 assay. Mean±SEM data from triplicate cultures are graphed. B. qRT-PCR analysis of Shh mRNA in similar treated hepatocytes (two doses of palmitate, 0.5 mM and 1 mM). Mean±SEM data from triplicate cultures are graphed. *p<0.05 and **p<0.01, BSA *vs*. palmitate.

3T3-L1 cells were differentiated into adipocytes as previously described (Zebisch et al., 2012), and differentiation confirmed with Oil red staining. Differentiated adipocytes were treated with 1 mM palmitate with or without 20 uM caspase-2 inhibitor Z-VDVAD-FMK (R&D Systems), for 48 hours. Mouse cholangiocyte 603B cell line and rat 8B myofibroblastic hepatic stellate cell line were cultured in DMEM with 10% fetal bovine serum. Rat 8B cells were treated with 1 mM palmitate or conditioned media from

hepatocytes treated with 1 mM palmitate, for 24 hours.

Cell viability was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies), apoptosis using ApoTox-Glo Triplex Assay (Promega) and proliferation using BrdU assay (Cell Signaling).

Statistics

Results are expressed as mean±SEM. Significance was established using student t-test and two-way ANOVA tests, with $p<0.05$ considered significant. Correlations were evaluated with non-parametric Spearman test.

RESULTS

The role of caspase-2 in the development of the metabolic syndrome

Caspase-2 deficient mice were protected from diet-induced obesity

Caspase-2 deficient mice and WT controls were fed standard chow diet or high fat, high sugar Western diet for 16 weeks. At the end of treatment, WT mice fed Western diet weighed 10% more than WT mice on chow diet. However, Western diet induced minimal additional weight gain in caspase-2 deficient mice (**Figure 27 and 28A**).

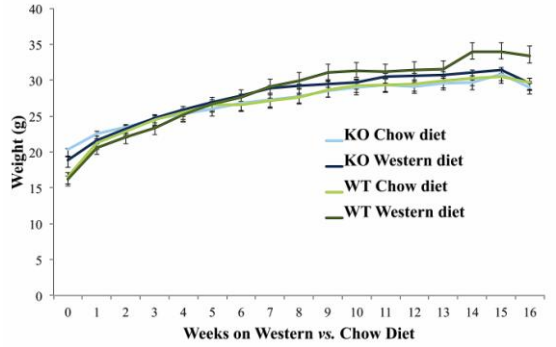


Figure 27. Caspase-2 deficient mice are protected from weight gain with Western diet.

Body weight curve in caspase-2 deficient mice and WT mice fed chow diet or Western diet.

As expected, Western diet doubled the amount of body fat, assessed by DXA, compared to chow-diet fed animals, and tripled the amount of abdominal adipose tissue. Western diet-induced adiposity was strikingly blunted in caspase-2 deficient mice (**Figure 28B**). This is noteworthy since, more than obesity itself, the distribution of body fat (abdominal adipose tissue *versus* subcutaneous) is known to be deleterious (Wajchenberg et al., 2002).

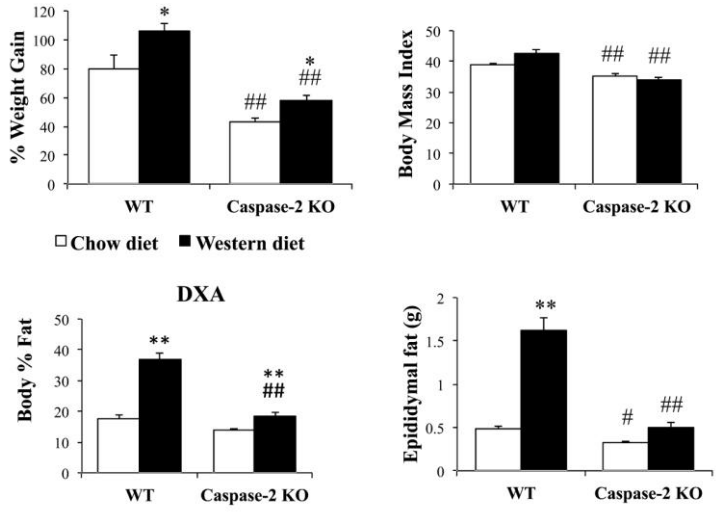


Figure 28. Caspase-2 deficient mice are protected from diet-induced obesity

A. Right panel: Percentage increase in weight in caspase-2 KO and WT mice fed chow *vs*. Western diet. Left panel: Body mass index. B. Body mass % of fat and epididymal fat weight. Results graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Differences in adiposity could not be explained by decreased food intake, since caspase-2 deficient mice ate 8% more solid food and drank 40% more sugared water than WT mice. In fact, WT mice had higher food efficiency, gaining more weight per gram of food ingested (**Figure 29**).

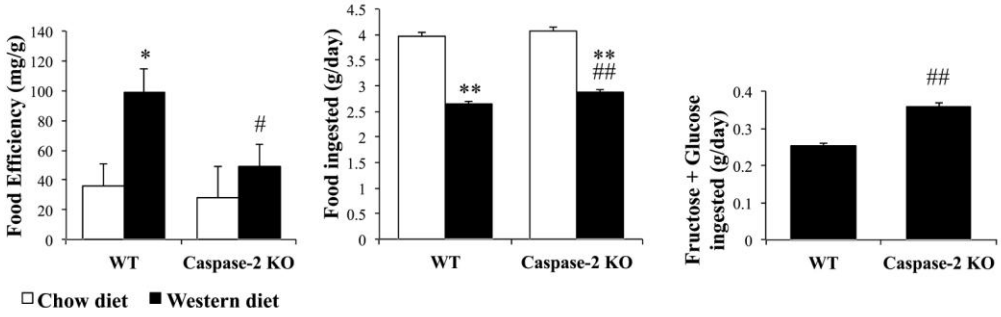


Figure 29. Caspase-2 deficient mice are protected from diet-induced obesity

Left panel: Food efficiency, in caspase-2 KO and WT mice fed chow *vs*. Western diet, analyzed from week 2 to week 15 of treatment. Right panels: food and high-corn syrup equivalent ingested during treatment. Results graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Caspase-2 deficient mice were protected from diet-induced impairments in glucose metabolism

Western diet resulted in glucose intolerance in WT mice, as evidenced by higher fasting glucose level, higher peak glucose after challenge, and delayed return to baseline. Also, WT mice on Western diet had a blunted response to insulin, indicative of IR. However, Western diet-fed caspase-2 deficient mice were normal in all these measures (**Figure 30A**). WT mice fed the Western diet developed overt T2DM, with fasting glucose levels of 200 mg/dL and HOMA-IR higher than 15, whereas caspase-2 deficient mice maintained normal glucose level (**Figure 30B**). As expected in a mouse model of IR (Yi et al., 2013), Western diet induced hyperplasia of pancreatic Langerhans islands in WT mice, but this expansion was significantly blunted in caspase-2 deficient mice (**Figure 30C and D**). T2DM is described as a combination of peripheral IR that surpasses the ability of the pancreas to increase insulin production, resulting in overwhelming stress to insulin-producing β-cells and poor insulin responsiveness (Kasuga, 2006). After 16 weeks on Western diet, WT exhibited extreme hyperplasia of Langerhans islands, but had fasting hyperglycemia and impaired late-response to glucose challenge, suggesting

insufficient insulin secretion and T2DM; but these manifestations of glucose and insulin dysfunction were largely absent in the caspase-2 deficient mice.

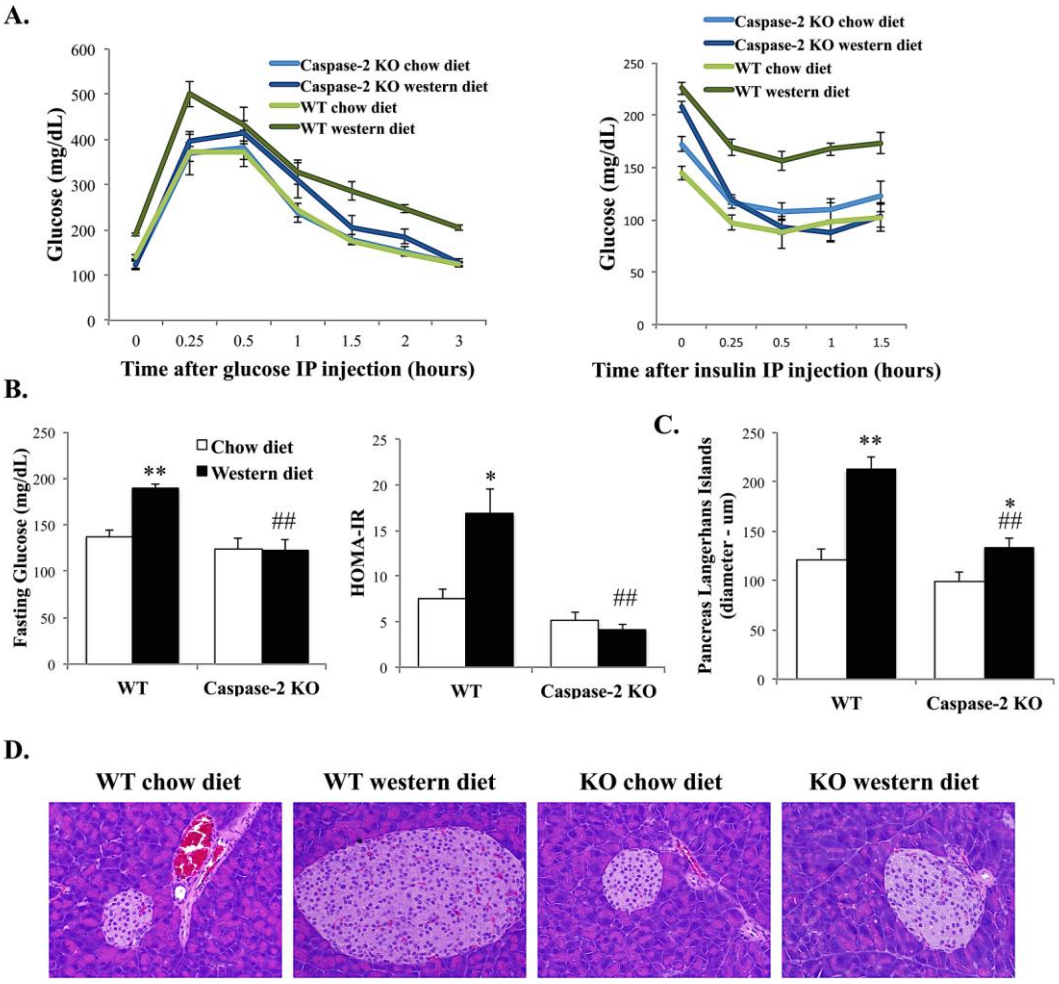


Figure 30. Caspase-2 deficient mice are protected from diet-induced diabetes mellitus

A. Right panel: Glucose tolerance test after 15 weeks on diet. AUROC Western diet WT *vs*. KO mice: 190 *vs*. 122, p=0.02. Left panel: Insulin tolerance test at 15 weeks on diet. AUROC 167 *vs*. 118, p=0.0002. B. Fasting glucose and HOMA at 15 weeks. C. Size of pancreatic Langherhans islands. D. Representative H&E sections (20x field) from pancreas. Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Caspase-2 deficient mice were protected from diet-induced dyslipidemia

In WT mice, Western diet induced mixed dyslipidemia with elevated total cholesterol, triglycerides and NEFA's. Caspase-2 deficient mice were protected from dyslipidemia, for all the lipids assessed (**Figure 31**). The failure of caspase-2 mice to increase circulating NEFA's with Western diet may be extremely relevant in the prevention of T2DM, since increased circulating NEFA's are known to decrease glucose transport into

muscle cells, and increase both liver gluconeogenesis and pancreatic β-cell dysfunction (Al-Goblan et al., 2014).

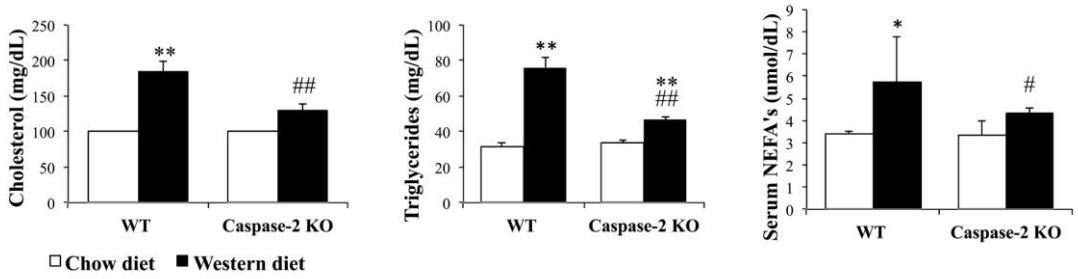


Figure 31. Caspase-2 deficient mice are protected from diet-induced dyslipidemia

Serum levels of total cholesterol, triglycerides and non-esterified fatty acids in WT and caspase-2 deficient mice fed chow diet *vs*. Western diet. Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Caspase-2 deficient mice reprogram adipose tissue to cope with energy surplus

Western diet perturbed adipose tissue homeostasis by deregulating adipokines, increasing expression of leptin and reducing adiponectin, in WT mice. This deregulation did not occur in caspase-2 deficient mice (**Figure 32**).

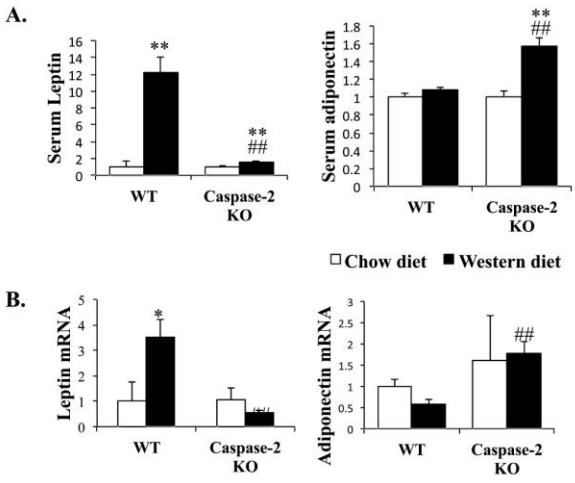


Figure 32. Caspase-2 deficient mice are protected from altered adipokine deregulation induced by Western diet.

Leptin and adiponectin serum levels (A) and qRT-PCR analysis in abdominal adipose tissue (B), in WT and caspase-2 deficient mice fed chow diet *vs*. Western diet. Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Even in mice fed chow diet, the abdominal adipose tissue from caspase-2-deficient mice was fundamentally different from that of WT mice, with an increased number of small adipocytes. On Western diet, adipocytes in WT mice markedly increased their size, but remained small in caspase-2 deficient mice (**Figure 33**). The difference in adipocytes size is highly relevant, since it has been shown that there is an adipocyte size threshold after which the risk of T2DM increases exponentially (Kloting and Bluher, 2014).

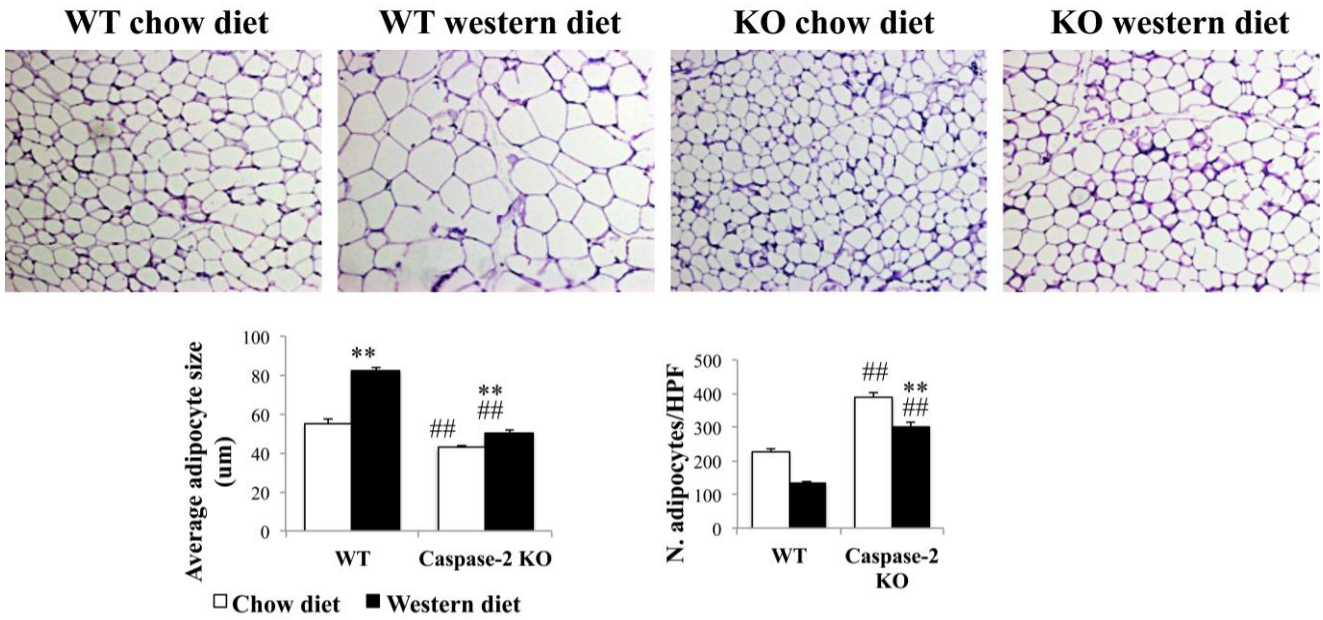


Figure 33. Adipose tissue is fundamentally altered in caspase-2 deficient mice

Upper panels: Representative photos from H&E staining in adipose tissue sections. Lower panels: average size of adipocytes and number of adipocytes per 10x field. Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Consistent with the increased number of smaller cells in caspase-2 mice, these cells also had increased expression of cyclins, at both mRNA and protein level, suggesting increased proliferative activity in the adipose cells of caspase-2 deficient mice (**Figure 34A**). Isolated adipose stem cells from caspase-2 deficient mice showed increased proliferation compared to WT cells (**Figure 34B**).

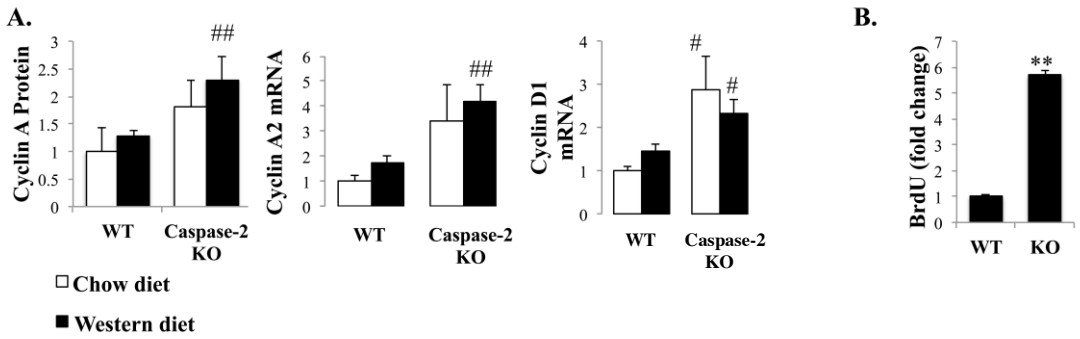


Figure 34. Adipose tissue in caspase-2 deficient mice is more proliferative

A. Cyclin expression (immunoblot and qRT-PCR) in WT and caspase-2 KO mice submitted to chow *vs*. Western diet. B. Proliferation (BrdU) in adipose tissue mesenchymal stem cells isolated from WT or caspase-2 KO mice. Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Caspase-2 deficient mice cope better with energy surplus by decreasing the efficiency of energy obtained by burning fat, through increased expression of mitochondrial uncoupling proteins (UCPs), suggesting browning of the adipose tissue (**Figure 35**). UCPs decrease the proton gradient across the mitochondrial inner membrane, decreasing the generation of ATP from burning glucose and FA. Browning of white adipose tissue, that is, genetic or pharmacological increase in UCP function, is protective against the development of obesity-related T2DM (Tao et al., 2014).

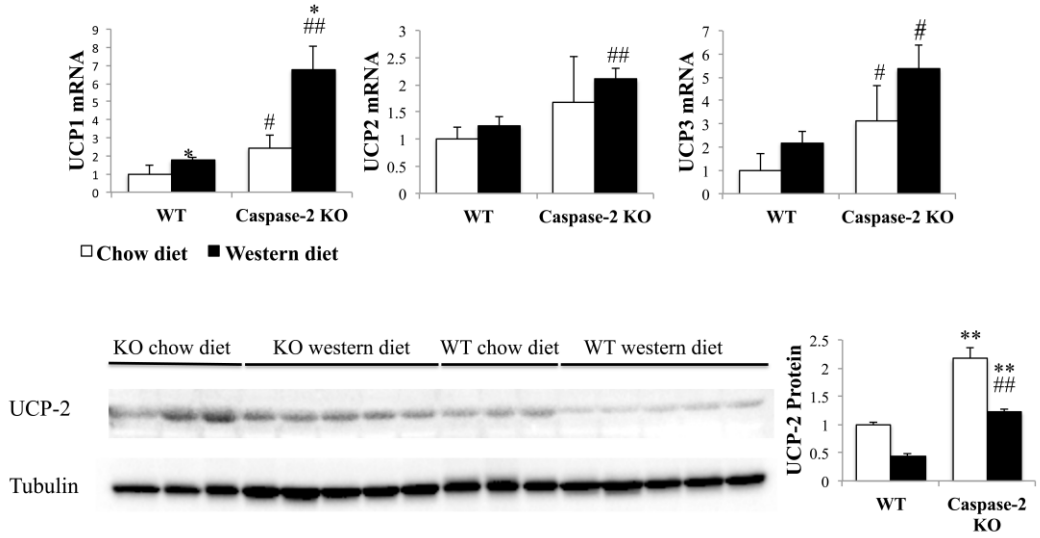


Figure 35. Evidence of browning in abdominal adipose tissue from caspase-2 deficient mice
Expression, evaluated by qRT-PCR (upper panels) and immunoblot (lower panels), of uncoupling protein expression (UCP) in abdominal adipose tissue from WT and caspase-2 KO mice submitted to chow *vs*. Western diet. Results are graphed as mean±SEM and normalized for WT chow diet. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

WT mice fed the Western diet had elevated levels of active, cleaved caspase-3 in abdominal fat, indicating that adipocyte hypertrophy is accompanied by increased cell death by apoptosis. However, there was no evidence of elevated apoptosis in abdominal adipose tissue from caspase-2-deficient mice (**Figure 36**).

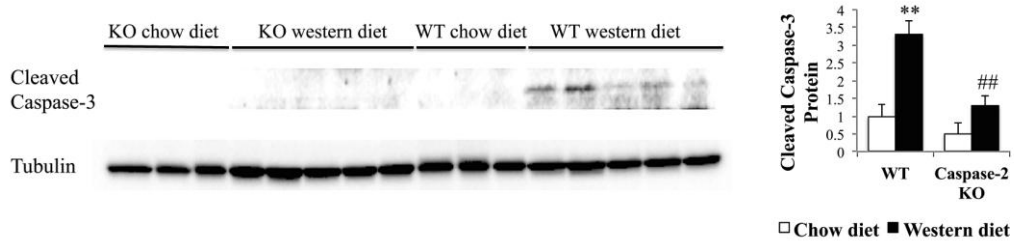


Figure 36. Caspase-2 deficient mice are protected from adipose tissue apoptosis

Cleaved caspase-3 expression in adipose tissue (immunoblot) from WT and caspase-2 KO mice submitted to chow *vs*. Western diet. Results are graphed as mean±SEM and normalized for WT chow diet. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Indeed, palmitate-induced lipoapoptosis in adipocytes in cell culture was prevented by simultaneous treatment with the caspase-2 inhibitor Z-VDVAD-FMK (**Figure 37**).

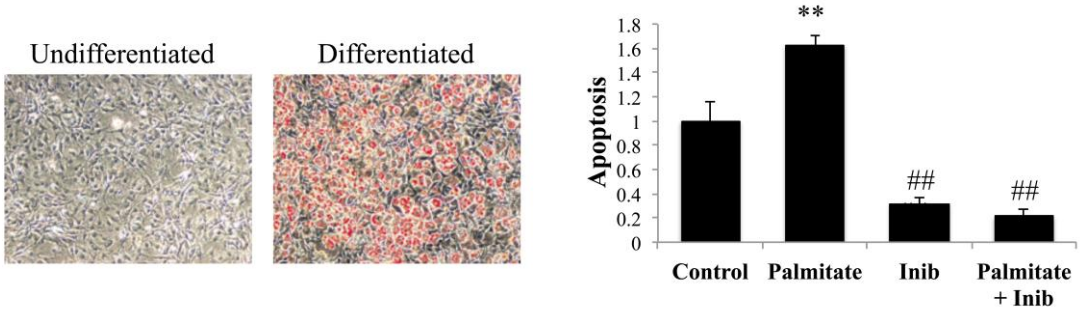
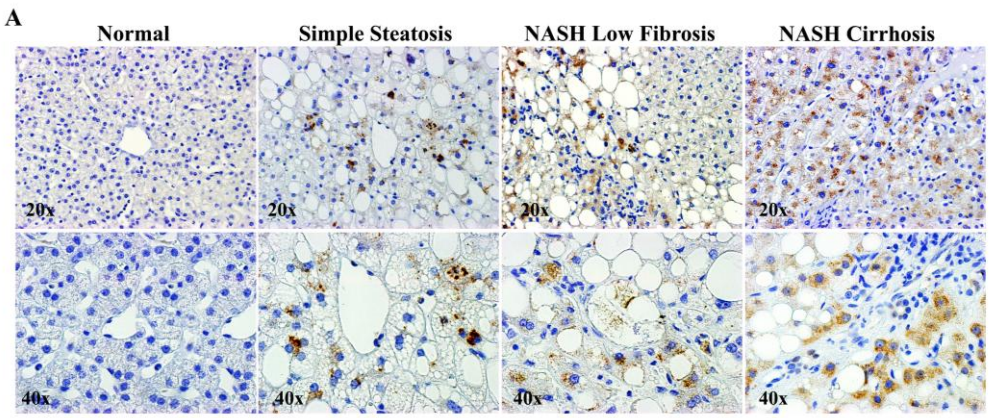


Figure 37. Inhibition of caspase-2 prevents adipocyte lipoapoptosis, *in vitro*.
Right panel: oil red staining demonstrating 3T3-L1 cells differentiation into adipocytes. Left panel: apoptosis (caspase 3/7 activity) in adipocytes treated with palmitate (1 mM) ± caspase-2 inhibitor (20 uM). Results are graphed as mean±SEM and normalized for control-treated adipocytes. **p<0.01 control *vs*. palmitate-treated cells; ##p<0.01 comparing cells treated with caspase-2 inhibitor *vs*. untreated cells.

The role of caspase-2 in the development of NAFLD

Patients with NASH demonstrate increased expression of caspase-2, with predilection for ballooned hepatocytes

Immunohistochemistry for caspase-2 in liver samples from NAFLD patients showed a dramatic increase in staining, as compared to normal controls **(Figure 38)**.



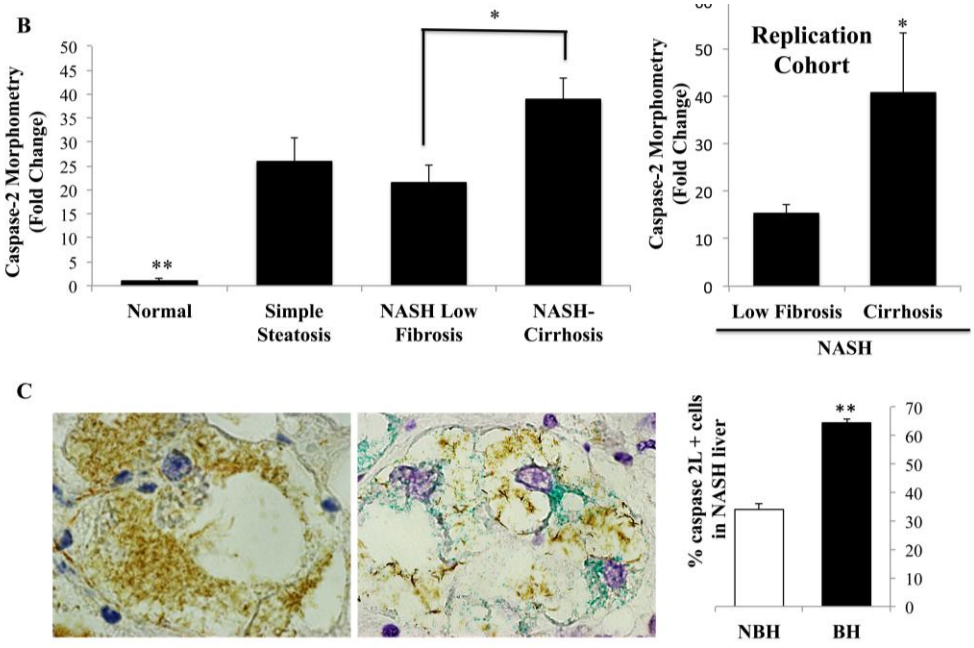


Figure 38. Increased caspase-2 staining in patients with NASH.

A. Caspase-2 immunohistochemistry (brown) in representative human healthy liver (n=4), patients with simple steatosis (n=4), NASH with no or mild fibrosis (n=5) and NASH-associated cirrhosis (n=5). B. Morphometric analysis of caspase-2 staining in the discovery cohort and in a replication cohort constituted by patients with NASH with low fibrosis (n=12) or cirrhosis (n=11). Results are expressed as fold change over data in healthy liver and graphed as mean±SEM. **p<0.01 all groups *vs*. normal liver, *p<0.05. C. Caspase-2 positive cells were counted in non-ballooned hepatocytes (NBH) and ballooned hepatocytes (BH) in 20 randomly selected fields/section in 2 patients with NASH. Mean±SEM are graphed (right panel). Representative photos showing ballooned hepatocytes with immunohistochemistry for caspase-2, left panel, or double immunohistochemistry for Sonic hedgehog (Shh, green) and caspase-2 (brown) in NASH, middle panel.

Patients with NASH-cirrhosis presented even higher expression. These findings were evaluated in a discovery cohort with samples from normal liver (n=4), patients with simple steatosis (n=4) and with NASH without/low fibrosis (n=5) or with NASH-associated cirrhosis (n=5). Immunohistochemistry studies were confirmed in a replication cohort enclosing patients with NAFLD and low fibrosis (n=12) or cirrhosis (n=11). Caspase-2 localized predominantly in hepatocytes and the ratio of positive/negative cells was higher in ballooned compared to non-ballooned hepatocytes. Staining was also more intense near areas of lobular inflammation and near fibrotic septa. In NASH, Sonic hedgehog (Shh) is expressed in lipotoxic hepatocytes, as opposed to healthy ones, and is particularly enriched in ballooned cells (Rangwala et al., 2011). Double

immunohistochemistry for Shh and caspase-2 showed frequent double-positive cells, with strong co-localization of Shh and caspase-2 in ballooned hepatocytes.

Low expression of caspase-2 in the healthy liver is highly induced in mouse models of NASH

To better characterize caspase-2 expression in the healthy liver, we performed immunohistochemistry of liver sections and western blot analysis of whole tissue lysate and observed minimal expression by those methods.

To better understand which liver cell types express caspase-2, we evaluated relative gene expression by qRT-PCR in quiescent cells: primary mouse fresh isolated hepatocytes, liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC), quiescent primary Kupffer cells after 24 hours in culture and a mouse cell line of cholangiocytes, 603B cells. We found that HSC are the cell type that less expresses caspase-2, whereas other cell types express roughly twice as much as hepatocytes (**Figure 39**).

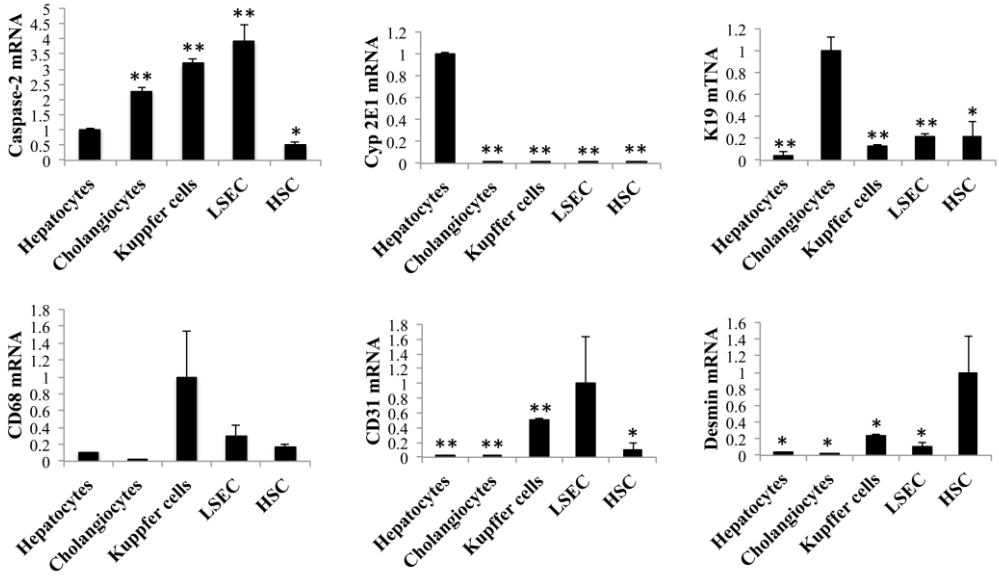


Figure 39. Expression of caspase-2 in the different cell types from the quiescent liver.

qRT-PCR analysis of mouse primary hepatocytes, Kupffer cells, liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC) and a cholangiocyte cell line (603B cells), for caspase-2 and cell-type specific markers. Results were graphed as mean±SEM and normalized to expression in hepatocytes (caspase-2) or the cell for which the marker is specific. *p<0.05 and **p<0.01 *vs*. cell specific for the marker evaluated.

Next we evaluated different animal models of NAFLD/NASH: dietary models, genetic models and mixed dietary-genetic models. We evaluated WT mice submitted to MCD diet for 8 weeks, which induces severe fibrosing NASH but does not associate with the

metabolic syndrome; and Western high-fat, high-sugar diet for 16 weeks, which induces the metabolic syndrome and severe steatosis with mild necroinflammatory activity and fibrosis stage. We found impressive up-regulation of caspase-2 protein expression in whole lysates by immunoblotting and by immunohistochemistry. The latter showed that caspase-2 expression was more pronounced in epithelial cells, namely hepatocytes but also in cholangiocytes. In contrast, up-regulation at the mRNA level was only mild (data not shown), which suggest important post-translational regulation of protein levels. Caspase-2 expression was higher in the dietary model that associated with more severe NASH. We performed a time-course analysis in the MCD dietary model and verified a time-course dependent increase in caspase-2 expression by immunohistochemistry (**Figure 40**).

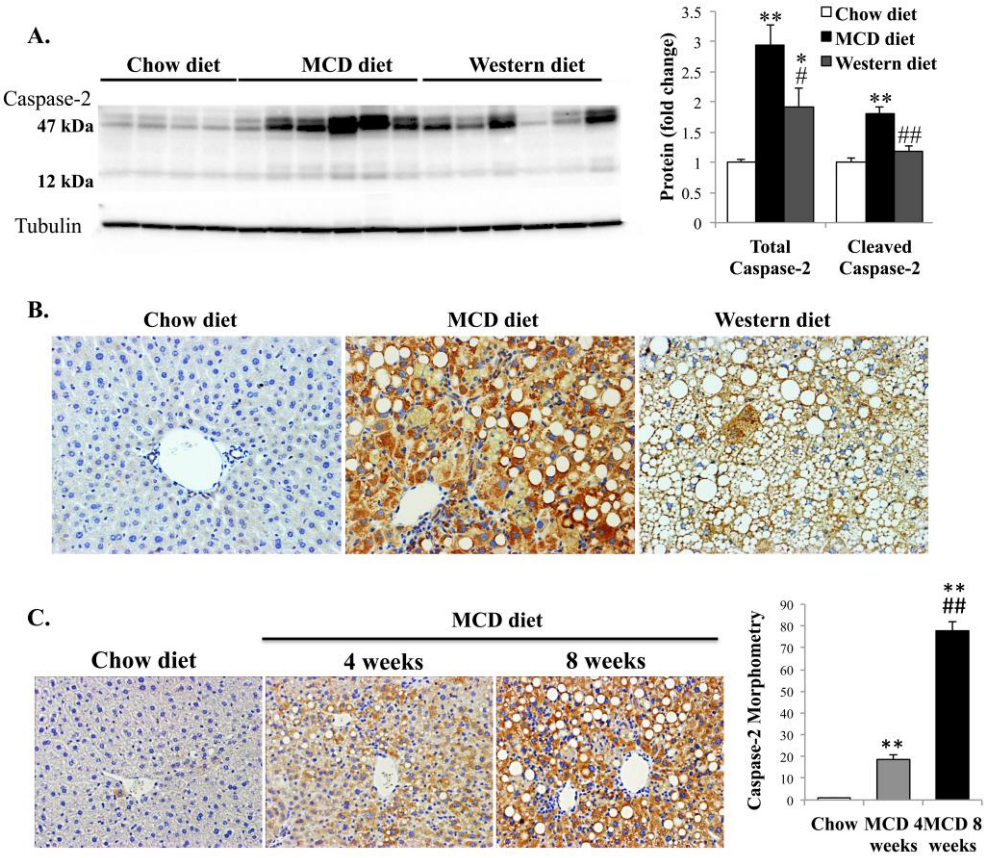


Figure 40. Expression of caspase-2 is increased in dietary mouse models of NASH.

A. Immunoblot for caspase-2 from whole liver extracts from mice fed with either chow diet, MCD diet for 8 weeks or Western diet for 16 weeks. B. Liver sections from representative WT mice the same diet as above, immune-stained for caspase-2. C. Time course of caspase-2 expression assessed by immunohistochemistry, in the livers from mice fed MCD diet, representative liver sections and morphometry. Results were graphed as mean±SEM and normalized to expression in chow-diet fed mice. *p<0.05, **p<0.01 interventional *vs*. chow diet; #p<0.05 and ##p<0.01 between interventional diets.

We further evaluated, by immunohistochemistry, caspase-2 expression in genetically and diabetic mice ob/ob (leptin deficient) and db/db (with a dysfunctional leptin receptor) fed chow diet or MCD diet. All models showed increased caspase-2 expression. Most importantly, we found a very strong correlation (R=0.818, p<0.001) between caspase-2 expression and severity of liver fibrosis assessed by Sirius red (**Figure 41**).

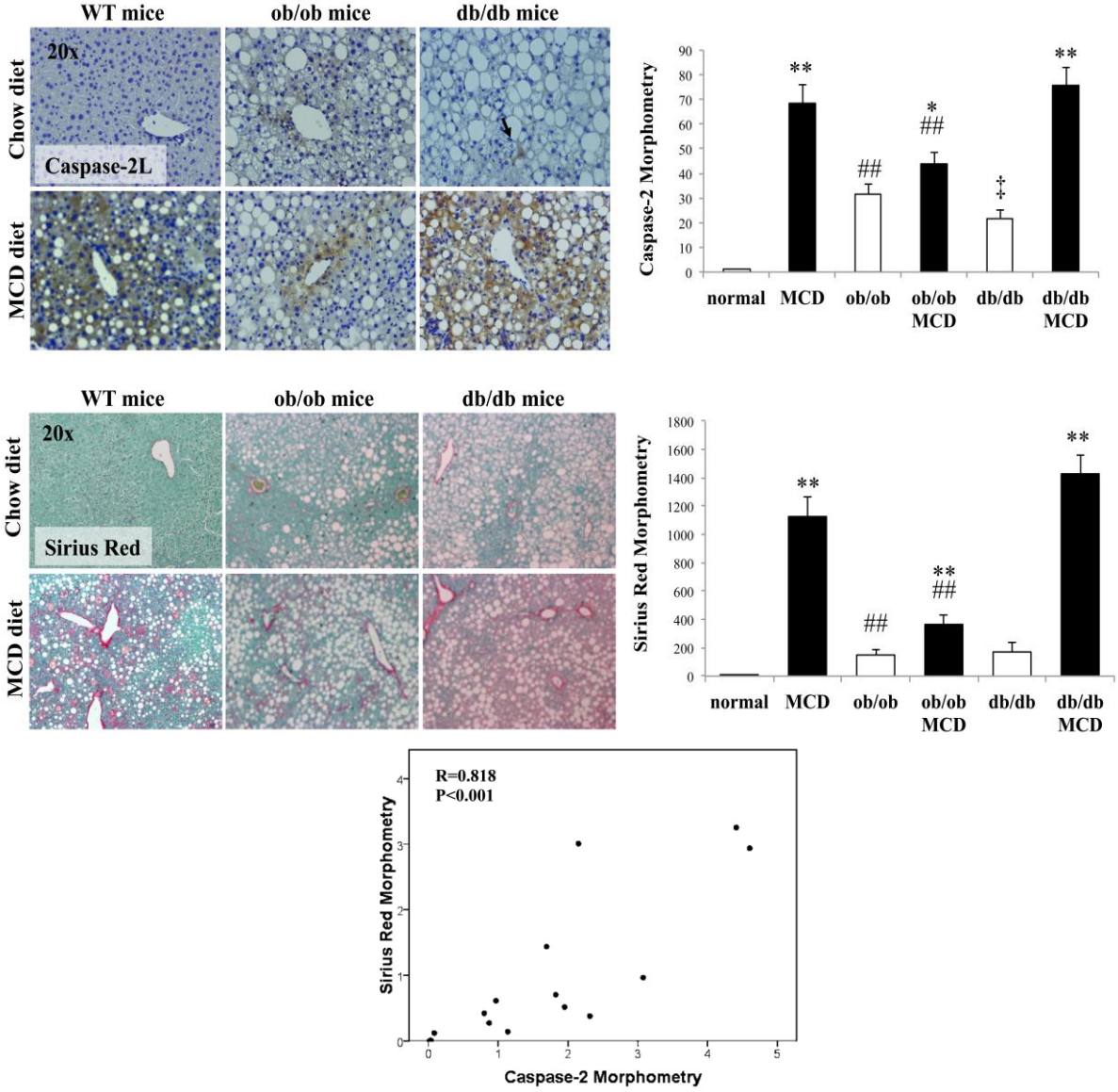


Figure 41. Increased caspase-2 levels in mouse models of NASH correlates with fibrosis.
Upper panel: caspase-2 immunohistochemistry in livers from representative WT mice, ob/ob and db/db mice fed regular chow or MCD diet. Morphometric analysis of caspase-2 staining in all mice (n=5/treatment group). Middle panels: Sirius red from liver section from the same mice and respective morphometric analysis. Results are expressed relative to data in chow-fed WT mice and graphed as mean±SEM.D. Lower panel: Spearman correlation between Sirius red morphometry and caspase-2 immunohistochemistry.

Caspase-2 deficient mice are protected from hepatic steatosis associated with the metabolic syndrome

Accumulation of fat in the liver, or nonalcoholic fatty liver disease (NAFLD), has been proposed as an additional component of the metabolic syndrome (Machado and Cortez-Pinto, 2014). Western diet induced massive hepatomegaly in WT mice, with an almost two-fold increase in liver mass, and 50% increase in liver-to-body weight ratio. However, caspase-2 deficient mice maintained normal liver weight on the Western diet (**Figure 42**). Hepatomegaly in WT mice was due to a 5-6 fold increase in liver fat content, as assessed by DXA (data not shown), oil red O staining and triglyceride measurement, but caspase-2 mice had strikingly lower fat accumulation in the liver (**Figure 42**).

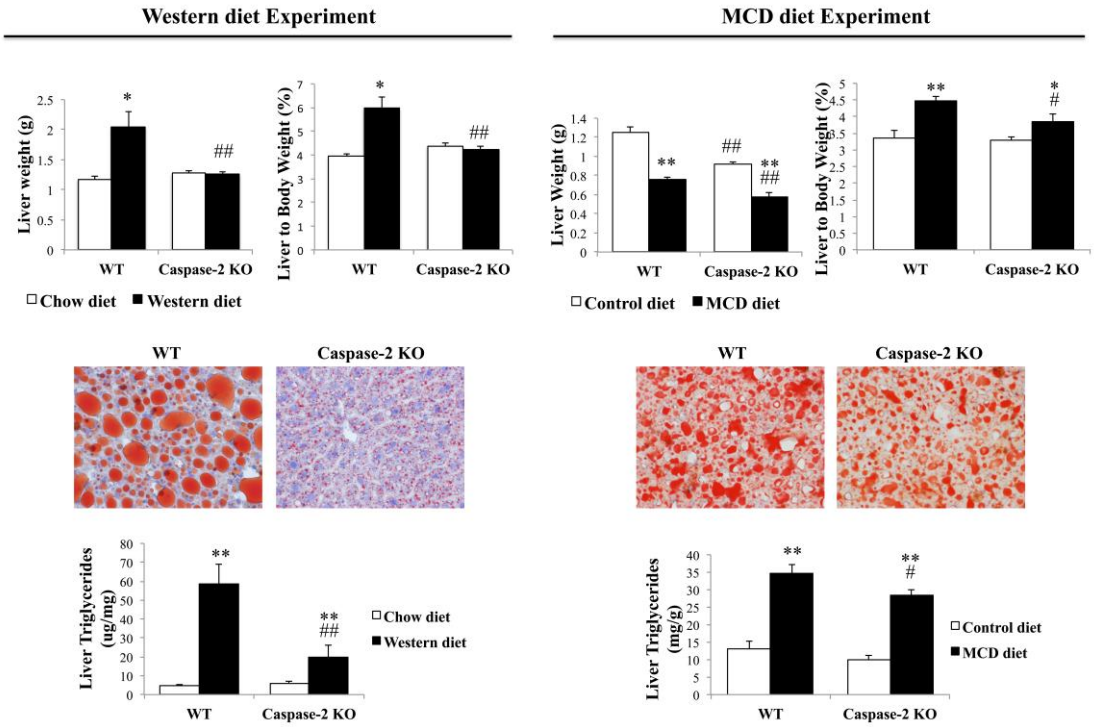


Figure 42. Caspase-2 deficient mice are protected from hepatic steatosis induced by Western diet, but not by MCD diet.

Evaluation of liver steatosis in both dietary models of NASH, Western and MCD diet, in WT and caspase-2 deficient mice. Upper panels: liver weight and liver-to-body weight ratio. Middle panels: representative photos from Oil red staining of liver sections (20x magnification). Lower panel: liver triglycerides content. Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. interventional diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Gene expression analysis of whole liver extracts showed that caspase-2-deficient mice modulate lipid metabolism towards less accumulation and higher consumption and export of fat, compared to WT. As compared to WT, Western diet-fed caspase-2 deficient mice

had lower expression of enzymes of *de novo* lipogenesis, and increased expression of enzymes in fatty acid β-oxidation, esterification and VLDL secretion (**Figure 43**).

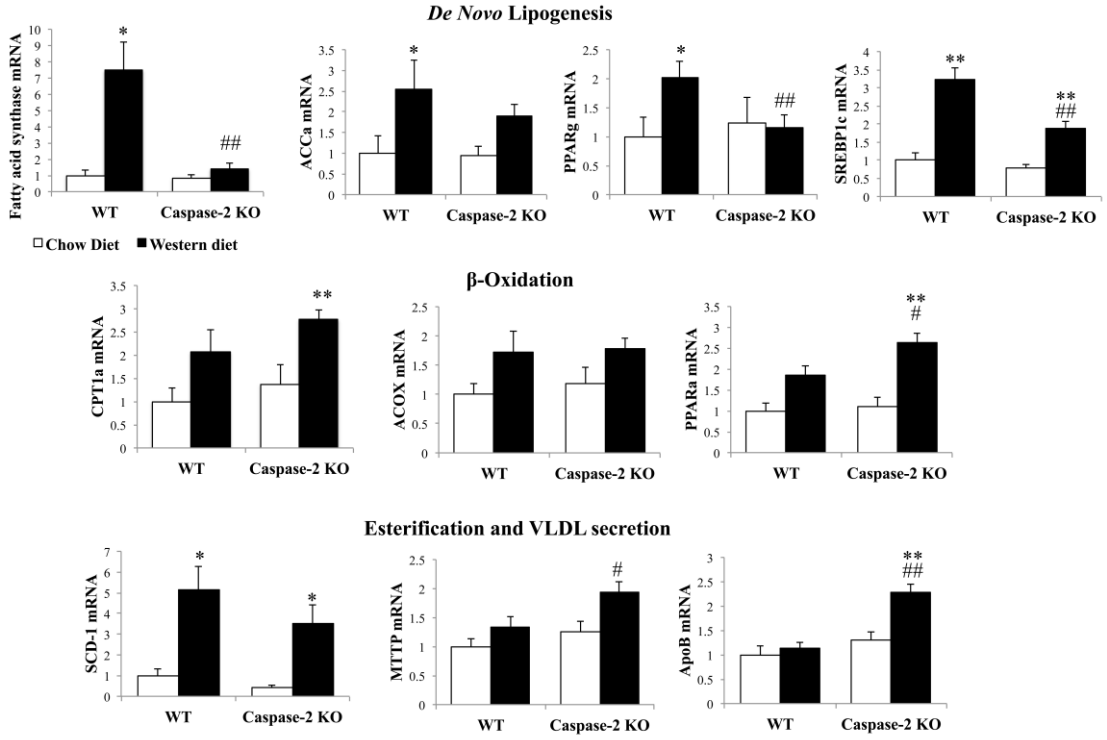


Figure 43. Caspase-2 deficient mice reprogram lipid metabolism, when submitted to energy surplus.

qRT-PCR analysis of liver RNA for genes involved in lipid metabolism, in WT and caspase-2 deficient mice submitted to chow or Western diet. Results are graphed as mean±SEM and normalized to chow-diet fed WT mice. *p<0.05, **p<0.01 chow *vs*. Western diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

In contrast with the results obtained when feeding Western diet, when caspase-2 deficient or WT control mice were fed MCD diet, the former had similar accumulation of lipids, as assessed by triglycerides quantification and Oil red O staining (differences between both genotypes were very mild, albeit statistically significant) (**Figure 42**). Of note, the mechanisms leading to MCD diet associated hepatic steatosis are different from the Western dietary model. MCD diet associates with an impairment in the export of lipids rather then increase import from adipose-tissue derived circulating FA.

The role of caspase-2 in NAFLD-associated liver injury

Caspase-2 deficient mice are protected from diet-induced NASH

Caspase-2 deficient mice were protected from liver injury in both dietary models of NASH, MCD and Western diet, as assessed by H&E staining (**Figure 44**). The protective effect of caspase-2 deficiency was confirmed by analysis of serum alanine and aspartate aminotransferase (ALT and AST) levels. In both models, aminotransferases levels were significantly lower in caspase-2 deficient mice. When WT mice were fed Western diet, the increase in aminotransferases levels was much milder than the increase induced by MCD diet. Interestingly, caspase-2 deficient mice showed normal levels of aminotransferases when fed Western diet (**Figure 44**).

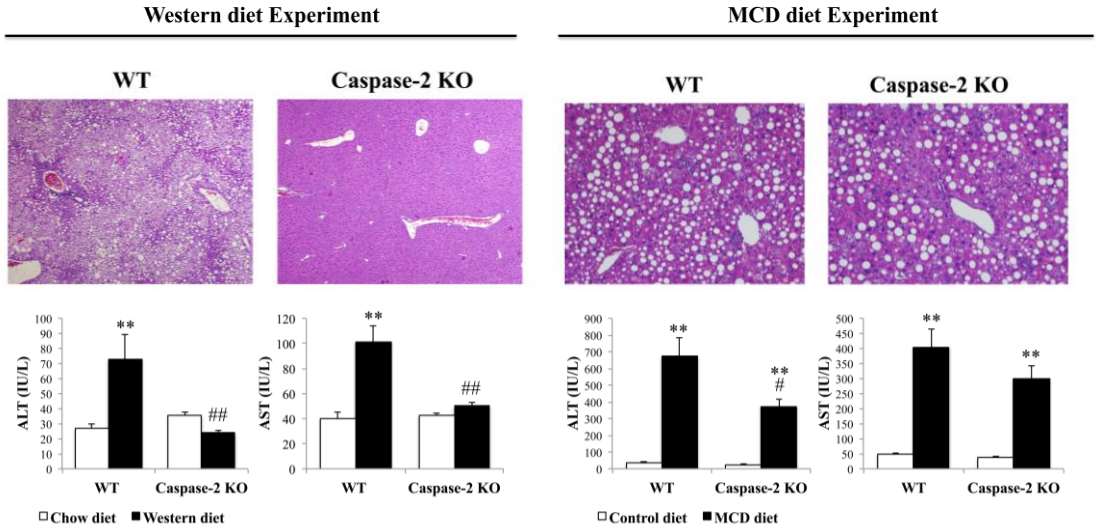


Figure 44. Caspase-2 deficient mice are protected from diet-induced NASH.

Evaluation of liver injury in both dietary models of NASH, Western and MCD diet, in WT and caspase-2 deficient mice. Upper panels: representative photos from H&E staining of liver sections (10x magnification). Lower panel: Serum levels of aminotransferases. Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. interventional diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Caspase-2 deficient mice are protected from hepatocyte lipoapoptosis

Apoptosis can be difficult to assess in liver samples, since acidophilic bodies (indicating apoptosis) can be phagocytized and disappear, resulting in the underestimation of hepatocyte apoptosis quantification (Machado and Cortez-Pinto, 2011). We took advantage of TUNEL assay to evaluate apoptosis, in both models. TUNEL is a method for detecting DNA fragmentation by labeling the terminal end of nuclei acids. Since DNA fragmentation results from apoptotic signaling pathways, TUNEL labels the cells that are

undergoing apoptosis (Negoescu et al., 1998). Caspase-2 deficiency significantly reduced, though it did not completely abolished, apoptosis as assessed by number of positive labeled cells per field, in the TUNEL assay (**Figure 45**). In the MCD diet model, which associates with higher levels of hepatocyte apoptosis as compared to the Western diet model, we also performed immunohistochemistry for activated caspase-3 a key downstream effector caspase in lipoapoptosis (Kakisaka et al., 2012). Livers from caspase -2 deficient mice showed less than half positive cells, compared to WT mice (**Figure 45**).

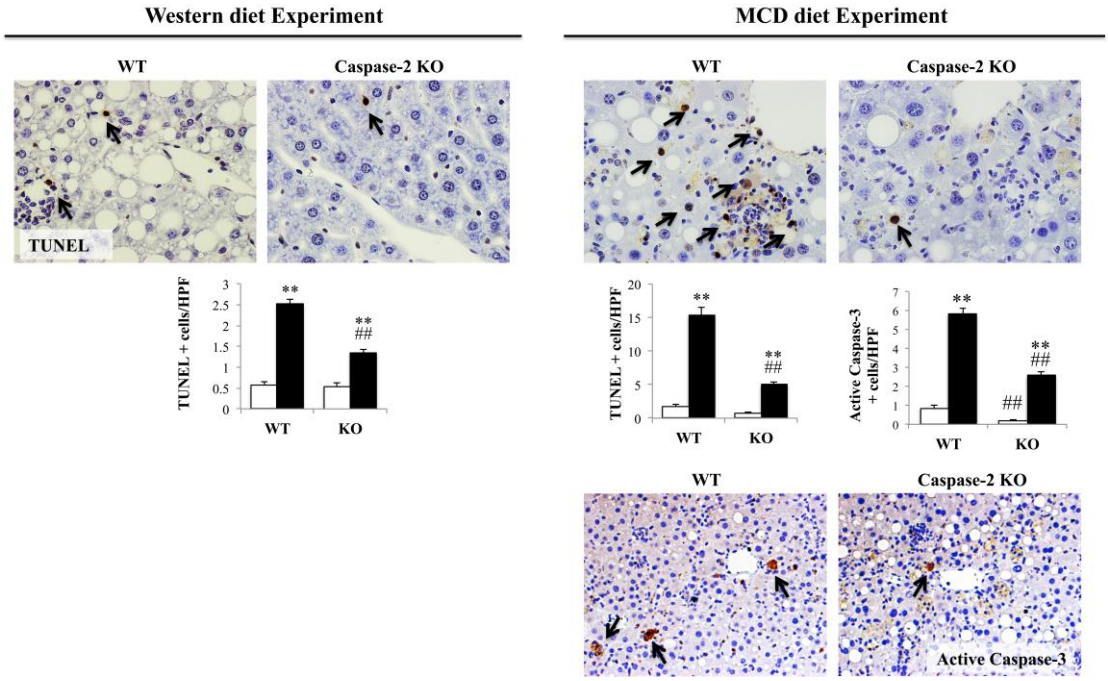


Figure 45. Caspase-2 deficient mice are protected from hepatocyte apoptosis in different dietary models of NASH.

Evaluation of hepatocyte apoptosis in both dietary models of NASH, Western and MCD diet, in WT and caspase-2 deficient mice. Upper panels: representative photos from TUNEL staining of liver sections (40x magnification). Middle panels: quantification of number of positive cells per HPF. Lower panels: representative photos from active cleaved caspase-3 immunohistochemistry of liver sections (20x magnification) Results are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. interventional diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

To confirm our *in vivo* results, we evaluated the effect of caspase-2 deficiency in lipoapoptosis *in vitro*. Primary hepatocytes isolated from WT and caspase-2 mice were treated in culture with palmitate 1 mM. In a preparatory time-dose curve experiment, we found that palmitate induced apoptosis in hepatocytes, with a maximum induction after 12 hours treatment in culture (**Figure 26**). After treating caspase-2 deficient and sufficient hepatocytes with palmitate during 12 hours, we found that the former were protected

from apoptosis, as assessed by activity of effector caspases, caspase-3 and -7. That protection from apoptosis translated in a 50% increase viability after 48 hours of treatment, as assessed by CCK-8 assay (**Figure 46**).

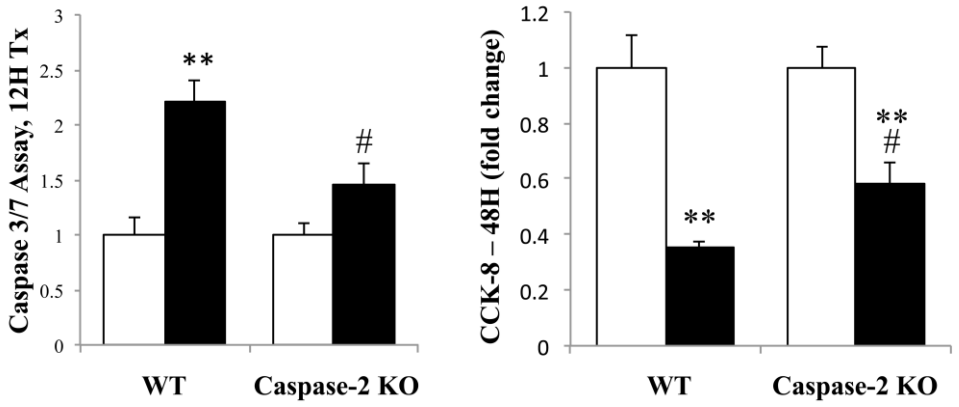


Figure 46. Caspase-2 deficient hepatocytes are protected from palmitate-induced apoptosis. Apoptosis assessed by Caspase-3/7 assay and viability assessed by CCK-8 assay, in primary hepatocytes from WT and caspase-2 deficient mice 12 hours and 48 hours respectively, after treatment with palmitate (1 mM, black bars) or bovine serum albumin (BSA, 2%, white bars). Mean±SEM data from triplicate cultures are graphed. Results shown are representative of triplicate experiments. **p<0.01, BSA *vs*. palmitate; #p<0.05, WT *vs*. caspase-2 KO mice.

Mild or no protection from hepatic inflammation in caspase-2 deficient mice submitted to NASH-inducing diets

Overall we did not find relevant differences in terms of hepatic inflammation, in both NASH-inducing diet models, in caspase-2 deficient mice as compared to WT mice. Both genotypes behaved similarly regarding inflammation as assessed by H&E liver staining and hepatic mRNA expression of the major pro-inflammatory cytokine TNF-α, as well as markers of classical macrophage activation (F4/80) or alternative activation (YM-1) (**Figure 47**). When macrophage activation markers were assessed by immunohistochemistry, the results were not concordant between diets, with a tendency for protection against macrophage activation in caspase-2 mice submitted to Western diet, and worsening macrophage-related inflammation when exposed to MCD diet.

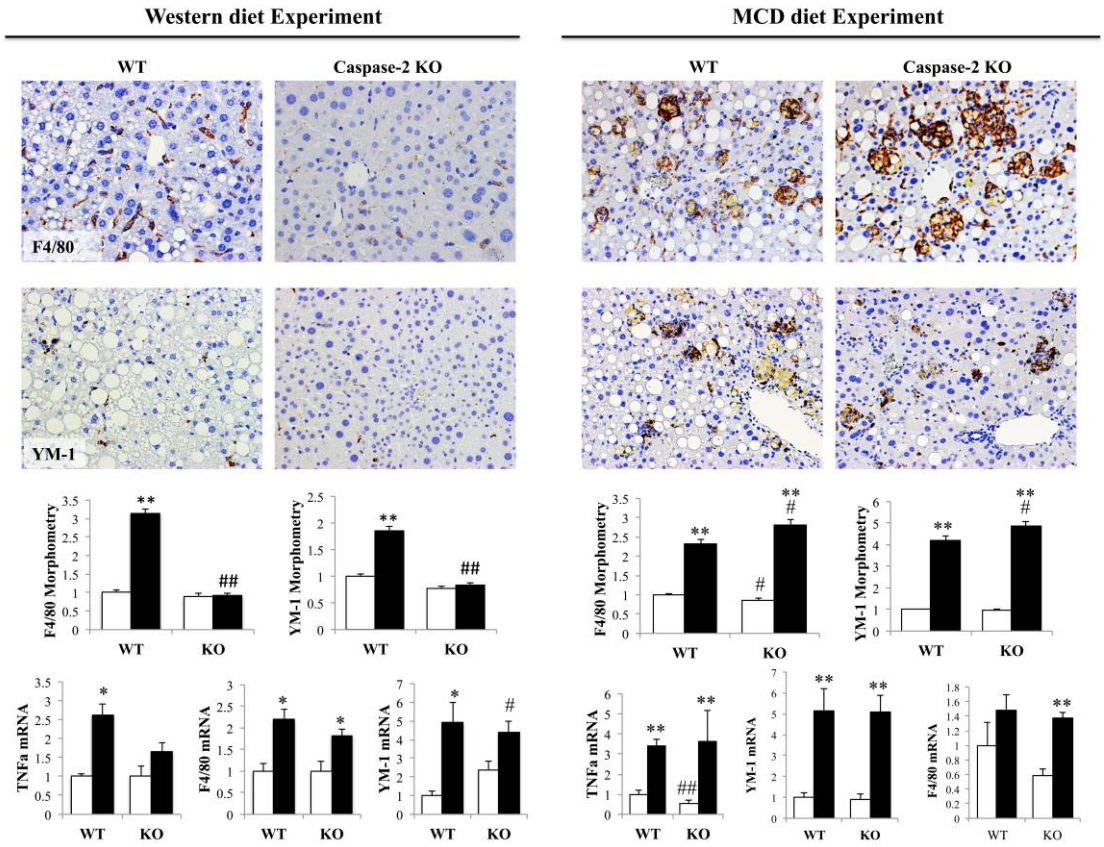


Figure 47. Caspase-2 deficient mice are not protected from hepatic inflammation when induced severe NASH.

Evaluation of hepatic inflammation in both dietary models of NASH, Western and MCD diet, in WT and caspase-2 deficient mice. Upper panels: representative photos from immunohistochemistry for markers of macrophage activation F4/80 (classical activation) and YM-1 (alternative activation), 20x magnification. Middle panels: morphometric analysis from immunohistochemistry for F4/80 and YM-1. Lower panel: qRT-PCR analysis of inflammatory genes. Results are graphed as mean±SEM and normalized for WT chow diet. *p<0.05, **p<0.01 chow *vs*. interventional diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

The lack of effect of caspase-2 deficiency in inflammation, despite a strong effect in liver injury is intriguing. In fact, as opposed to other forms of cell death such as necrosis, hepatocyte apoptosis is believed to instigate a much less important inflammatory reaction, though it can worsen inflammation (Machado and Cortez-Pinto, 2011; Yang et al., 2015). On the other hand, caspase-2 itself may have some anti-inflammatory actions through inhibition of NFkB pathway (Bouchier-Hayes and Green, 2012; Guha et al., 2010).

Caspase-2 deficient mice show less up-regulation of the hedgehog pathway when submitted to NASH-inducing diets

Our findings in humans and mice with NASH demonstrate that caspase-2 is increased during lipotoxicity and plays an important role in NASH-related lipoapoptosis. Lipotoxicity has been modeled by treating cultured hepatocytes with palmitate to induce lipoapoptosis (Kakisaka et al., 2012). Dying cells are known to activate their production of various pro-regenerative fibrogenic factors, including Shh (Jung et al., 2010; Rangwala et al., 2011; Ryoo et al., 2004). Moreover, blocking cell death after initiation of apoptosis prolongs Shh production (Kakisaka et al., 2012; Ryoo et al., 2004). To determine if caspase-2 directly influenced hepatocyte production of Shh ligands, we compared the effects of palmitate on Shh expression in primary hepatocytes from WT and caspase-2 deficient mice. Hepatocytes from caspase-2 deficient mice expressed less Shh ligand in response to a lipotoxic stimulus than WT mice, at both the mRNA and protein level **(Figure 48)**.

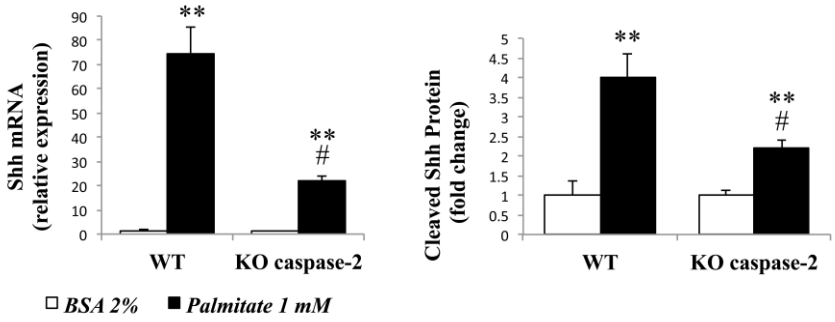


Figure 48. Lipotoxic caspase-2 deficient hepatocytes produce less Shh as WT hepatocytes.
qRT-PCR analysis of Shh mRNA (left) and Western blot analysis of Shh protein (right), in primary hepatocytes from WT and caspase-2 deficient mice 24 hours after treatment with palmitate (1 mM, black bars) or bovine serum albumin (BSA, 2%, white bars). Mean±SEM data from triplicate cultures are graphed. Results shown are representative of triplicate experiments. **p<0.01, BSA *vs*. palmitate; #p<0.05, WT *vs*. caspase-2 KO mice.

Because Shh protein cannot be localized by immunohistochemistry, in mouse liver, using available Shh antibodies, we compared serum levels of Shh in WT and caspase-2 deficient mice submitted to MCD diet to determine if caspase-2 deficiency also reduced Shh ligand production during lipotoxicity *in vivo*. Indeed, after MCD diet feeding, levels of Shh protein were significantly lower in caspase-2 deficient mice than similarly treated WT mice (3.8±2.3 *versus* 14.5±2.1pg/mL, P<0.007). Reduced Shh ligand exposure was accompanied by less activation of the hedgehog pathway in the livers of caspase-2

deficient mice. Compared to WT mice, caspase-2 deficient mice demonstrated lower levels of the hedgehog-regulated transcription factor, gli-2, by qRT-PCR, when fed MCD diet (**Figure 49**). Also, mRNA and protein expression of osteopontin, a profibrogenic gli-2-target gene, were significantly reduced in the caspase-2 deficient mice in both models of diet-induced NASH (**Figure 49**).

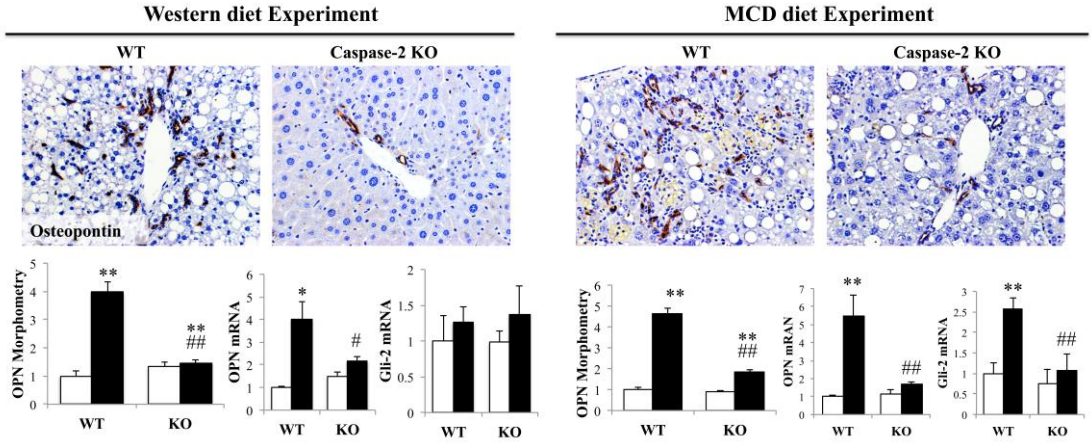


Figure 49. Decreased hedgehog pathway activation in caspase-2 deficient mice submitted to different dietary models of NASH.

Evaluation of hedgehog pathway in both dietary models of NASH, Western and MCD diet, in WT and caspase-2 deficient mice. Upper panels: representative photos from osteopontin (OPN) immunohistochemistry of liver sections (20x magnification). Lower panels: morphometric analysis of OPN immunohistochemistry and qRT-PCR analysis of gene expression of genes in the hedgehog pathway: OPN and gli-2. Results are graphed as mean±SEM, and normalized for WT chow diet. *p<0.05, **p<0.01 chow *vs*. interventional diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

The role of caspase-2 in NAFLD-associated fibrosis

Caspase-2 deficient mice are protected from NASH-related fibro-ductular reaction

During NASH, liver cell death triggers compensatory regenerative responses that lead to activation of the hepatic progenitor compartment, resulting in the ductular reaction (Jung et al., 2010). Because we found that capase-2 promotes NASH-related apoptosis we examined the effect of capase-2 deficiency on the ductular reaction. As expected (Richardson et al., 2007), both dietary models of NASH provoked a ductular reaction in WT mice, as demonstrated by induction of several markers of ductular-type progenitors,

including keratins 19 and 7, as well as Sox-9 (**Figure 50**). In caspase-2 deficient mice, all of these ductular reaction parameters were significantly reduced (**Figure 50**).

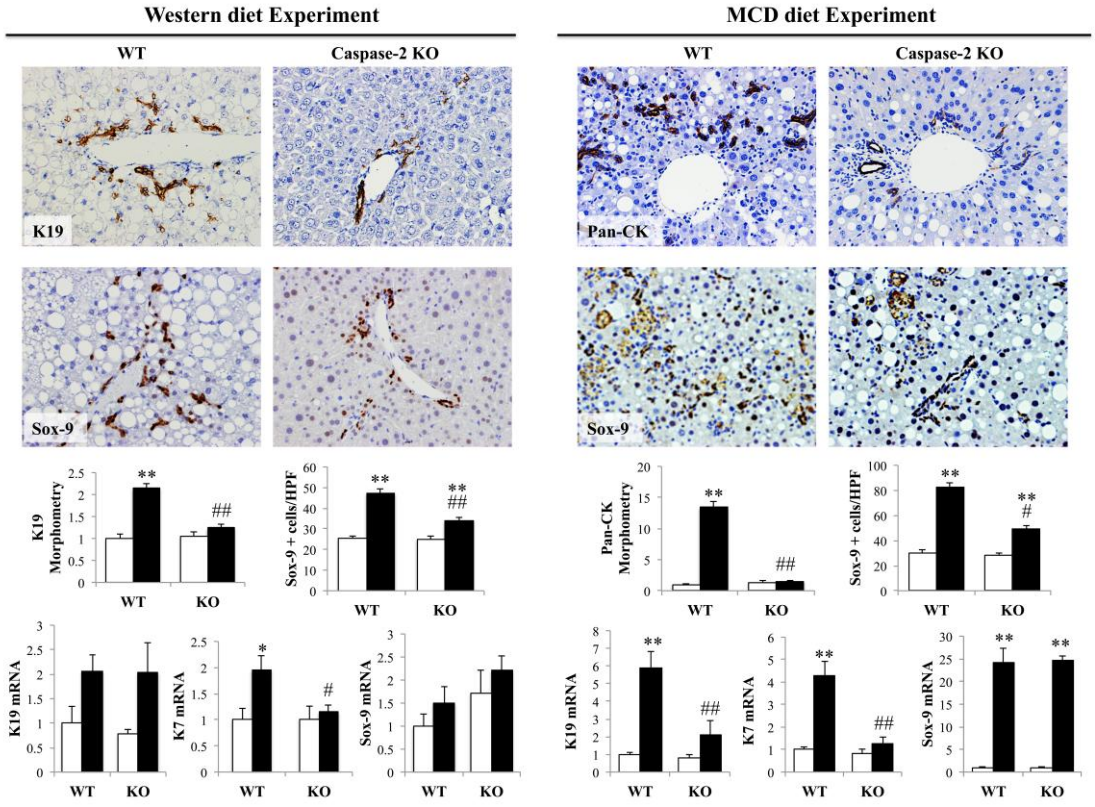


Figure 50. Caspase-2 deficient mice showed blunted ductular response when submitted to different dietary models of NASH.

Evaluation of ductular reaction in both dietary models of NASH, Western and MCD diet, in WT and caspase-2 deficient mice. Upper panels: representative photos from keratin 19 (K19)/pan-keratin (pan-CK) and Sox-9 immunohistochemistry of liver sections (20x magnification). Middle panels: morphometric analysis of the above immunohistochemistries. Lower panels: qRT-PCR analysis of gene expression of progenitor markers. Results are graphed as mean±SEM, and normalized for WT chow diet. *p<0.05, **p<0.01 chow *vs*. interventional diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

The blunted ductular response in caspase-2 KO seems to be a consequence of decreased apopototic cell death (**Figure 45**) and not a primary defect in regeneration, since liver mass and liver to body mass was similar in both genotypes when exposed to MCD diet (whereas difference in liver mass in the Western diet translate difference in fat content – **Figure 42**) and there was no evidence of liver failure, namely no increase in bilirubin levels (data not shown). Accordingly, we found strong significant correlations between apoptosis assessed by TUNEL and progenitor compartment expansion assessed by keratin-7 mRNA expression and Sox-9 immunohistochemistry (**Figure 51**), in the MCD model.

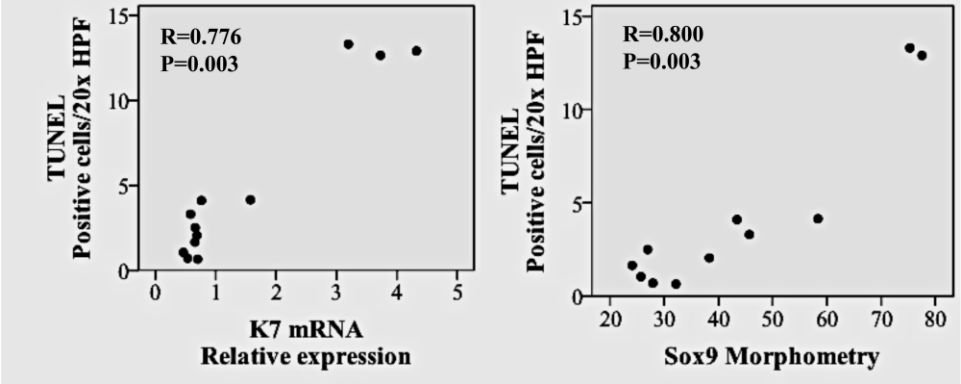


Figure 51. Apoptosis correlates with ductular reaction in mice with MCD diet-induced NASH. Apoptosis (as assessed by TUNEL assay) strongly positively correlates with ductular reaction (as assessed by keratin-7 gene expression and Sox-9 immunohistochemistry). Correlation evaluated with non-parametric Spearmen test.

Because lipoapoptosis and the ductular reaction are strongly linked to fibrosis stage in NASH (Richardson et al., 2007), we next compared the level of HSC activation and fibrosis severity in the two strains.

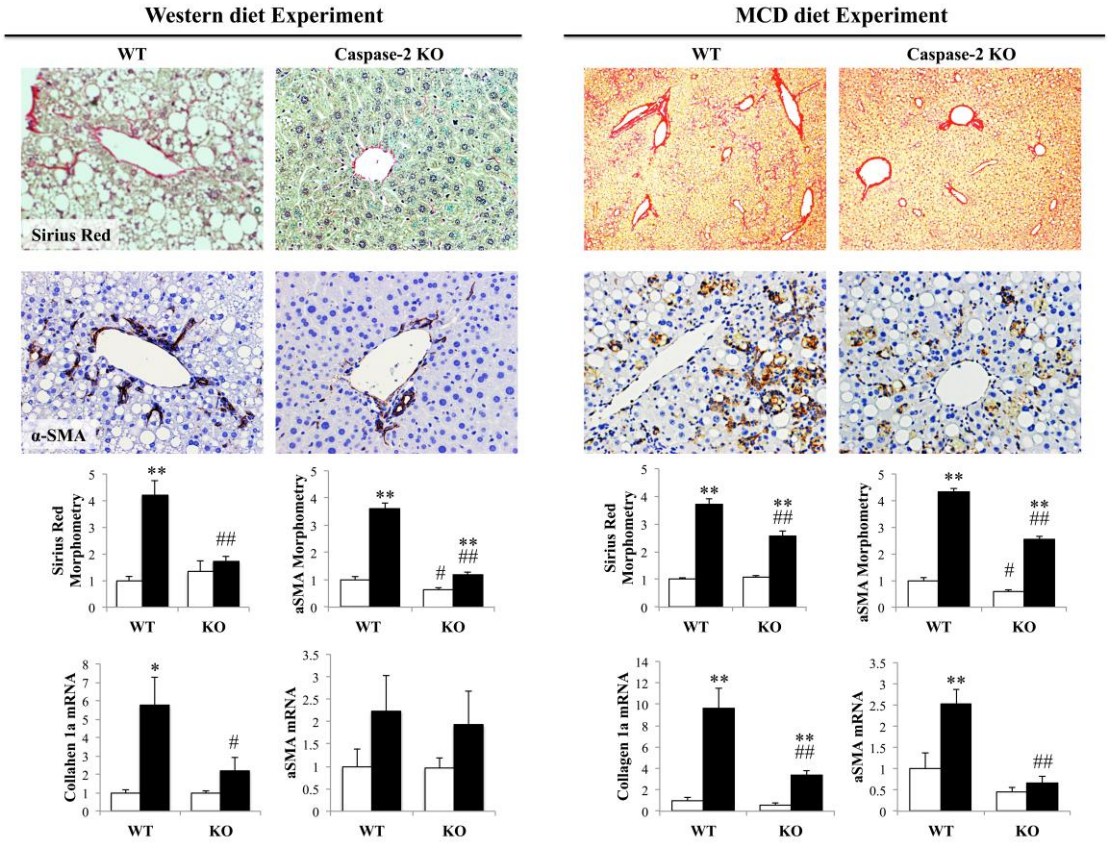


Figure 52. Caspase-2 deficient mice are protected from hepatic fibrosis when submitted to different dietary models of NASH.

Evaluation of fibrosis in both dietary models of NASH, Western and MCD diet, in WT and caspase-2 deficient mice. Upper panels: representative photos from Sirius red staining and α-smooth muscle actin (α-SMA) immunohistochemistry of liver sections (20x magnification). Middle panels: morphometric analysis of the above stainings. Lower panels: qRT-PCR analysis of fibrogenic genes expression. Results are graphed as mean±SEM, and normalized for WT chow diet. *p<0.05, **p<0.01 chow *vs*. interventional diet; #p<0.05, ##p<0.01 WT *vs*. KO mice.

Compared to WT mice, caspase-2 KO mice developed significantly less HSC activation and fibrosis when chronically challenged by either NASH-inducing diets, as assessed by Sirius red staining, and α-SMA immunohistochemistry, and qRT-PCR analysis of whole liver collagen-1α and α-SMA gene expression (**Figure 52**). Immunohistochemistry for collagen-1α in the MCD diet experiment and hydroxyproline content determination in the Western diet experiment confirmed those results (data not shown).

Mechanisms of protection from fibrogenesis in caspase-2 deficient mice

To better understand the role of caspase-2 deficiency in liver fibrogenesis, we characterized the dynamics of caspase-2 expression in primary HSC during culture-induced transition from quiescence (freshly isolated cells, represented as day 0 in culture, D0) into myofibroblastic HSC, that is, fully activated cells after 7 days in culture (D7). We found a dramatic decrease in caspase-2 expression upon HSC activation (**Figure 53**), suggesting that a direct effect of caspase-2 deficiency in HSC *per se* is unlikely to be important in preventing HSC activation.

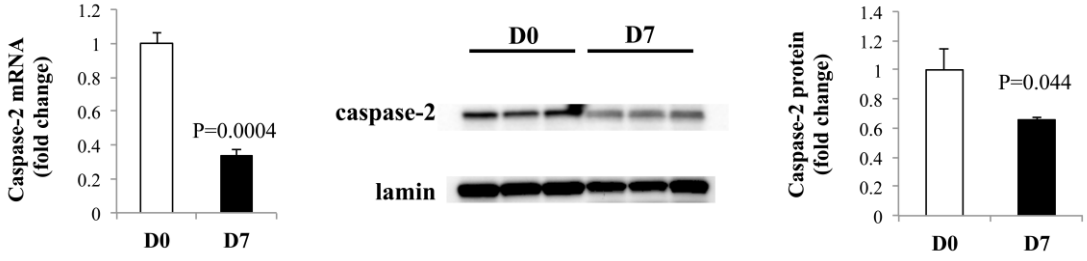


Figure 53. HSC activation associates with down-regulation of caspase-2 expression.
Changes in caspase-2 expression during culture-induced transition of primary rat quiescent hepatic stellate cells (HSC) (D0) into myofibroblastic HSC (D7) by qRT-PCR (left panel) and immunoblot (right panels). Data from triplicate cultures are graphed as mean±SEM, and normalized for quiescent (D0) HSC.

Concordant with this concept, primary HSC isolated from WT and caspase-2 deficient mice were able to fully activate in culture, as assessed by qRT-PCR analysis of markers

of HSC activation (α-SMA and collagen-α1a) *versus* quiescence (PPAR-γ), as well as content of lipid droplets using Oil Red O staining (**Figure 54**).

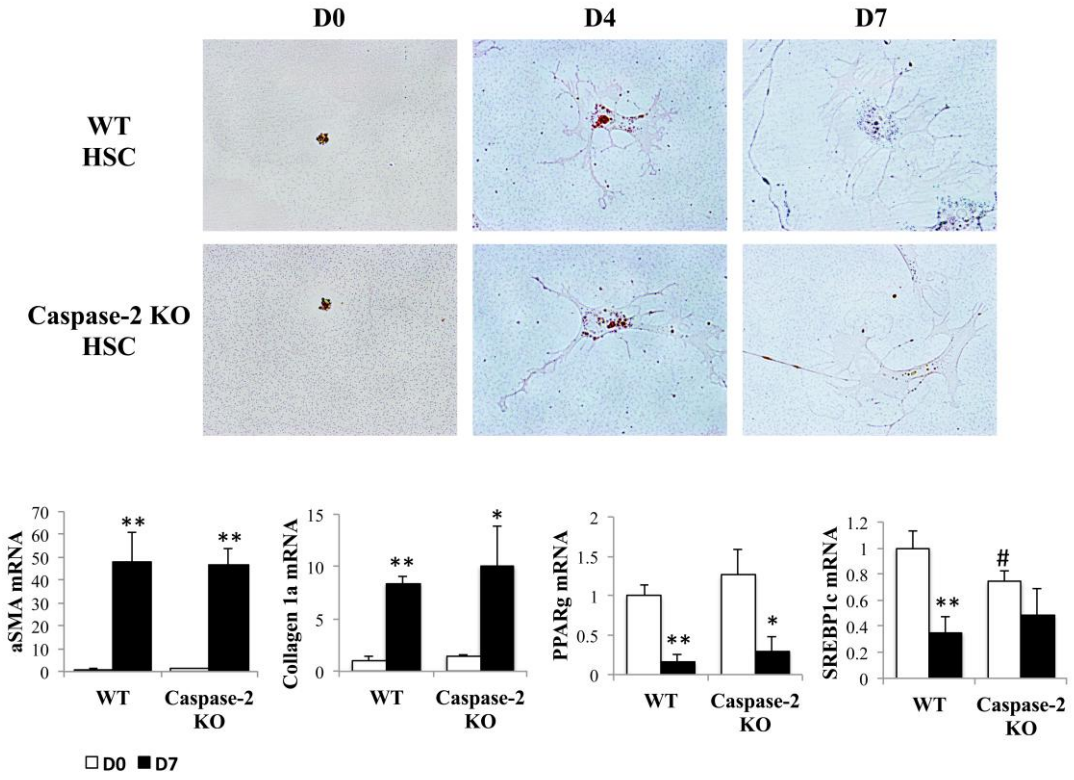


Figure 54. Caspase-2 deficient HSC can fully activate in culture.

Upper panels: representative photos from primary HSC isolated from WT and caspase-2 KO mice, freshly isolated (D0), after 4 and 7 days in culture (D4 and D7). Lower panels: changes in gene expression in markers of activation (α-SMA and collagen 1α) and quiescence (PPAR-γ and SREBP1c) in culture-induced activation of mouse HSC. Data from triplicate cultures are graphed as mean±SEM, and normalized for WT quiescent HSC.

Previously, we showed that caspase-2 deficient hepatocytes were protected from palmitate-induced ballooning and apoptosis. Concordant with the concept that hepatocyte apoptosis drives fibrogenesis, we found strong correlations between hepatocyte apoptosis assessed by TUNEL and fibrogenesis assessed by α-SMA and collagen-α1a, *in vivo*, in the MCD diet experiment (**Figure 55**).

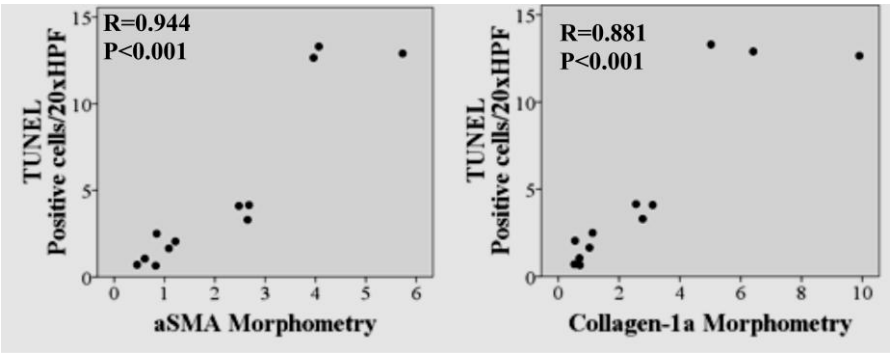


Figure 55. Apoptosis correlates with fibrosis in mice with MCD diet-induced NASH.
Apoptosis (as assessed by TUNEL assay) strongly positively correlates with fibrosis (as assessed by α-SMA and collagen-1α). Correlation evaluated with non-parametric Spearmen test.

Because dying cells release factors trophic to cells implicated in the repair response, such as HSC, we evaluated the expression of two fibrogenic factors, Shh and PDGF by *in vitro* lipotoxic, palmitate-treated, primary hepatocytes derived from WT and caspase-2 deficient mice. Caspase-2 deficient hepatocytes not only produced less Shh, but also produced less PDGF (**Figure 56**), a HSC trophic factor known to be important in human NASH (Moylan et al., 2014).

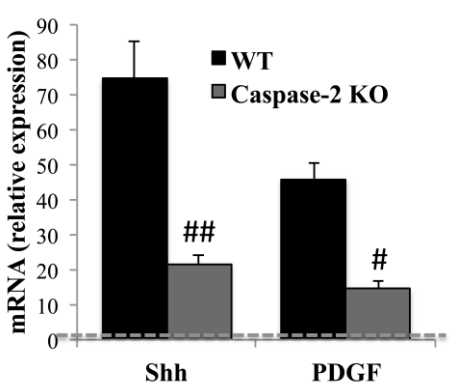


Figure 56. Decreased production of pro-fibrogenic factors from lipotoxic caspase-2 deficient hepatocytes.
Comparison of changes of gene expression of Shh and PDGF in primary hepatocytes treated with 1 mM palmitate, as compared to control treated hepatocytes, from WT and caspase-2 KO mice, normalized to its respective controls. #p<0.05, ##p<0.01 *vs*. WT or palmitate-treated group (right panels).

Interestingly, corroborating the concept that lipotoxic dying cells release hedgehog ligands activating that pathway, as well as PDGF, we found strong positive correlations between apoptosis assessed by TUNEL and hedgehog pathway activation assessed by expression of Gli-2 and osteopontin morphometry, as well as PDGF expression, in the MCD diet model (**Figure 57**).

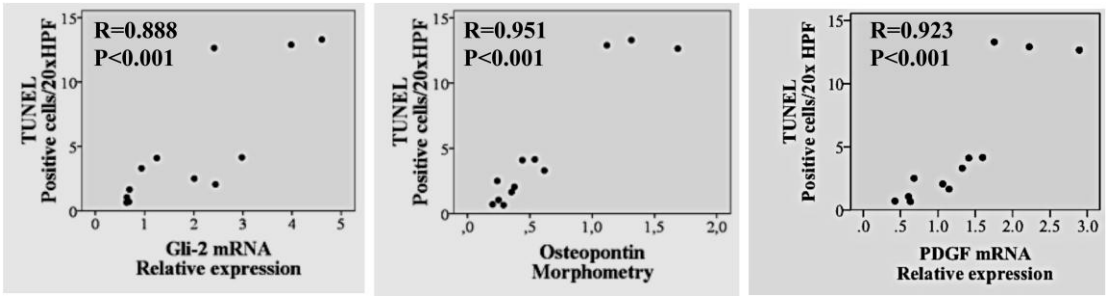


Figure 57. Apoptosis correlates with activation of pro-fibrogenic pathways, in mice with MCD diet-induced NASH.

Apoptosis (as assessed by TUNEL assay) strongly positively correlates with activation of hedgehog pathway (as assessed by mRNA expression of transcription factor gli-2 and osteopontin immunohistochemistry), and with mRNA expression of PDGF. Correlation evaluated with non-parametric Spearmen test.

Palmitate is also toxic to HSC, greatly reducing viability and increasing apoptosis in the rat 8B activated stellate cell line. Nonetheless, HSC grown in conditioned media from primary WT hepatocytes treated with palmitate (and which still contains palmitate) instead were protected from apoptosis and displayed almost completely restored HSC viability; however HSC viability was less protected when the conditioned media derived from caspase-2 deficient hepatocytes (**Figure 58**).

Also, as compared to palmitate alone, conditioned media from palmitate-treated WT hepatocytes induced hedgehog pathway activation and collagen production in 8B HSC, while these responses were blunted when using conditioned media from palmitate-treated caspase-2 deficient hepatocytes (**Figure 58**). Concordant with the concept that Shh and PDGF derived from lipoapoptotic hepatocytes activate HSC promoting fibrogenesis, we found strong correlations between hedgehog target gene, Gli-2, mRNA expression, as well as PDGF mRNA expression and fibrosis (α-SMA and collagen-α1a immunohistochemistry), in the MCD diet experiment (**Figure 52**).

In summary, in caspase-2 deficient mice, HSC have the potential to fully activate, but caspase-2 deficient hepatocytes incur less lipotoxic injury and release HSC trophic factors to a lesser extent, conferring relative protection from NASH-related fibrogenesis.

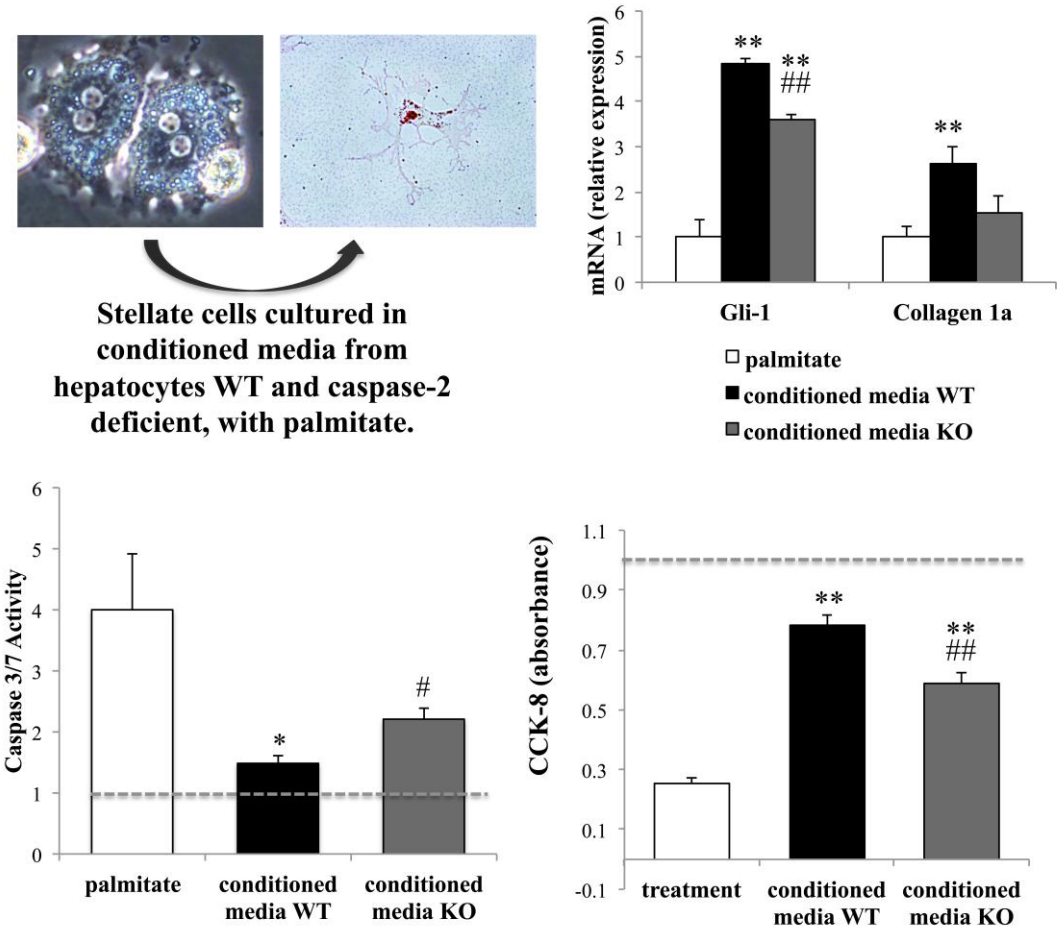


Figure 58. Caspase-2 deficiency blunts paracrine HSC viability/activation from lipotoxic hepatocytes.

Upper panels: qRT-PCR analysis of 8B rat HSC cell line treated either with 1 mM palmitate for 24 hours or from conditioned media from similarly treated primary hepatocytes from WT or caspase-2 KO mice. Lower panels show changes in apoptosis (caspase-3/7 activity, left) and viability (CCK-8 assay, left) compared to control group. Results from triplicate cultures are graphed as mean±SEM. *p<0.05, **p<0.01 chow *vs*. control group. #p<0.05, ##p<0.01 *vs*. WT (upper panel) or palmitate-treated group (lower panels).

DISCUSSION

In a mouse model of diet-induced metabolic syndrome, caspase-2 deficiency protects from the development of several key aspects of the metabolic syndrome, including central obesity, dyslipidemia, T2DM and NAFLD. We used a model that mimics a common Western diet, with 45% total fat enriched in saturated fats, supplemented with 0.2% cholesterol and with high fructose corn syrup equivalent to soft drinks.

The involvement of a protein known to regulate cell death and the cell cycle in also regulating whole-body metabolism is non-intuitive. Natural selection optimized mechanisms to cope with food deprivation. When presented with energy surplus, these adaptive mechanisms became deleterious and lead to the development of the metabolic syndrome. Caspase-2, the most evolutionarily conserved caspase (Bouchier-Hayes and Green, 2012), mediates programmed cell death in models of food deprivation through a mechanism requiring accumulation of toxic FA (Johnson et al., 2013). Interestingly, caspase-2 has two isoforms, a long isoform (caspase-2_L) that induces cell death and is expressed in most tissues, and a short isoform (caspase-2_S) that can antagonize cell death, and that is most highly expressed in brain, heart and skeletal muscle (Kumar et al., 1997). This suggests an ancient role for caspase-2 in preserving the most essential organs by sacrificing less critical organs and tissues in order to survive prolonged fasting. However, in modern times, energy surplus can also lead to accumulation of toxic lipids, leading to maladaptive caspase-2 activation.

We propose the following paradigm: Western diet leads to energy surplus and accumulation of toxic lipids in adipocytes. Overfilled and hypertrophic adipocytes overexpress and activate caspase-2 to induce cell stress and death, leading to the release of toxic mediators (such as adipokines), decreased uptake of NEFA's from blood, and release of NEFA's from dying cells. The net result is an increase in circulating NEFA's and perturbed adipokine secretion, promoting the metabolic syndrome. Supporting this hypothesis, caspase-2 deficient mice fed a Western diet, when compared to WT mice in similar conditions, show less activation of apoptosis in abdominal adipose tissue, normal adipokine secretion profile, decreased levels of circulating NEFA's, and protection from the development of IR/T2DM and the metabolic syndrome. Consistent with our findings, others have shown an increase in adipose tissue expression of caspase-2 in rats fed a HFD (Jobgen et al., 2009). Also, caspase-2 deficient mice were shown to be resistant to age-induced glucose intolerance (Wilson et al., 2015).

Unexpectedly, we found other mechanisms that may help maintain adipocyte homeostasis in response to excessive energy challenge. Abdominal adipose tissue from caspase-2 deficient mice is fundamentally different from that of WT mice: adipose stem cells lacking caspase-2 have a higher proliferative capacity, and as a consequence caspase-2 deficient mice have a higher number of small adipocytes, even when fed chow diet. Fat mass expansion in response to energy surplus can occur by hypertrophy (increase cell size) or hyperplasia (increase cell number). Only the latter is considered a healthy mechanism of fat storage (Rutkowski et al., 2015). An interesting study with twin pairs in which one was lean and the other obese showed that when the adipose tissue from the obese twin was hypoplastic (lower number of enlarged adipocytes), the obese twin had higher rates of IR, metabolic syndrome and NAFLD, whereas if the adipose tissue was hyperplastic, the obese twin did not have increased rate of metabolic disturbances as compared to the lean sibling (Heinonen et al., 2014).

Our study is the first report of increased proliferative capacity in untransformed somatic cells from caspase-2 deficient mice. It is well known that female caspase-2 deficient mice have an increased number of primordial follicles; however, that has been explained as a failure to induce apoptosis rather than increase in cell division (Bergeron et al., 1998; Nutt et al., 2005). Also, mouse embryonic fibroblasts from caspase-2 deficient mice have higher growth rates after oncogenic transformation as compared to wild type cells (Ho et al., 2009; Parsons et al., 2013). Here we show not only that untransformed adipose stem cells are more proliferative *in vitro*, but also that caspase-2 deficient mice display higher number of adipocytes in abdominal adipose tissue *in vivo* compared to WT mice, even under normal chow diet, and this difference persists after challenge with high-fat high-sugar diet. The mechanism conferring the proliferative advantage of caspase-2 deficient cells is not yet fully understood. It has been shown that caspase-2 activity is repressed during mitosis by an evolutionary conserved mechanism involving cyclin B1-mediated phosphorylation of serine 340 in the caspase-2 inter-domain region (Andersen et al., 2009), which suggests that inhibition of mitosis by caspase-2 may be a conserved phenomenon. In fact, caspase-2 has been proposed to regulate cell cycle checkpoints through regulation of p53 expression and function (Oliver et al., 2011). A positive feedback loop is generated since p53 is a transcription factor for the caspase-2 activation scaffold protein, p53-induced protein with a death domain (PIDD). On the other hand, caspase-2 negatively regulates the NFkB pro-survival and proliferative pathway, through cleavage and degradation of receptor-interacting serine/threonine-protein kinase 1 (RIP1)

(Guha et al., 2010) and competition for assembly with PIDD (Bouchier-Hayes and Green, 2012), both important in NFkB activation through NEMO.

Furthermore, white adipocytes from caspase-2 deficient mice show evidence of browning to more effectively cope with energy excess through UCPs. As a consequence, caspase-2 deficient mice have a larger number of adipocytes that are able to consume more energy. Fed a Western diet, these mice do not develop stressful enlargement of adipocytes and do not expand their fat mass. This is extremely important, since it has been well demonstrated that increased size/volume of adipocytes, particularly above a threshold, correlates with the development of T2DM (Kloting and Bluher, 2014). Caspase-2 can also regulate metabolism through other mechanisms not yet fully understood. For instance, it has been suggested that caspase-2 can regulate the expression of transcription factors FoxO1 and FoxO3a, important players in the insulin-signaling pathway (Shalini et al., 2012).

Several mechanisms can lead to activation of caspase-2 in adipocytes overloaded with FA, such as oxidative stress and ER stress (Jones et al., 2014). One potential link between Western diet/obesity and caspase-2 activation promoting the development of the metabolic syndrome may be decrease levels/activity of sirtuin-1 in visceral adipose tissue. Sirtuin-1 is a member of NAD^+-dependent histone deacetylase family, and acts both as an important energy status sensor (Chang and Guarente, 2014) and as a repressor of caspase-2 activity by deacetylating 14-3-3ζ and promoting its binding to caspase-2. Binding of 14-3-3ζ to caspase-2 prevents dephosphorylation of pSer135 and thus activation (Andersen et al., 2011). Increasing sirtuin-1 expression/activity, either by genetic approaches or treatment with sirtuin-1 specific activators, is protective against NAFLD and IR in HFD models (Banks et al., 2008; Feige et al., 2008). Conversely, HFD induces decreased levels of sirtuin-1 in adipose tissue through the cleavage of sirtuin by inflammasome-activated caspase-1 (Chalkiadaki and Guarente, 2012). Similarly, human obesity associates with low levels of sirtuin-1 in visceral adipose tissue (Pedersen et al., 2008). Whether caspase-2 effects on diet-induced development of metabolic syndrome is mediated in part by sirtuin-1 remains to be determined. Alternatively, it has been described that FA induce lysosomal permeabilization and cathepsin activation, resulting in mitochondrial dysfunction and adipocyte apoptosis. Intra-cytoplasmic levels and activity of cathepsin D and B were increased, in a time-dependent manner, in adipose tissue from mice fed a HFD (Gornicka et al., 2012; Masson et al., 2011). Interestingly, in

other models, such as treatment of pancreatic carcinoma cell lines with Bortezomib, cathepsins can induce apoptosis through activation of caspase-2 (Yeung et al., 2006).

We found a profound protection from hepatic steatosis in caspase-2 deficient mice fed Western diet, whereas almost no protection when fed MCD diet. This suggests that, although this is a whole-body gene knockout, the protection conferred to the development of hepatic steatosis results from deficiency of caspase-2 in the adipose tissue, and only to a lesser extent in the liver itself. In fact, as opposed to the Western diet model, MCD diet induces liver steatosis through a mechanism that does not involve increased import of NEFA's from the circulation/adipose tissue; rather it is the consequence of deficient export of fat from the liver. In the MCD model, caspase-2 deficient mice were not protected from hepatic steatosis, although they were protected from liver injury and fibrosis. In the Western diet model, caspase-2 deficiency improved adipose tissue physiology resulting in a decreased flow of circulating NEFA's to the liver, which can explain protection to develop NAFLD. Additionally, the livers from caspase-2 deficient mice cope better with energy surplus through metabolic reprograming, which results in decreased *de novo* lipogenesis, increased FA oxidation and increased fat export from the liver as lipoproteins, as compared to control mice. The mechanism of metabolic reprograming by caspase-2 probably relies in undiscovered non-apopototic functions of caspase-2. Indeed, it has been described that *in vitro* effects of SREBP-dependent lipogenesis is partially dependent on expression of caspase-2 (Logette et al., 2005a).
Regarding the role of caspase-2 in lipotoxicity-associated liver disease, we showed that caspase-2 expression is increased in NASH, and demonstrated the cell types in which that expression occurs, namely fatty hepatocytes and particularly, ballooned hepatocytes. Evidence for enrichment of caspase-2 in ballooned hepatocytes was intriguing to us because hepatocyte ballooning is a consequence of cytoskeletal disruption and caspase-2 has been shown to cleave certain cytoskeletal proteins (Vakifahmetoglu-Norberg et al., 2013). Accumulation of ballooned hepatocytes has been linked to fibrosis stage in NASH (Guy et al., 2012a), and we found that caspase-2-positive cells were enriched near fibrotic septa in both NASH patients and several animal models of NASH. Moreover, we discovered that the level of caspase-2 expression correlated strongly with the degree of fibrosis, suggesting that the caspase-2-positive hepatocytes themselves might be contributing to the fibrogenic process, though we cannot exclude that activated HSC, inflammatory cells and progenitors may release factors that decrease hepatocyte viability

(e.g. TGF-β) with activation of caspase-2. Interestingly, these findings were noted in obese, diabetic models of NASH, as well as NASH models that are characterized by weight loss and insulin sensitivity, suggesting that caspase-2 induction in hepatocytes and related fibrogenesis are driven by hepatocyte lipotoxicity *per se*, independently of associated co-morbidities.

The possibility that caspase-2 induction might be directly linked to lipotoxicity is intriguing because lipoapoptosis is considered a key driver of NASH pathogenesis and progression (Feldstein et al., 2003). A plausible model for caspase-2 activation in fat-laden hepatocytes is suggested by the fact that FA decrease the free CoA/acyl-CoA ratio, resulting in reduced activation of CAMKII and consequent decreases in CAMKII-mediated phosphorylation of caspase-2 at pSer135. Because such phosphorylation typically inactivates caspase-2, its activity might increase as FA accumulate (McCoy et al., 2013). Caspase-2 may also be activated by TNF-α. In endothelial cells, for example, TNF-α induces apoptosis through TNFRSF1A-mediated activation of caspase-2 (Espin et al., 2013). Though this pathway has not been studied in hepatocytes, TNFRSF1A is expressed by all cell types, and TNF-α is increased in obesity and NAFLD, two diseases in which this cytokine is believed to play a major role (Syn et al., 2009a). Lastly, similarly to the adipose tissue in obesity, sirtuin-1 expression is decreased in human NAFLD (Castro et al., 2013), which may further explain caspase-2 activation.

Liver cell death is a key factor that differentiates NASH from hepatic steatosis (Machado and Cortez-Pinto, 2011). The present study provides a novel *in vivo* function for caspase-2 and clarifies the role of caspase-2 in NASH-related lipoapoptosis. We showed that caspase-2 deficiency partially protected from lipoapoptosis *in vitro*, when culturing primary hepatocytes with the saturated fatty acid palmitate. Furthermore, *in vivo*, caspase-2 deficient mice had significantly fewer cells with activated caspase-3, as well as significantly reduced numbers of TUNEL-positive cells during NASH. Caspase-2 deficiency protected mice from hepatocyte apoptosis despite diet-related increases in hepatic steatosis and TNF-α production, suggesting that caspase-2 is an important down-stream effector of liver cell death due to these factors during NASH.

In two different mouse models of NASH, caspase-2 deficiency consistently conferred protection from hepatic fibrogenesis. To elucidate the mechanisms by which caspase-2 deficiency confers protection for hepatic fibrosis, we first showed that caspase-2 is not

necessary for the activation of HSC. Hence we hypothesized that caspase-2 deficiency could prevent fibrogenesis through down-regulation of hepatocyte apoptosis. Previous correlation human data have shown that the level of hepatocyte death strongly predicts the severity of liver fibrosis in NASH (Feldstein et al., 2003). Various mechanisms have been proposed to explain these observations: HSC can engulf apoptotic bodies, which induces its activation and profibrogenic potential (Canbay et al., 2003b; Jiang et al., 2009; Zhan et al., 2006); Kupffer cells can also phagocytize apoptotic bodies, which increases activation and release of profibrogenic mediators (Canbay et al., 2003a); and dying hepatocytes release profibrogenic mediators that act on nearby HSC (Arshad et al., 2012; Jung et al., 2010; Rangwala et al., 2011). Hedgehog ligand is one such pro-fibrogenic, death-associated molecular signal. Hedgehog ligands stimulate outgrowth of stromal cells that are typically associated with wound healing, such as progenitors and myofibroblasts (Fleig et al., 2007; Syn et al., 2009b). In flies, experimental interventions that delay the demise of cells that have initiated apoptosis prolongs Hedgehog ligand production, thereby amplifying local accumulation of factors that exacerbate fibrogenic repair (Ryoo et al., 2004). Previous works from Diehl' and Gores' labs have provided evidence that a similar process contributes to fibrosis progression during NASH. Triggering apoptosis by inhibiting nuclear localization of NFkB provoked mammalian hepatocytes to produce Shh *in vitro* and linked this to hepatic accumulation of hedgehog-responsive progenitors and myofibroblasts, as well as liver fibrogenesis, in mice with genetically impaired nuclear localization of NFkB in hepatocytes (Jung et al., 2010). Also, human ballooned hepatocytes are particularly enriched with hedgehog ligands and accumulation of hedgehog-positive hepatocytes strongly correlates with fibrosis severity in human NASH (Guy et al., 2012b; Rangwala et al., 2011). Furthermore, ballooned hepatocytes were shown to be deficient in caspase-9, thereby identifying a mechanism that likely delayed the death of such cells and prolonged their production of hedgehog ligands (Kakisaka et al., 2012). This helped to explain why Shh expression was particularly enriched in ballooned hepatocytes (Guy et al., 2012b; Rangwala et al., 2011), and why accumulation of ballooned hepatocytes was a robust predictor of fibrosis severity in NASH (Guy et al., 2012a). We showed that primary hepatocytes treated with lethal doses of palmitate produce Shh ligand, a well-established trigger of lipoapoptosis, in WT hepatocytes (Wei et al., 2006). Other groups found similar data in hepatocytes deficient in caspase-9 (Kakisaka et al., 2012). We also showed, for the first time, that lipotoxic dying hepatocytes additionally up-regulate the expression of PDGF, another potent fibrogenic

factor that has direct effects in HSC (Kocabayoglu et al., 2015; Liu et al., 2014). PDGF production by healthy hepatocytes is minimal, being highly produced by platelets, endothelial cells, cholangiocytes and Kupffer cells (Omenetti et al., 2008; Wynn and Barron, 2010). Concordant with PDGF production by lipoapoptotic hepatocytes, PDGF is up-regulated in human NASH, and correlates with the severity of liver disease (Moylan et al., 2014; Wright et al., 2014).

Moreover, we showed that palmitate is not only toxic to hepatocytes but also to HSC. However, dying lipotoxic hepatocytes protect HSC from palmitate-induced toxicity in association with release of pro-fibrogenic factors, allowing them to activate and elicit a wound-healing response. Importantly, we found that caspase-2 deficient hepatocytes were protected from palmitate-induced lipotoxicity, and hence released less alarm signals, such as Shh and PDGF. As a consequence, lipotoxic hepatocytes from caspase-2 deficient mice were less effective in stimulating nearby HSC to activate, which may explain why caspase-2 deficient mice are protected from liver fibrosis when subjected to NASH-inducing diets.

In conclusion, caspase-2 is a new potential target to address obesity and its associated comorbidities: metabolic syndrome, IR/T2DM and NAFLD. These diseases are the major killers of the Western world, and approaches to treat or prevent them will have tremendous implications on overall human health.

A FAILED APPROACH TO MODULATE CASPASE-2 THROUGH VITAMIN SUPPLEMENTATION, IN THE MANAGEMENT OF NAFLD

INTRODUCTION

Coenzyme A – more than a master regulator of metabolism

Coenzyme A (CoA) is an essential co-factor required for 4% of all known enzymes, being a master regulator of energy metabolism (Jackowski and Leonardi, 2014; Leonardi et al., 2005). CoA was first described in 1947 as a factor required for the enzymatic acetylation of sulfanilamide by liver homogenates (Lipmann et al., 1947). This discovery won Fritz Lipmann the Nobel Prize, 6 years latter (Martinez et al., 2014). CoA contains an active thiol group that is able to form a thioester bond with organic acids, which makes CoA the major carrier of acyl groups, including the short-chain acetyl (Jackowski and Leonardi, 2014).

CoA is a multifaceted factor. CoA and its derivatives are required for the catabolism of proteins, carbohydrates and lipids, allowing energy extracted from food to be used in the form of acetyl-CoA (Davaapil et al., 2014). It is particularly important for FA oxidation (acyl-CoA and acetyl-CoA) and amino acids metabolism (propionyl-CoA and succinyl-CoA). It is also involved in anabolic reactions, namely in the biosynthesis of FA, ketone bodies and cholesterol (malonyl-CoA and HMG-CoA), as well as the biosynthesis of steroid hormones and the neurotransmitter acetylcholine (acetyl-CoA) (Martinez et al., 2014).

Not only CoA and its metabolites act as co-factors, but they can also modulate the action of several enzymes and transcription factors (Jackowski and Leonardi, 2014). An example of the former is the inhibition of CPT-1 by malonyl-CoA. This inhibitory effect is responsible for the inhibition of FA oxidation during lipogenesis (Foster, 2012). Interestingly, acyl-CoA can directly bind and modulate the transcriptional activity of hepatocyte nuclear factor (HNF)-4α, a main hepatocyte transcription factor (Hertz et al., 1998). Indeed, in the last decades, the known functions of CoA surpass its effect in metabolism, and we now know that CoA has a privileged place in regulating many signaling proteins and signal transduction pathways (Davaapil et al., 2014).

Long-chain-acyl-CoA, but not free CoA, can inhibit glucose-stimulated insulin release by pancreatic β-cells (Larsson et al., 1996). Long-chain-acyl-CoA can bind directly to the ATP-sensitive potassium channel, inducing a conformational change, which allows the channel to remain open, and hence active, for longer duration (Branstrom et al., 2007).

This hampers cell depolarization and activation of voltage-dependent calcium channels, and hence inhibits insulin secretion (Bokvist et al., 1999).

CoA and acyl-CoA can also differentially regulate the activity of different isoforms of protein kinase C (PKC), with many predicted metabolic consequences (Stasia et al., 1987; Yaney et al., 2000).

Another important kinase to be regulated by CoA, particularly by free CoA, is CaMKII (McCoy et al., 2013b). Activation of CaMKII depends on the phosphorylation of the residue Thyr286, induced by calcium/calmodulin binding (Hudmon and Schulman, 2002). CoA (and to a much lower extent acetyl-CoA) can bind specifically to a patch of positively charged residues (Lys292, Arg296 and Lys300) in the calmodulin-binding domain. It is believed that CoA binding increases the conformational flexibility of the protein, facilitating the interaction with calcium/calmodulin and subsequent activation (McCoy et al., 2013b). The actions of CoA on CaMKII are particularly important because active CaMKII mediates phosphorylation with inactivation of caspase-2 (Huang et al., 2014; McCoy et al., 2013a; Nutt et al., 2005).

Long-chain-acyl-CoA can also abrogate platelet aggregation induced by ADP and thrombin. That inhibition is believed to be the result of a direct interaction with the membrane P2Y1 receptor (Lascu et al., 1988; Lin et al., 1976). On the other hand, CoA-glutathione heterodimer (CoA-GSS, CoA-glutathione disulfide) potentiates vasoconstriction by increasing proliferation of vascular smooth muscle cells. That effect is believed to be a consequence of direct binding and activation of purinergic P2 receptors (Jankowski et al., 2000). Those effects in extracellular membrane receptors raise the question of how does CoA and its derivatives reach the extracellular space. CoA and its derivatives are large charged molecules, and hence cannot cross the plasma membrane (Davaapil et al., 2014). One explanation could be that CoA could function as DAMP, in a similar way as ATP, being released by damaged or dying cells. In that way, CoA could control the function of ATP during injury, maintaining a balance between the blood sparing effect of ATP on vasoconstriction, but simultaneously limiting the blood clot formation (Davaapil et al., 2014).

Finally, CoA derivatives are essential for post-translational acetylation/acylation of proteins, which can have tremendous impact on protein function (Yang and Seto, 2007). Those are reversible modifications that neutralize positively charged amino acids such as lysine, modulating protein function through modifying subcellular localization, stability, enzymatic activity and assembly of regulatory complexes. Thousands of acetylated

proteins are involved in cellular processes such as chromatin remodeling and gene transcription, cytoskeletal arrangement, metabolic regulation and cell signaling, among other yet unknown processes (Davaapil et al., 2014). As examples of the role of CoA derivatives in controlling important cell functions, acetyl-CoA can regulate expression of autophagy genes through histone acetylation. Acetyl-CoA acts like a sensor of cellular energy repletion, inhibiting autophagy (Eisenberg et al., 2014; Marino et al., 2014). Histone acetylation by acetyl-CoA can also regulate cell-cycle progression through its effects in the expression of cyclins, being a possible mechanism to explain increased cyclins expression with nutrient repletion (Shi and Tu, 2013; Siudeja et al., 2011).

The body distribution of CoA translates its metabolic functions. The liver has the highest CoA content, followed by kidney, adrenal glands and heart (Reibel et al., 1982). Its subcellular distribution reflects the plethora of processes in which CoA is involved. CoA concentration is higher in the mitochondria (2-5 mM), followed by peroxisomes (0.7 mM) and lastly by the cytosol (0.05-0.14 mM) (Horie et al., 1986; Williamson and Corkey, 1979). CoA levels are tightly regulated, though flexible, allowing for metabolic flexibility, that is adaptation of the utilization of nutrients according to their availability (Jackowski and Leonardi, 2014). Accordingly, fasting, glucagon, glucocorticoids and hypolipidemia drugs increase CoA levels (Berge et al., 1983; Horie et al., 1986; Kerbey et al., 1977; Reibel et al., 1981; Smith and Savage, 1980), whereas insulin, glucose, pyruvate and FA decrease CoA levels (Berge et al., 1984; Robishaw et al., 1982).

CoA levels can be regulated through its synthesis and degradation. To synthesize CoA, cells need three substrates: pantothenic acid, cysteine and ATP (Robishaw and Neely, 1985). The first step of CoA biosynthesis, and also the rate-limiting one, is the phosphorylation of pantothenate by PanK (pantothenate kinase) (Rock et al., 2000) (**Figure 59**). PanK has several isoforms, with a complex pattern of tissue and subcellular distribution. For example, PanK-1α localizes exclusively in the nucleus, whereas PanK-1β has cytosolic localization and associates with clathrin-coated vesicles and recycling endosomes, and Pank-3 is exclusively cytosolic (Alfonso-Pecchio et al., 2012). Pank-4 is believed to be a pseudogene.

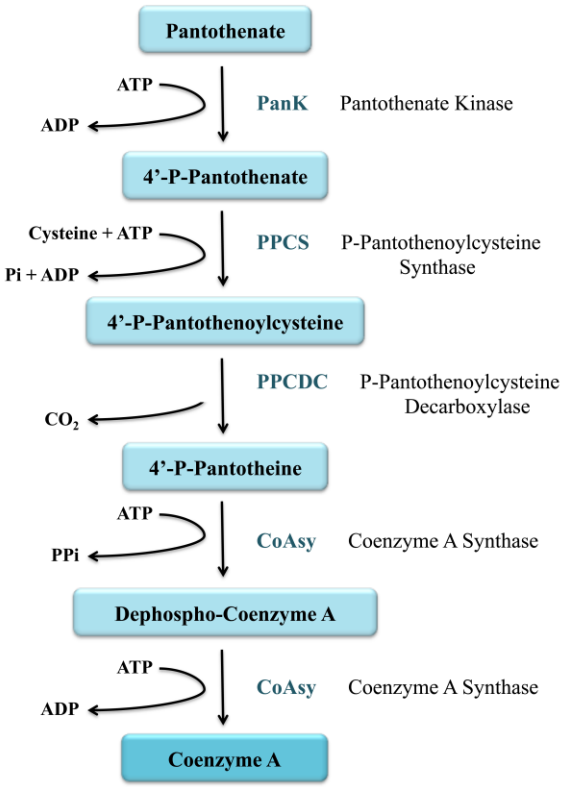


Figure 59. Coenzyme A biosynthetic pathway.

The CoA biosynthetic machinery seems to assemble in the cytosol in response to extracellular cues and stress, and newly synthesized CoA is afterward transported to the different subcellular compartments, in a highly regulated fashion (Martinez et al., 2014).

Pank enzymes are regulated through feedback inhibition by CoA species and through activation by long-chain acylcarnitines and acylethanolamines (Zhang et al., 2005). However, not all PanK enzymes respond similarly to this regulation. For example, Pank-2 and -3 are more sensitive than Pank-1. Roughly, both the isoform expression and regulatory properties correlate with the total amount of CoA in the different tissues. Liver, heart and kidney, that have high CoA levels, express preferentially Pank-1, whereas brain and skeletal muscle, that have lower CoA levels, express preferentially Pank-2 or -3 (Dansie et al., 2014).

Mice with either genetic Pank-1 deficiency or chemical Pank-1 inhibition, not only show lower levels of CoA in the liver, but also most importantly, do not increase CoA production in response to fasting. Those mice develop fasting hypoglycemia as a consequence of decreased FA β-oxidation, perturbed gluconeogenesis and ketogenesis. Moreover, those mice develop hepatic steatosis after fasting for 48 hours (Leonardi et al., 2010; Zhang et al., 2007).

CoA degradation is controlled by enzymes that regulate the bioavailability of the phosphopantetheine precursor, which is metabolized through the action of phosphatases and pantetheinases (Naquet et al., 2014). Panthetheinase is a ubiquitous enzyme, which hydrolyses D-panthetheine into cysteamine and pantothenate (Dupre and Cavallini, 1979; Wittwer et al., 1983). The mouse has two pantotheinases genes, vanin-1 and -3 (Vnn1 and Vnn3) (Martin et al., 2001). Vnn1 is ubiquitous, whereas Vnn3 expression is restricted to the hematopoietic lineage, particularly neutrophils (Naquet et al., 2014).

Coenzyme A can potentially affect the development and progression of NAFLD/NASH through its metabolic and regulatory effects. The link with caspase-2 activation is particularly appealing, since we showed that caspase-2 is a key regulator of the pathogenesis of liver steatosis and NASH. CoA status will be described in the next experimental protocol. We will also describe a strategy that intended to modulate liver CoA content, in an animal model of NASH.

Rational and aims for the experimental study

Although NAFLD/NASH is growing to become the liver pandemic in the Western world, an effective therapy for NASH is still not available. Recent large, randomized, placebo-controlled trials identified two agents that seem to improve histology in patients with NASH: vitamin E (Sanyal et al. 2010) and obeticholic acid (Neuschwander-Tetri et al., 2015; Sanyal et al., 2010). Vitamin E improved hepatocellular ballooning and inflammation, but it failed to improve fibrosis (Sanyal et al., 2010). Also, safety issues with vitamin E preclude its widespread use. Obeticholic acid seems to improve liver histology, including fibrosis, however it worsened the metabolic profile in patients with NASH (Neuschwander-Tetri et al., 2015). Hence, it is imperative to search for better NASH therapies.

NASH is characterized by increased hepatocyte apoptosis (Feldstein et al., 2003; Machado and Cortez-Pinto, 2011; Ribeiro et al., 2004). Caspase-2 induces apoptosis when lipids accumulate in the context of starvation (Johnson et al., 2013). Our work further showed that caspase-2 activation is also a pivotal player in NASH-related lipotoxicity (Machado et al., 2015b). We demonstrated increased caspase-2 expression and correlated this with fibrosis stage, in human NASH and several NASH animal models. Moreover, caspase-2

deficient mice were protected from diet-induced NASH, developing less hepatocyte cell death and fibrosis than wild type mice that were fed the same diets (Machado et al., 2015b). Caspase-2 activation is regulated by free coenzyme-A (CoA) content and free CoA/acyl CoA ratio. Decreases in free CoA/acyl CoA ratio occur during hepatic lipogenesis. This may activate caspase-2 during NASH by inhibiting the CaMKII-dependent phosphorylation of caspase-2 at serine-135, which inactivates the caspase (McCoy et al., 2013b). Interestingly, either fasting or HFD and T2DM have been shown to decrease the free CoA/acyl-CoA ratio in hepatocytes (Horie et al., 1986). This may explain why both starvation and energy-surplus promote caspase-2 activation and suggests that approaches to replenish free CoA might reduce caspase-2 activation in these conditions.

CoA is a cofactor of 4% of all known enzymes and is required for intermediary metabolism (Leonardi et al., 2005). CoA can be synthesized from pantothenate, cysteine, and ATP (Robishaw and Neely, 1985). Pantothenate (from the Greek "panthos" meaning "everywhere") is the water-soluble vitamin B5 (Spry et al., 2008). Because mammalian cells cannot synthesize pantothenate, they need vitamin B5 to be provided by the diet or generated by the intestinal microbiome, in order to synthesize CoA (Daugherty et al., 2002). Pantothenic acid deficiency rarely occurs because most foods are rich in vitamin B5 (Hodges et al., 1958). When dietary pantothenate is depleted in animals, however, liver levels of CoA decline, and serum levels of triglyceride and FFA increase, suggesting that hepatic lipid metabolism has become de-regulated (Wittwer et al., 1990). Consistent with this concept, inhibiting the first enzyme in the synthesis of CoA from pantothenate also causes hypertriglyceridemia. Further, blocking the synthesis of CoA from pantothenate leads to mitochondrial dysmorphology similar to human NASH, impairs hepatic β-oxidation of FA, induces hepatic steatosis, and increases hepatic gluconeogenesis (Noda et al., 1991; Ohsuga et al., 1989; Zhang et al., 2007). These observations suggested to us that the generation of CoA from pantothenate might be impaired in NASH, reducing hepatic stores of free CoA and thereby promoting excessive activation of caspase-2 and increased hepatocyte apoptosis.

Aims:

1. To describe the hepatic CoA metabolism in different dietary rodent models of NASH.
2. If disturbed CoA metabolism is found (aim 1), we aim to perform a pre-clinical study to evaluate the effect of treatment with vitamin B5 (pantothenate) and a cysteine precursor (N-acetylcysteine), substrates for CoA biosynthesis, in animal models of NASH.

Contribution of the PhD candidate:

The candidate had the idea to evaluate disturbances of CoA metabolism in NASH, as well as the role of manipulating CoA metabolism as a therapeutic approach in NASH. Under close orientation and guidance by Prof. Anna Mae Diehl, the candidate planned all the experiments, executed them and interpreted them. The candidate also wrote the article (which was edited by Prof. Anna Mae Diehl) that presented the data described in this chapter.

METHODS

Animal Studies

Male wild-type (WT) mice C57Bl/6 were obtained from Jackson Laboratory and fed either chow diet (Picolab® Rodent diet 20, #5053), MCD diet (MP Biomedicals, #960439) for 8 weeks or Western diet (45% high-fat diet, enriched in saturated fat, supplemented with 0.2% cholesterol and high-corn syrup equivalent in the drinking water, TD.120330 22% HVO + 0.2% cholesterol diet, Teklad Research) for 16 weeks (diet specifications in **Supplemental Tables 1 and 2**). Two separate experiments were done, the first compared chow-fed diet (n=6), MCD diet-fed (n=8) and Western diet-fed (n=8) mice; the second experiment studied 30 mice divided into 3 groups: chow diet, MCD diet and MCD diet treated with Pantothenate and N-acetylcysteine. Pantothenate (Sigma C8731) was administered IP in a solution in PBS, at a dose of 250 mg/kg three times per week. The control group was injected with same volume of PBS. N-acetylcysteine (Sigma, A9165) was administered in the drinking water, resulting in an estimated consumption of 250 mg N-acetylcysteine /kg body weight/day.

Animal care and procedures were approved by the Duke University Institutional Animal Care and fulfilled National Institutes for Health and Duke University IACUC requirements for humane animal care.

Histopathological analysis

Formalin-fixed, paraffin-embedded liver biopsies were cut into 5 μm serial sections, were stained with H&E and evaluated for severity of NAFLD.

For immunohistochemistry, sections were deparafinized with xylene and rehydrated. Slides were incubated in 3% hydrogen peroxide/methanol. Antigen retrieval was performed by heating in 10 mM sodium citrate (pH 6.0) or pepsin digestion, according to the primary antibody. Horseradish peroxidase-conjugated IgG secondary antibody was used. Antigens were demonstrated by diaminobenzidine (K3466, Dako). Tissue sections were counterstained with Mayer's Hematoxylin (S3309, Dako). Morphometric analysis was done with Metamorph Software (Molecular Devices Corporation, Downingtown, PA), in randomly chosen sections (20x magnification, 20 fields/sample). Immunohistochemistry antibodies are specified in **Supplemental Table 3**.

Serum and tissue analysis

Liver enzymes, alanine and aspartate aminotransferases (ALT and AST) were determined colorimetrically (Biotrom Diagnostics Inc, #398917 and #50726, respectively). Free CoA was quantified in flash frozen liver samples with Coenzyme-A detection kit (Abcam, ab102504) according to manufacturer protocol. Triglycerides were measured in the liver with Triglyceride Colorimetric Assay kit (Cayman Chemical Company: #10010303). Liver hydroxyproline content was colorimetrically quantified in flash frozen liver samples, as previously described (Syn et al., 2009). Caspase-3 activity was evaluated in 200 ug of whole liver cytosolic extracts, with Caspase-3 Colorimetric Activity Assay kit (EMD Millipore, APT 165), according to manufacturer protocol.

Molecular Studies

mRNA quantification by Real-time Reverse Transcription-PCR (RT-PCR). Total RNA was extracted from livers using TRIzol (Invitrogen). RNA was reverse transcribed to cDNA templates using random primer and Super Script RNAse H-Reverse Transcriptase (Invitrogen) and amplified. Semiquantitative qRT-PCR was performed using iQ-SYBR Green Supermix (Bio-Rad) and StepOne Plus Real-Time PCR Platform (ABI/Life Technologies), as previously described (Michelotti et al., 2013). For primers, see **Supplemental Table 4**.

Western Blotting. Total proteins were extracted from whole liver using RIPA buffer (Sigma). Proteins were separated by electrophoresis on 4%-20% Criterion gels (BioRad), transblotted into polyvinylidene-difluoride membranes, and incubated with the following primary antibodies specified in **Supplemental Table 5**.

Statistics

Results were expressed as mean±SEM. Significance was established using Mann-Whitney tests, with significance $p<0.05$. Correlations evaluated with Spearman coefficient.

RESULTS

Disturbances of CoA metabolism in NAFLD

CoA metabolism is perturbed in mouse models of NASH

Our group previously showed that caspase-2 is up-regulated in the livers of patients with NASH and correlates with the severity of fibrosis (Machado et al., 2015b). Also, animal models of NASH suggest a crucial role for caspase-2 in the pathogenesis of NASH, particularly in regulating cell death and fibrogenesis (Machado et al., 2015b). Because it has been reported that decreased free CoA promotes caspase-2 activation (McCoy et al., 2013b), we evaluated free CoA content and metabolism in a mouse model of NASH induced by feeding MCD diet. As compared to mice fed chow diet, mice fed with MCD diet for 8 weeks, developed a marked decrease in free CoA liver content (**Figure 60**).

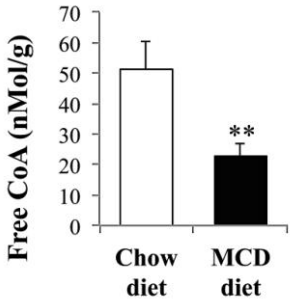


Figure 60. Mice with diet-induced NASH show decreased hepatic CoA levels.

Free CoA liver content in WT mice fed chow (n=6) or MCD diet (n=8). Results were graphed as mean±SEM. **p<0.01 chow *vs*. MCD diet.

Decreased free CoA content might reflect a shift in the free CoA/acyl CoA ratio that accompanies hepatic accumulation of FFA in this model (Machado et al., 2015c). A decrease in CoA synthesis could also be involved.

Therefore, we used qRT-PCR (**Figure 61A**) and Western-blot (**Figure 61B**) to evaluate the liver expression of enzymes in the CoA synthetic pathway, including PANK1, the rate-limiting enzyme in this process.

A marked decrease in PANK expression was observed. This finding was intriguing because a feedback inhibition of PANK expression by acyl-CoA has been described (Karasawa et al., 1972; Rock et al., 2002; Zhang et al., 2005). Lastly, we evaluated the expression of enzymes in the CoA degradation pathway, vanin-1 and vanin-3. qRT-PCR demonstrated a significant up-regulation of these genes (**Figure 61A**).

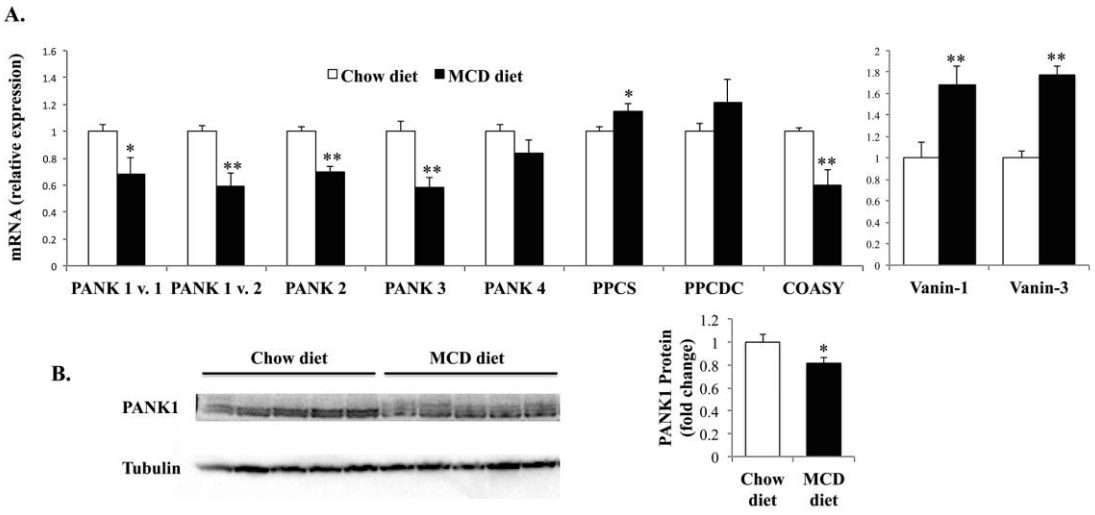


Figure 61. Mice fed NASH-inducing diet show perturbations of CoA metabolism.

A. qRT-PCR analysis of whole liver mRNA for genes in the CoA synthetic pathway and CoA degradation pathway, in mice fed chow or MCD diet. B. Immunoblot for liver PANK-1. Results were graphed as mean±SEM and normalized to expression in chow-diet fed mice. *p<0.05, **p<0.01 chow *vs*. MCD diet.

We found similar changes in CoA metabolism in a different model of NASH caused by feeding Western diet for 16 weeks (**Figure 62**).

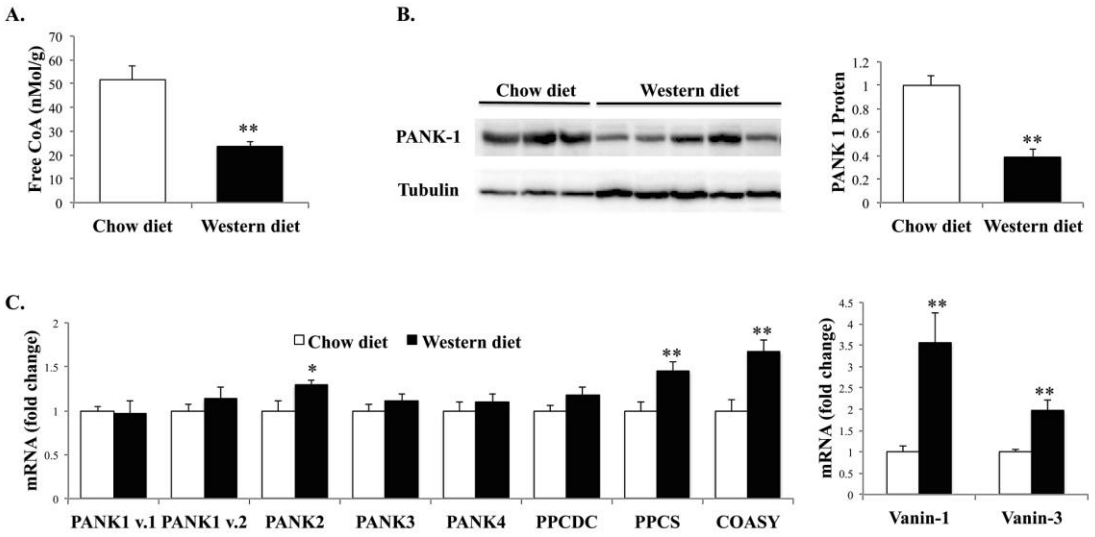


Figure 62. Mice with Western diet-induced NASH show perturbations of CoA metabolism.

A. Free CoA liver content in WT mice fed chow (n=8) or Western diet (n=8). B. Immunoblot for liver PANK-1. C. qRT-PCR analysis of whole liver mRNA for genes in the CoA synthetic pathway and CoA degradation pathway. Results were graphed as mean±SEM and normalized to expression in chow-diet fed mice. *p<0.05, **p<0.01 chow *vs*. Western diet.

Thus, in two mouse models of NASH, we found a profound decrease in liver free CoA content. Two potential mechanisms for this were also demonstrated, i.e., decreased synthesis of free CoA and increased CoA degradation.

To assess whether or not CoA metabolism might be similarly altered in human NASH, we examined a previous microarray analysis of NASH patients (Moylan et al., 2014) and found CpG hypermethylation and decreased PANK-1 gene expression in patients with advanced fibrosis (i.e. fibrosis grade 3-4, n=32), as compared to those with mild fibrosis (i.e. fibrosis grade 0-1, n=40). Thus, the human data support the findings in pre-clinical models: both suggest that free CoA stores can decline during NASH.

Hepatic free CoA content negatively correlates with apoptosis and liver injury

In mice fed either chow diet or MCD diet, we found strong negative correlations between hepatic levels of free CoA and evidence of cell death by apoptosis as assessed by ALT serum levels, TUNEL immunohistochemistry and caspase-3 activity (**Figure 63**). Consistent with the concept that free CoA inhibits caspase-2 activity and protein stability, we found an inverse correlation between free CoA and expression of activated caspase-2 protein on Western blot (**Figure 63**).

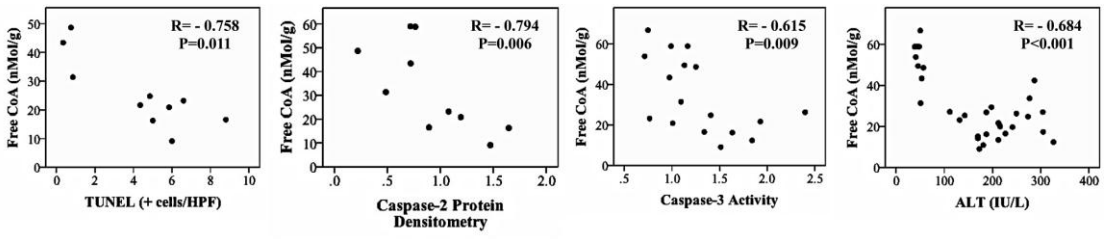


Figure 63. Hepatic free CoA content negatively correlates with apoptosis and liver injury.

In mice fed either chow diet or MCD diet, correlations between free CoA liver content and apoptosis markers in whole liver (TUNEL, densitometry for caspase-2 assessed by western blot, caspase-3 activity) and ALT serum levels. Correlations evaluated with Spearman Coefficient.

We also found strong negative correlations between free CoA content and the fibroductular reaction (**Figure 64A**), proinflammatory cytokine TNF-α, and macrophage markers F4/80 and YM-1 (**Figure 64B**).

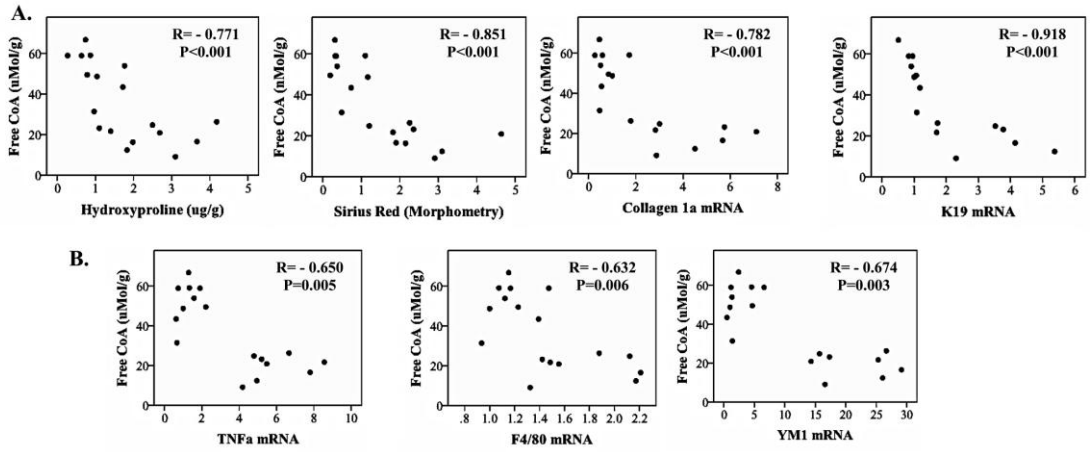


Figure 64 – Hepatic free CoA content negatively correlates with fibroductular response and inflammation.

In mice fed either chow diet or MCD diet, correlations between free CoA liver content and markers of fibroductular reaction (hydroxyproline content, Sirius red morphometry, collagen 1a and K19 expression by qRT-PCR analysis) (A), and inflammatory markers TNF-a, F4/80 and YM-1 expression by qRT-PCR analysis (B). Correlations evaluated with Spearman Coefficient.

Treatment with CoA precursors, vitamin B5 and N-acetylcysteine, as a therapeutic approach in NAFLD

Treatment with pantothenate and N-acetylcysteine does not protect from liver injury in the MCD model

Because we found decreased levels of free CoA and evidence for decreased CoA synthesis in mouse models of NASH, we performed a second experiment to evaluate the effect of treatment with the CoA precursors, pantothenate (P) and N-acetylcysteine (NAC) (Daugherty et al., 2002), on liver injury. Treatment with CoA precursors did not prevent weight loss in the MCD model and caused a mild increase in liver-to-body ratio (**Figure 65A**). Such hepatomegaly might reflect the tendency for increased liver triglyceride accumulation in P+NAC-treated mice (**Figure 65D**).

Furthermore, treated and untreated mice submitted to MCD diet showed similar liver injury as assessed by aminotransferases serum levels (**Figure 65B**) and evaluation of H&E stained liver sections (**Figure 65C**). Treatment also did not improve inflammation as assessed by qRT-PCR analysis of pro-inflammatory genes (TNF-α, F4/80 and YM-1) in whole liver. In fact, those transcript levels were higher in MCD diet-fed mice treated with P+NAC than untreated MCD diet-fed mice.

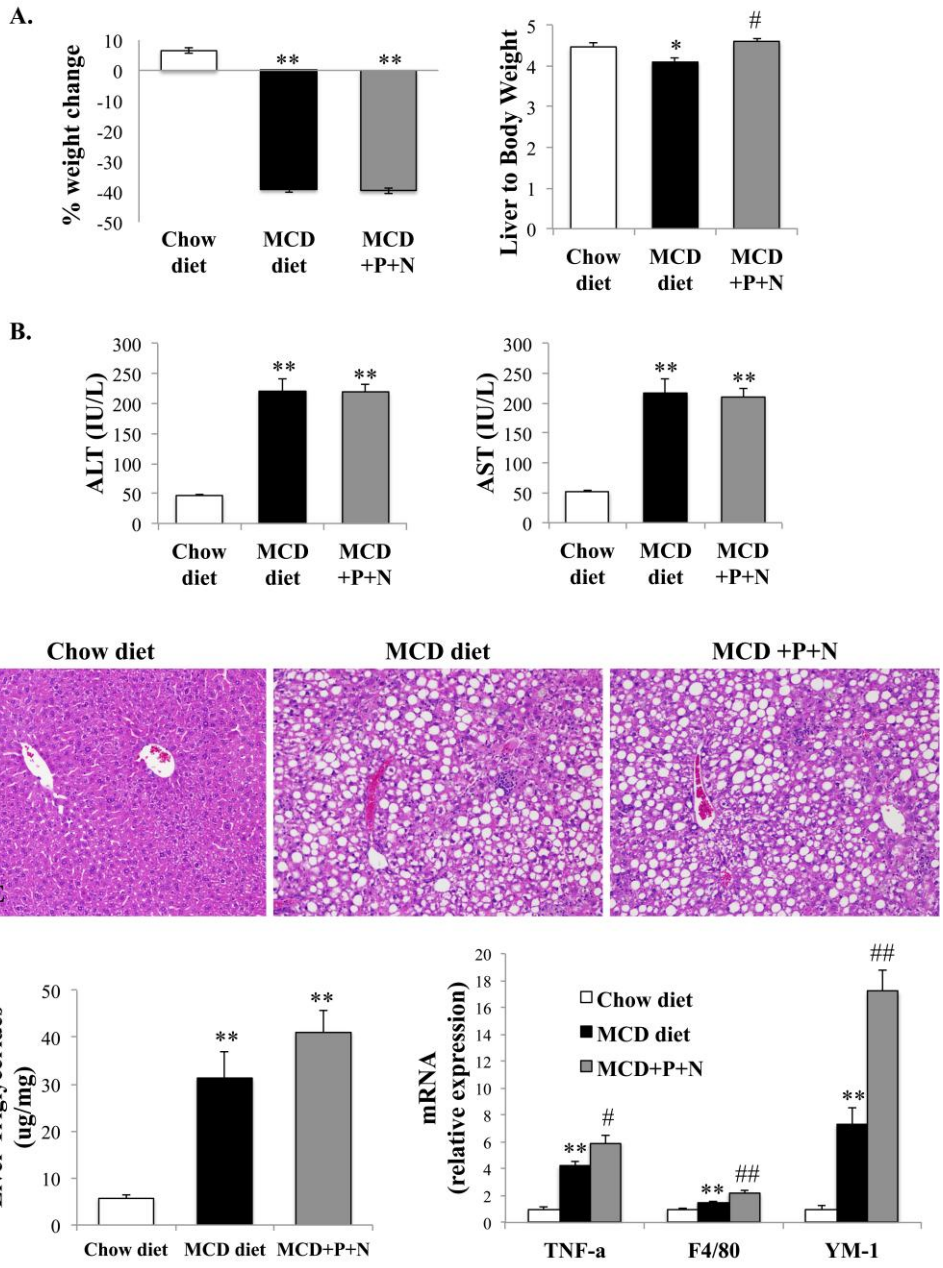


Figure 65. Treatment with Pantothenate and N-acetylcysteine does not protect from liver injury in the MCD model.

WT mice (n=30) were divided in 3 groups: chow diet and MCD diet with or without treatment with pantothenate and N-acetylcysteine. A. At the end of treatment, weight loss and liver to body weight ratio were recorded. B. Aminotransferase serum levels. C. Representative liver sections stained with H&E (20xd field). D. Right panel: Quantification of liver triglycerides content. Left panel: qRT-PCR analysis of whole liver mRNA for inflammatory genes, normalized for chow diet-fed mice. Results were graphed as mean±SEM. *p<0.05, **p<0.01 *vs*. chow diet. #p<0.05, ##p<0.01 *vs*. MCD diet.

Treatment with pantothenate and N-acetylcysteine does not protect from fibroductular response in the MCD model

In human NASH, the level of injury does not independently dictate liver-related morbidity or mortality. Rather, fibrosis is the most important factor that predicts worse liver outcomes (Angulo et al., 2015a; Ekstedt et al., 2015). Hence, it is the repair response to lipotoxicity that dictates prognosis (Angulo et al., 2015b). Taking that into consideration, we evaluated the effect of P+NAC on the ductular reaction (i.e., accumulation of ductular-appearing progenitors and myofibroblasts) because fibrosis severity in NASH patients and animal models of NASH strongly correlates with the intensity of the ductular reaction (Richardson et al., 2007). Compared to untreated mice, treated mice were not protected from liver fibrosis, as assessed by hydroxyproline content (**Figure 66A**) or qRT-PCR/immunohistochemistry analysis of the fibrogenic markers α-SMA, collagen-1a and desmin (**Figure 66B and C**).

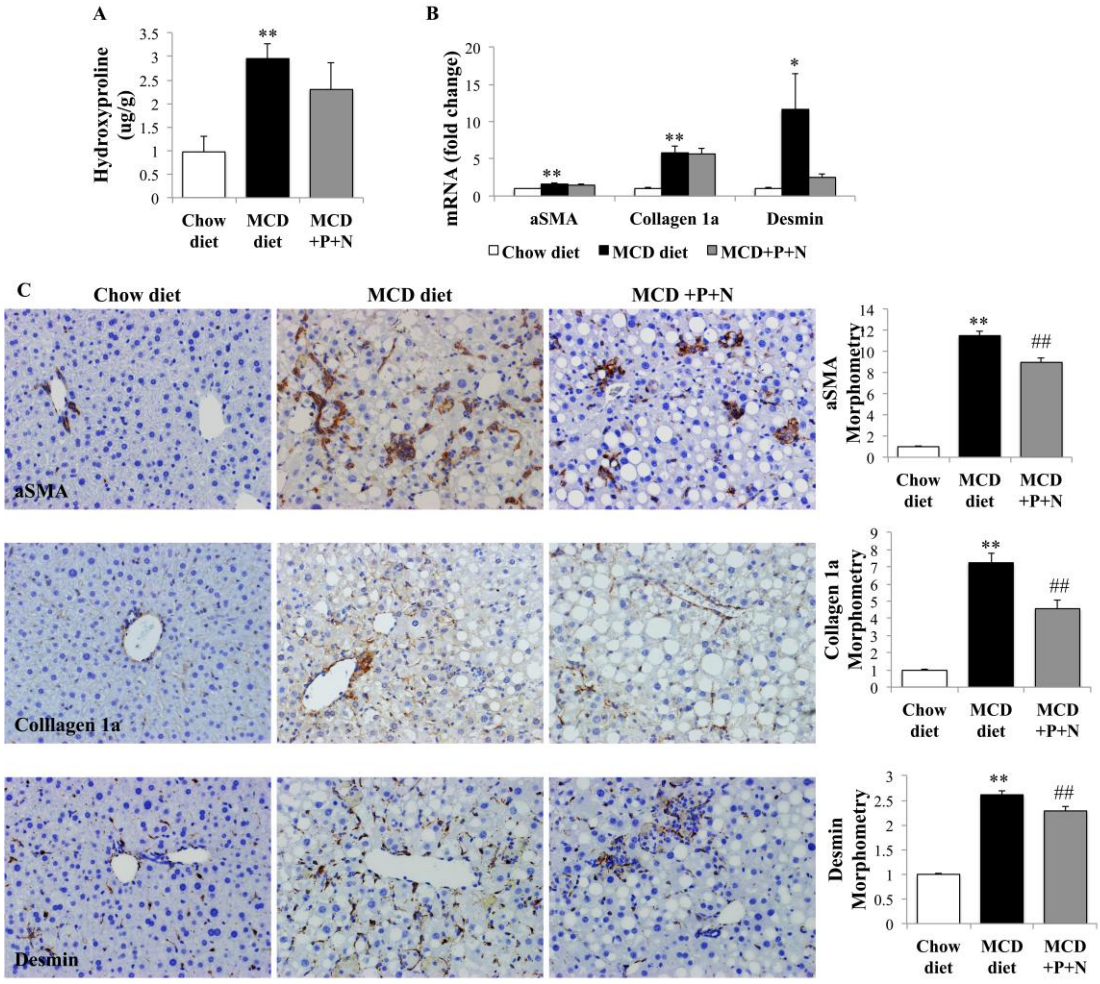


Figure 66. Treatment with Pantothenate and N-acetylcysteine does not protect from fibrosis, in the MCD model.

A. Hydroxyproline liver content determination in the same mice as figure 65. B. qRT-PCR analysis of whole liver mRNA for genes associated with fibrogenesis. C. Liver sections from representative mice stained for markers of fibrosis (α-SMA, collagen-1a and desmin) (left panels) and respective morphometry (right panels). Results were normalized for chow-diet mice and graphed as mean±SEM. *p<0.05, **p<0.01 *vs*. chow diet. ##p<0.01 *vs*. MCD diet.

The progenitor reaction of treated and untreated mice was also similar, i.e., P+NAC did not diminish MCD diet-related induction of the progenitor markers, K7 and K19, as evaluated by qRT-PCR and immunohistochemistry (**Figure 67A and B**).

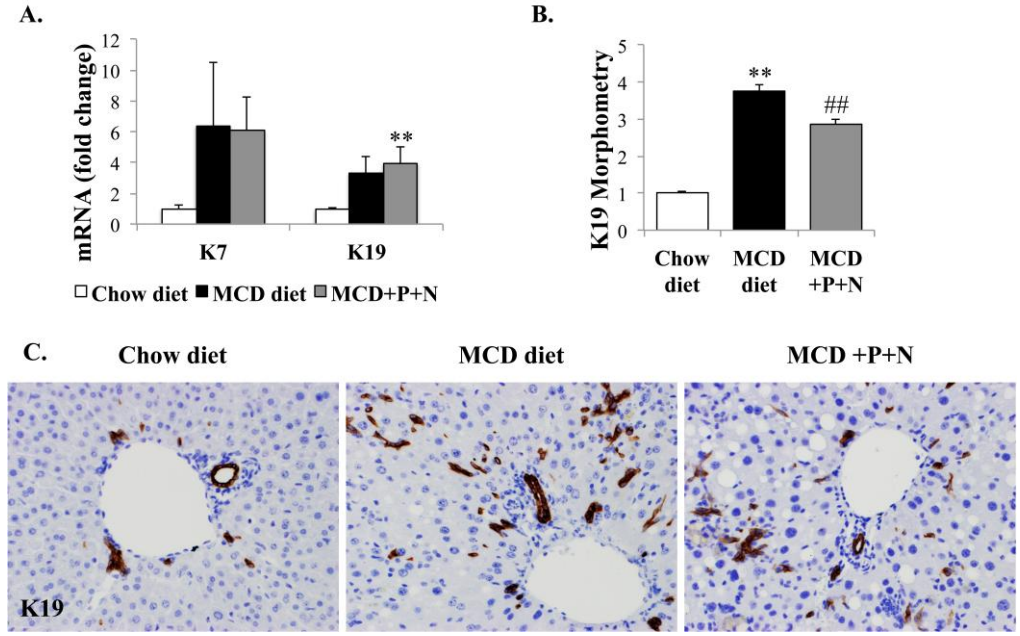


Figure 67. Treatment with Pantothenate and N-acetylcysteine does not protect from the ductular reaction in the MCD model.

A. qRT-PCR analysis of whole liver mRNA for genes associated with ductular reaction/progenitor markers, in the same mice as previously. C. Liver sections from representative mice stained for the progenitor markers of K19 and B. respective morphometry. Results were normalized for chow-diet mice and graphed as mean±SEM. **p<0.01 *vs*. chow diet. ##p<0.01 *vs*. MCD diet.

Treatment with Pantothenate and N-acetylcysteine does not reverse caspase-2 up-regulation or impairment in CoA metabolism, in the MCD model

Since we gave precursors of CoA, which promotes caspase-2 inactivation (McCoy et al., 2013b), and caspase-2 seems to have a crucial role in NASH pathogenesis (Machado et al., 2015a), we evaluated caspase-2 induction (by qRT-PCR) and activation (by Western-blot analysis for cleaved caspase-2) in whole liver extracts. Treatment with P+NAC did not protect mice from MCD diet-related induction or activation of caspase-2 (**Figure 68**).

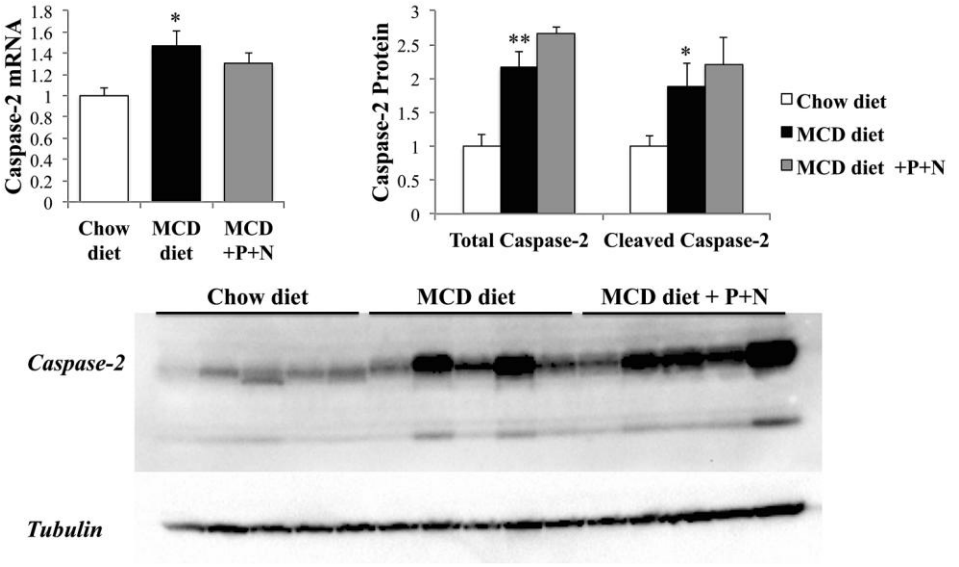


Figure 68. Treatment with Pantothenate and N-acetylcysteine does not reverse caspase-2 up-regulation, in the MCD model.

Whole liver expression of caspase-2 assessed by qRT-PCR and western-blot, in the same mice as previously. Results were normalized for chow-diet mice and graphed as mean±SEM. *p<0.05, **p<0.01 *vs*. chow diet.

In retrospect, this finding was not unanticipated because we found that treatment with CoA precursors did not increase the hepatic content of free CoA, or reverse the MCD diet-related down-regulation of rate-limiting enzymes in the CoA synthesis pathway in our model (**Figure 69**).

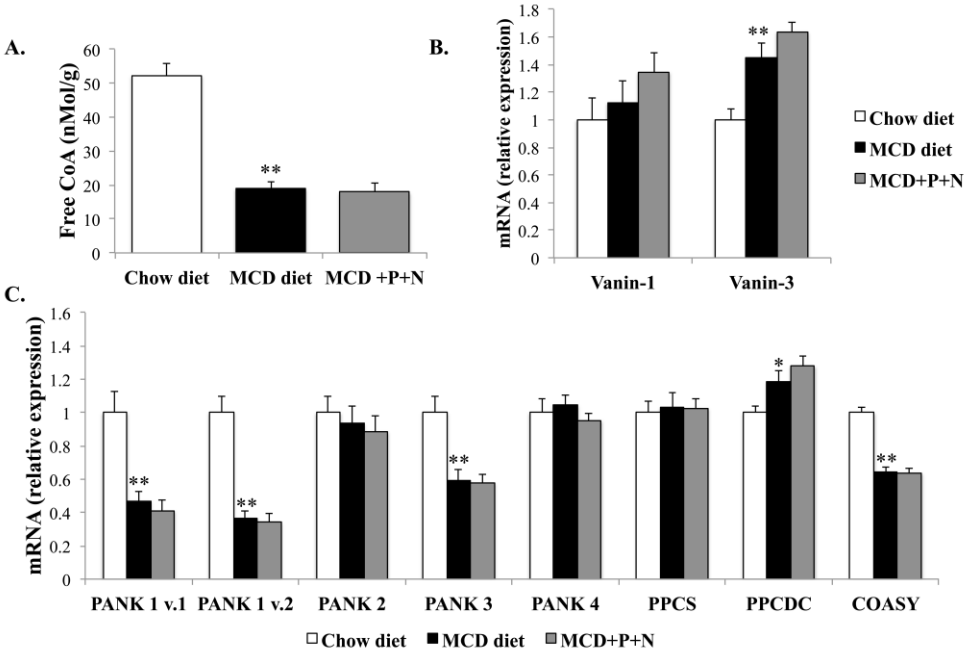


Figure 69. Treatment with Pantothenate and N-acetylcysteine does not correct CoA metabolism, in the MCD model.

A. Free-CoA liver content in the same mice as previously. B and C. qRT-PCR analysis of whole liver for genes in the CoA synthetic and degradation pathways. Results were normalized for chow-diet mice and graphed as mean±SEM. *p<0.05, **p<0.01 *vs*. chow diet.

Treatment with Pantothenate and N-acetylcysteine does not decrease MCD-induced oxidative stress

Oxidative stress is believed to play a crucial role in the pathogenesis of human NASH (Alkhouri et al., 2014; Ucar et al., 2013). Antioxidant enzymes, such as superoxide dismutase 1 and 2 (SOD-1 and -2) neutralize toxic hydroxyl radicals, nitric oxide radicals, and superoxide anions, converting them into H_2O_2, which is further detoxified by GPx and catalase. Thus, oxidative stress typically triggers compensatory induction of SOD and GPx. N-acetylcysteine provides a source of cysteine to replenish stores of reduced glutathione and thus, NAC has antioxidant properties that reduce the stimulus for antioxidant enzyme induction (Pereira-Filho et al., 2008). NAC has also been reported to reduce hepatic steatosis and inflammation in a HFD rat model of NAFLD (Thong-Ngam et al., 2007).

In mice submitted to MCD diet, however, treatment with NAC did not improve mRNA expression of anti-oxidant enzymes (**Figure 70**).

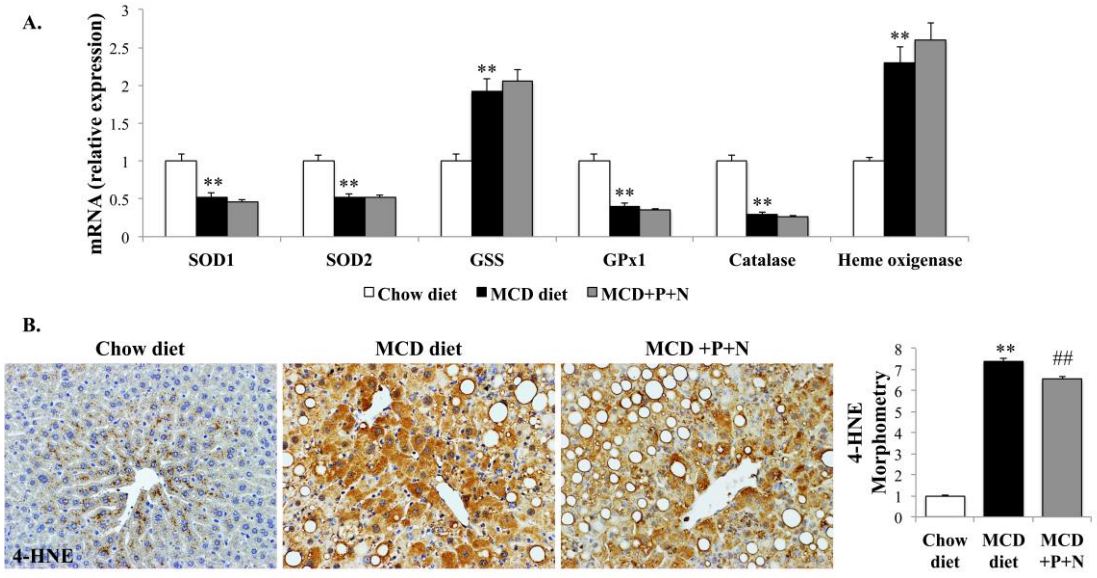


Figure 70. Treatment with Pantothenate and N-acetylcysteine does not decrease MCD-induced oxidative stress.

A. qRT-PCR analysis in whole liver for enzymes involved in oxidative stress in the same mice as previous figures. B. Liver sections from representative mice stained for markers of the oxidative stress

marker 4-hydroxynonenal (4-HNE) (left panel) and respective morphometry (right panel). Results were normalized for chow-diet mice and graphed as mean±SEM. *p<0.05, **p<0.01 *vs*. chow diet.

Also, it did not improve oxidative stress as assessed by gene expression of heme oxigenase [known to be up-regulated in response to oxidative stress (Choi and Alam, 1996)] and by immunohistochemistry for 4-HNE, a byproduct of lipid peroxidation.

These findings suggest that supplementing NAC was not sufficient to overcome the negative impacts of CoA depletion on hepatic redox balance in MCD diet-fed mice.

DISCUSSION

Our study showed that the hepatic content of free CoA is significantly decreased in two animal models of NASH. We also discovered that hepatic levels of free CoA strongly and inversely correlate with liver caspase-2 activation, apoptosis, inflammation, the intensity of fibrogenic repair responses such as the ductular reaction, and liver fibrosis severity. In addition, we identified two potential mechanisms that may contribute to the reduced free CoA content in mice with NASH, namely down-regulation of key enzymes in the biosynthetic pathway of CoA from pantothenate, and up-regulation of enzymes in the CoA degradation pathway. Reciprocal changes in free CoA synthesis and degradation could confound the increased demand for free CoA to generate the acyl-CoA required for FA metabolism. Preliminary evidence for decreased expression of the first enzyme in CoA biosynthesis was also documented in NASH patients with severe fibrosis, relative to those with mild fibrosis suggesting that free CoA synthesis may also be impaired in human NASH. Decreases in the free CoA/acyl-CoA ratio has critical consequences for lipid and glucose metabolism, since this regulates key metabolic reactions catalyzed by acyl-CoA synthetase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase (Robishaw and Neely, 1985). Importantly, decreases in free CoA are also known to activate caspase-2 (McCoy et al., 2013b), a pivotal pro-apoptotic protein in the pathogenesis of NASH (Machado et al., 2015b). In an attempt to restore free CoA levels during NASH, we treated MCD diet-fed mice with pantothenate and N-acetylcysteine, precursors of CoA. However, this approach did not reduce liver injury or improve fibrosis. Treatment failure might have reflected the fact that our protocol was unable to normalize hepatic free CoA content in this model. Consistent with its failure to improve free CoA levels, treatment did not blunt the induction of caspase-2, a key driving force for liver apoptosis in NASH pathogenesis. We cannot exclude the possibility that the treatment failed because of the specificities of the protocol. However, in an experimental model of NASH induced by HFD in mice with perturbed ketogenesis, *ex vivo* liver perfusion with pantothenate and L-cysteine restored CoA levels (Cotter et al., 2014). We used a high dose of pantothenate, which has been shown by others to be effective in improving insulin resistance and dyslipidemia in a hypothalamic mouse model of obesity (Naruta and Buko, 2001), but that is three times lower the toxic dose (Shibata et al., 2005). Furthermore, we used a protocol for N-

acetylcysteine that has resulted in improvement of oxidative stress in other diseases mouse models (Wang et al., 2013).

The fact that administering pantothenate did not correct free CoA levels in our mice suggests that CoA levels are tightly regulated. Early studies showed that adult rodents maintain stable CoA levels when the dietary supply of pantothenic acid is drastically reduced or increased (Karasawa et al., 1972). This suggests that pantothenate levels *per se* may not be rate-limiting factor for CoA biosynthesis, or that tissue levels of pantothenate typically exceed those that are required for CoA synthesis. Indeed, tissue pantothenate levels are maintained by compensatory changes in urinary excretion of vitamin B5 when dietary ingestion of pantothenate is altered (Tahiliani and Beinlich, 1991). CoA synthesis itself is highly regulated at the level of the first enzyme of the pathway, pantothenate kinase (PANK). There is a strong feedback inhibition of PANK expression and activity by acetyl-CoA and acyl-CoA (Karasawa et al., 1972; Robishaw and Neely, 1985; Rock et al., 2002; Zhang et al., 2005). Our results suggest that supplemental pantothenate might be unable to fuel CoA synthesis during NASH because PANK expression is severely suppressed during NASH. Further, newly synthesized CoA from pantothenate would be rapidly esterified with FA, further inhibiting PANK. Consistent with this hypothesis, treatment with pantothenate did not correct the down-regulation of PANK expression in MCD diet-fed mice. Despite the present dismal results, a modified approach for supplementing pantothenate might have some merit in NASH. Concomitant administration of L-carnitine and pantothenate was reported to overcome the feedback inhibition of PANK (Robishaw and Neely, 1985; Thurston and Hauhart, 1992). This success might reflect the fact that L-carnitine can accept acyl groups from acyl-CoA, thereby importing acyl groups into mitochondria to facilitate fatty acid β-oxidation and liberating CoA (Mitchell et al., 2008). Further research is needed to evaluate the effects of supplemental pantothenate plus carnitine in NASH. Enthusiasm for this approach should be tempered by the present evidence that treatment with pantothenate and N-acetylcysteine exacerbated MCD diet-related accumulation of mRNAs encoding TNF-α (a major pro-inflammatory cytokine) and macrophage activation markers, F4/80 (a marker of classical activation) and YM-1 (a marker of alternative activation). This finding was unexpected, but might be explained by the fact that pantothenate is essential for the growth of pathogenic microorganisms and thus, may have altered the gut microbiome (Spry et al., 2008). Another surprising result was the fact that we failed to detect improvements in hepatic

oxidative stress in mice that were treated with N-acetylcysteine, which has known anti-oxidant properties (Pereira-Filho et al., 2008).

In conclusion, free CoA is reduced in NASH. This may contribute both to metabolic impairment and caspase-2 activation. Hence, strategies to restore free-CoA levels may be beneficial in NASH. Administration of CoA precursors, pantothenate (vitamin B5) and N-acetylcysteine, was unable to correct the reduced hepatic free CoA content in the MCD diet mouse model of NASH. We believe that the lack of efficacy in our study is explained by the NASH-related down-regulation of the rate-limiting enzyme for CoA biosynthesis that cannot be overcome simply by increasing substrate levels alone. Further studies are needed to determine if adding carnitine supplements to pantothenate can overcome this obstacle and thereby restore levels of free CoA, reduce caspase-2 activation, and prevent NASH.

CONCLUSION

NAFLD, in its primary form, can be considered the hepatic manifestation of obesity and the metabolic syndrome, which explains why NAFLD is the hepatic pandemic of this century (Loomba and Sanyal, 2013). In fact, in 2014, almost 2 billion persons worldwide were overweight and more than half a million obese (WHO, 2015), which represents a two fold increase regarding the prevalence of obesity in 1980, when NAFLD was first described (Ludwig et al., 1980).

The liver responds to chronic injury in a monotonous way. Similarly to other chronic liver diseases, such as induced by alcohol (Mathurin and Bataller, 2015), virus (Nguyen and Hu, 2014) and parasites (Lambertucci, 2014), not all patients with NAFLD will have progressive liver disease. In fact, only 10-20% of patients with metabolic ectopic fat accumulation in the liver show evidence of hepatocellular damage, inflammation and cell death, a.k.a. NASH. Moreover, only 20% of patients with NASH are expected to develop liver cirrhosis and end-stage liver disease (Machado and Cortez-Pinto, 2014b). Also, in NAFLD, again similarly to other liver diseases, rather than the degree of hepatocellular injury or the severity of the inflammatory response, it is the presence and severity of fibrosis that dictates the liver outcome (Angulo et al., 2015a; Ekstedt et al., 2015). In other words, it is not the level of injury that predicts liver prognosis, rather the individual response to that injury. When facing epithelial injury, the liver initiates a regenerative/repair response. When that response is sustained and overly active, it does not restore normal architecture of the liver, rather it induces scarring and fibrogenic responses that can lead to cirrhosis and end-stage liver disease (Machado and Diehl, 2014; Machado et al., 2015a).

Considering NAFLD a disease associated with excessive mortality as compared to the general population (Adams et al., 2005; Ekstedt et al., 2015; Vernon et al., 2011), we have to evaluate not only liver disease, but most importantly, global mortality and particularly cardiovascular mortality, the main cause of death in patients with NAFLD (Lazo et al., 2011). This excessive cardiovascular mortality [two fold increased compared to the general population (Adams et al., 2005)] has been explained by the fact that NAFLD develops in the context of important cardiovascular risk factors, such as obesity, IR/T2DM and the metabolic syndrome (Porepa et al., 2010; Wang et al., 2013). However, we now know that NAFLD increases cardiovascular risk independently of other risk factors (Assy et al., 2010; Ekstedt et al., 2006; Stepanova and Younossi, 2012; Targher et al., 2007; Wong et al., 2011). Also, NAFLD increases the risk of developing the cardiovascular risk factors itself, such as IR/T2DM (Bae et al., 2011; Cicero et al., 2013; Ekstedt et al., 2006;

Fraser et al., 2009; Sung and Kim, 2011; Yamada et al., 2010). Ectopic fat accumulation in the liver is a consequence, but also a cause, of whole-body dysmetabolism (Machado and Cortez-Pinto, 2014a).

NAFLD is a growing health threat because it is a highly prevalent disease, potentially progressive, to which an effective treatment is still lacking (Machado and Cortez-Pinto, 2014a). As such, it should be a priority in Hepatology to better understand the pathogenesis of NAFLD and to develop new therapeutic approaches for NAFLD.

To have a clinical impact in the liver burden and treatment of NAFLD, basic/translational research could focus on common mechanisms of liver repair/scarring when facing liver injury. This strategy has the potential advantage of being transversal to other chronic liver diseases. Alternatively, research could focus on mechanisms of liver injury, and particularly lipotoxicity. This strategy, particularly if those mechanisms could be holistically integrated in the context of the metabolic syndrome, can potentially impact global morbi-mortality in patients with NAFLD. Whichever strategy is applied, in order to evaluate liver outcomes, the best approach is to evaluate the repair response and most importantly, the presence and severity of liver fibrosis.

The work developed for this thesis adds important knowledge in the pathogenesis of NAFLD and of the metabolic syndrome, and suggests cues to study new therapeutic approaches. We first dedicated to evaluate the better tools to model NAFLD/NASH. There are many animal models of NASH (Anstee and Goldin, 2006; Charlton et al., 2011; Diehl, 2005; Takahashi et al., 2012), but such range of possibilities comes with uncertainties when choosing an animal model. Because we specifically wanted to study the effects of caspase-2, for which a genetically deficient mouse has already been extensively characterized and studied (Bergeron et al., 1998), we were particularly interested in a dietary mouse model of NASH. Two dietary mouse models are most commonly used, feeding MCD and Western diet, both being described as inducing fibrosis (Itagaki et al., 2013; Mells et al., 2012), the most desired histological parameter, since it can be used as proxy of adverse liver outcome (Angulo et al., 2015a; Ekstedt et al., 2015). Although each model has its advocates, a true head-to-head comparison between both models was still not available, and hence the choice of a model would not truly be evidenced-based. In this context, we performed a head-to-head comparison between MCD diet and Western diet NASH mouse models, applying the diets at the same time, in the same facility, in the same mouse genetic background and gender, matching the animals for age at sacrifice. We confirmed some of the empiric understanding of those animal models, and found

interesting new information that helped us planning our experiments, and may help clarifying the field (Machado et al., 2015c). Both dietary models induce severe ectopic fat accumulation in the liver, worse when feeding Western diet. However, the MCD model induces much worse liver injury, with defective regeneration (translating in a decrease in liver mass, despite the accumulation of fat), important cell death/apoptosis, and a robust scarring response with development of significant liver fibrosis. In opposition, Western diet induced minimal liver injury and trivial liver fibrosis. As such, in order to study the liver outcome and the effect of an intervention/genetic susceptibility to develop clinically relevant liver disease, the MCD diet model is more appropriate. However, the MCD diet model does not result from energy surplus and systemic metabolic derangement, on the contrary, it associates with cachexia and peripheral hypersensitivity to insulin. In the MCD diet model, fat accumulation in the liver is a result of impairment in liver fat export, whereas in the Western diet model, fat accumulation is due to higher liver fat import (mainly fat derived from the adipose tissue) and increased liver lipogenesis, resembling metabolic human NAFLD (Donnelly et al., 2005; Tamura and Shimomura, 2005). As a consequence, if we wish to study NAFLD in a more holistic context of the metabolic syndrome, the Western diet model should be the preferred one. Because both animal models are truly complementary, we used both animal models in our research.

We decided to study the role of caspase-2 in NAFLD/NASH and more broadly in energy surplus induced adiposopathy, after recent exciting data being published regarding the role of caspase-2 in lipotoxicity-associated apoptosis. Caspase-2 was shown to be the link explaining apoptosis induced by cellular FA accumulation. This was described in cellular extracts from *Xenopus* oocytes, as well as in human hepatoma cell lines (Johnson et al., 2013). We anticipated important implications of this discovery to our field, since obesity, metabolic syndrome IR/T2DM and NAFLD can simplistically be explained as the result of lipotoxicity in the adipose tissue, whereas NASH as the consequence of lipotoxicity in the liver. Suggesting the role of caspase-2 in the pathogenesis of human NAFLD/NASH, we found increased expression of caspase-2, by immunohistochemistry, in the livers of patients with NAFLD. Importantly, caspase-2 expression positively associated with the severity of liver fibrosis, suggesting a role in the progression of liver disease (Machado et al., 2015b). Similarly to human NAFLD, we consistently found increased hepatic caspase-2 expression (at the mRNA level and at the protein level, by immunoblotting and immunohistochemistry) in different genetic and dietary mouse models of NAFLD/NASH

(Machado et al., 2015b). Those preliminary results encouraged us to further study the role of caspase-2 in the pathogenesis of NAFLD and obesity-associated metabolic syndrome.

We applied both MCD and Western diets to WT and caspase-2 deficient mice. We found that caspase-2 deficient mice were strikingly protected from most features of the metabolic syndrome induced by a Western-type diet. Caspase-2 deficient mice had an impressively lower body fat percentage, did not develop IR/T2DM or dyslipidemia. Also, contrary to WT mice, caspase-2 deficient mice developed minimal, not clinically relevant, liver ectopic fat accumulation, a.k.a NAFLD. Those findings associated with a fundamentally different adipose tissue. Caspase-2 deficient mice harbored a more proliferative adipose tissue that does not respond to energy surplus with hypertrophy, it is protected from lipotoxicity-associated apoptosis (*in vitro* and *in vivo*) and shows evidence of browning, with increased expression of mitochondrial uncoupling proteins. Hence, caspase-2 deficient mice are more efficient in dealing with fat overload, and in that way are protected from adiposopathy-associated metabolic syndrome and NAFLD. We further demonstrated that caspase-2 deficiency protects from hepatic lipotoxic injury, independently of the presence of the metabolic syndrome and of the route of fat accumulation. Hepatocytes deficient in caspase-2 were protected from FA induced apoptosis (*in vitro* and *in vivo*). As a consequence, in the liver of mice deficient in caspase-2, NASH induced a softer repair response and there was protection from liver fibrosis. Protection from liver fibrosis did not seem to be the result of a direct effect in HSC, rather, a consequence of less hepatocellular injury with lower release of alarm signals to trigger a wound healing response, in other words, lower pro-fibrogenic paracrine effect from fat-filled hepatocytes in HSC. The aggregate data places caspase-2 as a potential target to treat NAFLD and, more broadly, obesity and its consequences.

Lastly, we tried a therapeutic approach to modulate caspase-2, which failed to accomplish the desired results. Depletion of free CoA potentiates caspase-2 activation, *in vitro* (McCoy et al., 2013b). NAFLD results in accumulation of FA, which have the potential to consume CoA, with the accumulation of its derivative acyl-CoA. Accordingly, we found decreased hepatic free CoA content in different animal models of NASH. That was accompanied with an important down-regulation of enzymes in the CoA biosynthetic pathway and up-regulation of enzymes in the CoA degradation pathway. In severe human NASH, as compared to mild NAFLD, we also found a down-regulation in the expression of the rate-limiting enzyme in the CoA biosynthetic pathway, PANK1. With this preliminary data, we tested the hypothesis that supplementation with CoA precursors,

pantothenate and cysteine, could increase hepatic free CoA content, abrogate caspase-2 activation and protect from progression/fibrosis in NASH. To study this hypothesis, we submitted WT mice to MCD diet and treated them with pantothenate and N-acetylcysteine. This approach failed to improve liver outcome, which was consistent with being ineffective in restoring free CoA levels and in abrogating caspase-2 activation. We explained these negative results by the fact that adding the precursors was not enough to overcome the down-regulation of key enzymes in the CoA biosynthetic pathway. However, other approaches to modulate CoA levels could be of interest in treating NAFLD, and it remains to be determined the role of adding carnitine to this strategy, being however expected to block the repression of the CoA biosynthetic pathway.

In conclusion, we unraveled a non-intuitive role of caspase-2, an apoptotic protein, in the development of metabolic diseases associated with energy surplus/obesity, namely the metabolic syndrome, IR/T2DM and NAFLD/NASH, the serial killers of modern times. Caspase-2 as a therapeutic target for those conditions may have a huge impact on global health.

SUPLEMENTAL TABLES

Supplemental Table 1 – Characteristics of the diets

	Chow diet	Western diet
Reference	Picolab© Rodent diet 20, #5053	TD.120330, 22% HVO + 0.2% cholesterol diet, Teklad Research
Kcal/g of diet	4.7	4.6
% Fat as calories	13	45.3
% CH as calories	62	37
% Proteins as calories	24	17.7

Supplemental Table 2. Composition of the experimental diets

	MCD diet	Western diet*
Reference	MP Biomedicals, #960439	TD.120330, 22% HVO + 0.2% cholesterol diet, Teklad Research
Formula (g/Kg)	Sucrose (455.3) Corn Starch (200.0) Corn Oil (100.0) Alphacel Non-Nutritive Bulk (30.0) Mineral Mix, AIN-76, (35.0) Calcium Phosphate, dibasic (3.0) L-Alanine (3.5) L-Arginine HCl (12.1) L-Asparagine Monohydrate (6.0) L-Aspartic Acid (3.5) L-Cystine (3.5) L-Glutamic Acid (40.0) Glycine (23.3) L-Histidine HCl (4.5) L-Isoleucine (8.2) L-Leucine (11.1) L-Lysine HCl (18.0) L-Phenylalanine (7.5) L-Proline (3.5) L-Serine (3.5) L-Threonine (8.2) L-Tryptophan (1.8) L-Tyrosine (5.0) L-Valine (8.2) Vitamin E, DL-alpha tocopherol acetate (250 IU/g) (0.484) Vitamin A Palmitate (250,000 IU/g) (0.0792) Vitamin D_3, cholecalciferol (400,000 IU/g) (0.0055) Ethoxyquin (0.02) Biotin (0.0004) Calcium Patothenate (0.0661) Folic acid (0.002) Inositol (0.1101) Menadione (0.0496) Niacin (0.0991) p-Aminobenzoic acid (0.1101) Pyridoxine HCl (0.0220) Riboflacin (0.022) Thiamine HCl (0.022)	Casein (230.0) DL-Methionine (3.4) Sucrose, fine ground (211.7098) Corn Starch (80.0) Maltodextrin (140.0) Vegetable Shortening, hydrogenated (Primex) (220.0) Soybean Oil (10.0) Cholesterol (2.0) Cellulose (50.0) Mineral Mix, AIN-93G-MX (94046) (46.0) Calcium Phosphate, dibasic (3.3) Niacin (0.042) Calcium Pantothenate (0.0224) Pyridoxine HCl (0.0098) Thiamin HCl (0.0084) Riboflavin (0.0084) Folic Acid (0.0028) Biotin (0.0003) Vitamin B_{12} (0.1% in mannitol) (0.035) Vitamin E, DL-alpha tocopherol acetate (500 IU/g) (0.1) Vitamin A Palmitate (500,000 IU/g) (0.0112) Vitamin D_3, cholecalciferol (500,000 IU/g) (0.0028) Vitamin K_1, phylloquinone (0.0011) Choline Bitartrate (3.3) TBHQ, antioxidant (0.046)

	Vitamin B12 (0.1% trit.) (0.0297)
	Ascorbic acid (1.0166)
	Corn Starch (3.4503)

*This diet is a modification of TD.06303 to add 0.2% cholesterol. We also add 42 g/L glucose and fructose in the drinking water (55% fructose and 45% glucose, w/w).

Supplemental Table 3 – Primaries Antibodies for Immunohistochemistry

Antibody	Host	Company	Catalog #	Retrieval	Dilution
4-Hydroxynonenal	Rabbit	Abcam	Ab46545	Citrate	1:250
α-SMA	Rabbit	Abcam	Ab32575	Citrate	1:400
Caspase-2_L	Rabbit	Santa Cruz	Sc-626	Citrate	1:3000
Cleaved Caspase-3	Rabbit	Cell Signaling	#9664	Citrate	1:50
Collagen-1αa	Rabbit	Abcam	Ab292	Citrate	1:300
Desmin	Rabbit	Abcam	Ab15200	Citrate	1:400
F4/80	Rat	AbD Serotec	MCA497GA	Citrate	1:150
K19	Rat	Develpmental Studies Hybridoma Bank	Troma III	Pepsin + EDTA	1:150
Osteopontin	Goat	R&D systems	AF808	Pepsin	5μg/mL
Pan-CK	Mouse	Invitrogen	08-4132	Pepsin	1:200
Sox-9	Rabbit	EMD Millipore	Ab5535	Citrate	1:4000
Shh	Rabbit	Abcam	Ab19897	Citrate +	1:6000
YM-1	Rabbit	Stem Cell Technologies	#01404	Citrate	1:4000

α-SMA, alpha smooth muscle actin; K19, Keratin 19; Shh, Sonic hedgehog

Table 4 – RT-PCR primers for analysis

Gene	*Primer forward*	*Primer reverse*
α-SMA	GTGGGGGACGAAGCGCAGAG	GGCCTTAGGGTTCAGCGGCG
α-Fetoprotein	TCGTATTCCAACAGGAGG	AGGCTTTTGCTTCACCAG
ACC-α	AGGAGGACCGCATTTATCGAC	TGACCGTGGGCACAAAGTT
ACOX	ATGCCTTTGTTGTCCCTATC	CCATCTTCAGGTAGCCATTATC
Adiponectin	TCTCCAGGAGTGCCATCTCT	GTTGCAAGCTCTCCTGTTCC
ApoB	GGACTGTCTGACTTCCATATTC	AAGACTTGCCACCCAAAG
ATF-4	GCTATGGATGATGGCTTGG	CGAAACAGAGCATCGAAGT
ATF-6	CGAAGGGATCATCTGCTATTAC	CTTCATAGTCCTGCCCATTG
Caspase-2	CAATGCTAACTGTCCAAGTCTA	GGGATTGTGTGTGGTTCTT
Catalase	AGATGGAGAGGCAGTCTATT	AGATCTCGGAGGCCATAAT
CD3	GATGCGGTGGAACACTTT	CTCTACACTGGTTCCTGAGA
CD20	TCTACGACTGTGAACCATCTA	GTTTCTGGAAGAAGGCAGAG
CD31	AACAGAGCCAGCAGTATGAGGACC	ACCACTCCAATGACAACCACCG
CD68	CCTTGACCTGCTCTCTCTAA	ACCAGGCCAATGATGAGA
CD115	CTGTATGTCTGTCATGTCTCTG	GCTATTGCCTTCGTATCTCTC
CPT-1a	TCCACCCTGAGGCATCTATT	ATGACCTCCTGGCATTCTCC
COASY	CCATCAATAGGAAGGTCCTG	CACAGCCACATCCATCTC
Col1α1	TTCCCTGGACCTAAGGGTACT	TTGAGCTCCAGCTTCGCC
Cyclin D1	TAGGCCCTCAGCCTCACT	CCACCCCTGGGATAAAGCAC
Cyclin D2	CCCGACTCCTAAGACCCAT	TTCAGCTTACCCAACACTACCA
Cyclin A2	GGCTGCACCAACAGTAAA	GGGTCAGCATCTATCAAACTC
Cyp2E1	AGGCTGTCAAGGAGGTGCTA	GGAAGTGTGCCTCTCTTTGG
Desmin	TACACCTGCGAGATTGATGC	ACATCCAAGGCCATCTTCAC
F4/80	CAACACTCTCGGAAGCTATTAT	GAATTCCTGGAGCACTCATC
FAS	CTGCGGAAACTTCAGGAAATG	GGTTCGGAATGCTATCCAGG
GADD153	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
Gli-1	CCTCCTCCTCTCATTCCACA	CTCCCACAACAATTCCTGCT
Gli-2	CCCCATCACCATTCATAAGC	CTGCTCCTGTGTCAGTCCAA
GPR-78	CGAAGGGATCATCTGCTATTAC	CTTCATAGTCCTGCCCATTG
GPx-1	ACTACACCGAGATGAACGA	GACGTACTTGAGGGAATTCAG
GSS	GCCTCCTACATCCTCATGGA	CCACATGCTTGTTCATCACC
Heme Oxig.	GCTCGAATGAACACTCTGG	GTTCCTCTGTCAGCATCAC
Hip	AATTATGCCGCTACCTCTCG	CAGCATGTACCATTGCAACC
Ihh	CTCAGACCGTGACCGAAATAAG	TGGGCCTTGGACTCGTAATA
IL-6	GACCTGTCTATACCACTTCAC	GTGCATCATCGTTGTTCATAC
K-7	TAGAGTCCAGCATCGCAGAG	CACAGGTCCCATTCCGTC
K-19	GTGAAGATCCGCGACTGGT	AGGCGAGCATTGTCAATCTG
Leptin	TGACACCAAAACCCTCATCA	CCAGGTCATTGGCTATCTGC
Ly6G	TTCCTGCAACACAACTACC	GATGGGAAGGCAGAGATTG
MCP-1	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG
MTTP	TCTCACAGTACCCGTTCTT	TCTTCTCCGAGAGACATATCC
Osteopontin	TGCACCCAGATCCTATAGCC	CTCCATCGTCATCATCATCG
PANK1 v.1	GAGAGAGTCCATCAGCAAAG	GAGAAAGTTCCCAACAAACAC
PANK1 v.2	CTTATCTGAGCCTAACTCCATTC	CCACCGATATCCATACCAAAC
PANK2	CTCTGCTTCTGGTGAACATC	GACAGCAGAGACCAAAGAAA
PANK3	AGGAGGTGGTGCTTACAA	CCATTGAAACTGACGGAATCTA
PANK4	GTGGAGCCTACAAGTTCAAG	CTCATGCGGGATGTTCTTC
Patch	ATGCTCCTTTCCTCCTGAAACC	TGAACTGGGCAGCTATGAAGTC

PDGF	GACTACCTGCACCGGAACAA	GTCCAACATGGGCACGTAA
PPAR-α	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
PPAR-γ	AGGCCGAGAAGGAGAAGCTGTTG	TGGCCACCTCTTTGCTCTGCTC
PPCS	CCTGAACACAAGATCCACTC	CTCCAGCTTAAAGGAAACTACA
PPCDC	AACAACAGAGAGAGCCAAAC	GCAGGTCAATGTGGAGAAC
S9	GACTCCGGAACAAACGTGAGGT	CTTCATCTTGCCCTCGTCCA
SCD-1	CGTCTGGAGGAACATCATTC	AGCGCTGGTCATGTAGTA
Shh	CTGGCCAGATGTTTTCTGGT	GATGTCGGGGTTGTAATTGG
SOD-1	GAGACCTGGGCAATGTGACT	GTTTACTGCGCAATCCCAAT
SOD-2	CCGAGGAGAAGTACCACGAG	GCTTGATAGCCTCCAGCAAC
Sox-9	CAGCAAGAACAAGCCACAACGTCAA	TTGTCCGTTCTTCACCGACTTCCT
SREBP-1c	GCTACCGGTCTTCTATCAATG	GCAAGAAGCGGATGTAGTC
Vanin-1	ATCCCAGACCCTCAAGTAAA	CATGTTCGCCACAACATAGA
Vanin-3	GAGGCTTTGCTCCTGATAAA	CTGGTGAAGATCCAACCATAG
TGF-β	TTGCCCTCTACAACCAACACAA	GGCTTGCGACCCACGTAGTA
TNF-α	TCGTAGCAAACCACCAAGTG	AGATAGCAAATCGGCTGACG
UCP-1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
UCP-2	ATGGTTGGTTTCAAGGCCACA	CGGTATCCAGAGGGAAAGTGAT
UCP-3	CGAATTGGCCTCTACGA	TGTAGGCATCCATAGTCCC
XBP-1	TCCGCAGCACTCAGACTATG	ACAGGGTCCAACTTGTCCAG
YM-1	TCTGGTGAAGGAAATGCGTA	AATGATTCCTGCTCCTGTGG

α-SMA, α-Smooth muscle actin; ACC-α, Acetyl-CoA Carboxylase-α; ACOX, Acyl Coenzyme A Oxidase; ApoB, Apolipoprotein B; ATF-4 and -6, Activating Transcription Factor-4 and -6; COASY, CoA synthase; Col1α1, Collagen-α1; CPT1a, Carnitine Palmitoyltransferase 1A; Cyp2E1, cytochrome P450 2E1; FAS, Fatty Acid Synthase; GPR-78, G protein-coupled receptor 78; GPx1, Glutathione Peroxidase; GSS, Glutathione Synthetase; Hip, Hedgehog Interacting Protein; Ihh, Indian Hedgehog; IL-6, Interleukin-6; K7, Keratin 7; K19, Keratin 19; Ly6G, Lymphocyte Antigen 6G; MCP-1, Monocyte Chemoattractant Protein-1; MTTP, Microsomal Triglyceride Transfer Protein; PANK, Pantothenate kinase; PDGF, Platelet Derived Growth Factor; PPAR-α, Peroxisome Proliferator-Activated Receptor-α; PPAR-γ, Peroxisome Proliferator-Activated Receptor-γ; PPCDC, Phosphopantothenoylcysteine carboxylase; PPCS, 4-Phosphopantothenoylcysteine synthase; SCD-1, Stearoyl-CoA Desaturase; SOD-1 and -2, Superoxide Dismutase-1 and-2; Shh, Sonic Hedgehog; SREBP-1c, Sterol Regulatory Element Binding Protein-1c; TGF-β, Transforming Growth Factor-β; TNF-α, Tumor Necrosis Factor-α; UCP, uncoupling protein, XBP-1, X-box protein-1;

Supplemental Table 5 – Primaries Antibodies for Western blot

Antibody	Host	Company	Catalog #	Dilution
α-Tubulin	Mouse	Abcam	Ab4074	1:5000
Caspase-2	Mouse	Cell Signaling Technology	#2224	1:500
Cleaved Caspase-3	Rabbit	Cell Signalling	#9661	1:500
Cyclin A	Rabbit	Santa Cruz Biotechnology	Sc-596	1:500
Gli-2	Rabbit	Genway	GWB-CE7858	1:500
Shh	Mouse	Santa Cruz Biotechnology	Sc-9024	1:1000
UCP-2	Goat	Santa Cruz Biotechnology	Sc-6525	1:500

Shh, Sonic hedgehog; UCP, uncoupling protein.

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