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Die Nicht-alkoholische Fettlebererkrankung – ein globales Gesundheitsproblem mit unterschätztem Risikoprofil

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1. Inhaltsverzeichnis

2. Einführung

Die nicht-alkoholische Fettlebererkrankung, englisch *non-alcoholic fatty liver disease* (NAFLD), ist eine mittlerweile global weit verbreitete Gesundheitsproblematik (1). Sie gilt als hepatische Manifestation des metabolischen Syndroms (siehe Tabelle 1), die aktuelle Datenlage spricht jedoch dafür, dass die NAFLD mitursächlich für das metabolische Syndrom bzw. dessen Komponenten ist (2–4). Der Terminus NAFLD umfasst dabei ein Spektrum an Erkrankungsstadien von der reinen Steatose (Leberverfettung), bezeichnet als nicht-alkoholische Fettleber (NAFL), über die nicht-alkoholische Steatohepatitis (NASH) bis hin zu daraus resultierender Fibrose und hepatozellulärem Karzinom (5). In den folgenden Abschnitten werden Genese, Pathophysiologie und Epidemiologie kurz zusammengefasst.

Tabelle 1: Definition des metabolischen Syndroms nach aktuellen Kriterien der *International Diabetes Federation* (IDF).

Kriterium	**Bedingung**
Adipositas[1]	Frauen Taillenumfang > 80 cm, Männer > 94 cm (Europa); Alternativ BMI > 30
Hyperglykämie[2]	Nüchternblutglukose > 100 mg/dl / 5,6 mmol/l oder Typ 2 Diabetes mellitus
Dyslipidämie[2]	HDL < 50 mg/dl / 1,25 mmol/l (Frauen); < 40 mg/dl / 1,05 mmol/l (Männer); oder Therapie eingeleitet
Erhöhte Triglyzeride	> 150 mg/dl / 1,7 mmol/l oder Therapie eingeleitet
Hypertonie[2]	Systolischer Blutdruck > 130 mmHg und diastolischer Blutdruck > 85 mmHG oder behandelte Hypertonie

1: Nach den Kriterien der IDF gilt (abdominelle) Adipositas als Grundvoraussetzung, diese muss gegeben sein; 2: Für das Vorliegen eines metabolischen Syndroms müssen zusätzlich zur Adipositas mindestens 2 der anderen Kriterien erfüllt sein.

2.1. Ursache der nicht-alkoholischen Fettlebererkrankung

Die NAFLD basiert letztlich auf einem Überangebot an Kalorien im Organismus. Der sogenannte westliche Lebensstil, der sich zunehmend in allen industrialisierten Gesellschaften auf der Welt verbreitet (6–8), beinhaltet einerseits die schnelle und allzeitige Verfügbarkeit von hochkalorischen Nahrungsmitteln, andererseits die Reduktion an körperlicher Arbeit und körperlichen Freizeitaktivitäten, einen *sedentary life style* (9). Durch Überkonsum an Zucker und Fetten im Alltag in Kombination mit der permanenten Nutzung von Auto, Fahrstuhl, Rolltreppe, häufig sitzender beruflicher Tätigkeit und einer Freizeitgestaltung vor dem Fernseher oder Computer wird ein deutlicher Kalorienüberschuss bei der Aufnahme gegenüber dem Verbrauch erreicht. Die

überschüssigen Kalorien speichert der Körper unter anderem als Fett (Triglyzeride) in den Adipozyten im Fettgewebe (10). Sofern die Speicherkapazität von Adipozyten jedoch überschritten wird, kann eine Kompensation durch zwei Mechanismen erfolgen. Es kann eine Hyperplasie im Fettgewebe erfolgen, oder eine Hypertrophie der Fettzellen (11). Letzteres ist mit einer stärkeren Veränderung des freigesetzten Adipokinprofils (erniedrigtes Adiponektin, erhöhtes Leptin und Ghrelin) und der vermehrten Freisetzung von Lipidkomponenten in die Zirkulation, in erster Linie von freien Fettsäuren (FFA), assoziiert (12,13). Adipokine sind Fettgewebshormone, die, neben weiteren Effekten, Appetit und Insulinsensitivität regulieren (14–18). Die Veränderungen im Fettgewebe haben verschiedene Konsequenzen, einerseits führt die erhöhte Verfügbarkeit von Fettsäuren zu ektoper Ablagerung von Fett in den Muskeln und im Pankreas sowie in der Leber (3,19). Gleichzeitig entwickelt sich eine Insulinresistenz im Fettgewebe, in der Muskulatur und in der Leber, die vermutlich zu einem erheblichen Anteil durch reduzierte Adiponektin-Konzentration im Blut gefördert wird (20,21). Eine gesteigerte *de novo* Lipogenese in der Leber, verursacht durch konstant erhöhte Blutkonzentrationen an Glukose, Lipiden und Fettsäuren, führt zur Insulinresistenz der Hepatozyten, der parenchymalen Leberzellen. Dies stellt letztlich die Ursache für die Leberverfettung, die Steatose der Leberzellen dar (22).

2.2. Pathophysiologie der NAFLD und der NASH

Die Steatose in den Leberzellen wird häufig in zwei Aspekten unterschätzt. Der erste und klinisch relevante Aspekt ist, dass eine reine Steatose der Leber, ohne Entzündung und Fibrose harmlos wäre. Dass dem nicht so ist, wird in folgenden Kapiteln eruiert. In der Tat stellt jedoch die NASH als progressive Form der NAFLD mit Entzündung und Ballonierung der Hepatozyten ein größeres Risiko für leberbezogene Morbidität und Mortalität dar, als die reine Steatose (1). Der zweite häufig fehlerhaft interpretierte Aspekt der Lebersteatose ist, dass es sich hierbei um eine reine Speicherung von überschüssigen Lipiden in den Hepatozyten handeln würde, für die im Fettgewebe nicht mehr ausreichend Kapazität vorhanden wäre. Tatsächlich stammt der größte Teil der in den Hepatozyten gespeicherten Triglyzeride und Fettsäuren aus der *de novo* Lipogenese (3,23,24). Unter Überangebot von Fettsäuren und Lipiden produzieren Leberzellen also aus Glyzerin und Fettsäuren Lipide zur Speicherung. Das Glyzerin hierfür wird aus Glukose, Fructose oder über Pyruvat aus theoretisch allen Substraten des Citratzyklus generiert (25,26). Die gesteigerte *de novo* Lipogenese basiert also auf einem Überangebot aller Nährstoffkomponenten, inklusive Glukose und Fructose. Der Haupt-Speicherort für Glukose ist die Leber, in Form von Glykogen. Die Kapazität der Glykogen-Einlagerung beträgt 10 % der Leberzellmasse, also bei einem Organ von 1,5 kg bis zu 150 g Glykogen (27). Wesentlich für die Lipidhomöostase in der Leber sind vier Prozesse: Aufnahme, *de novo* Lipogenese, Fettsäure-Oxidation und Export als Lipoproteinpartikel (28). Für die NAFLD gibt es Daten, die Veränderungen an allen vier Prozessen zeigen, sowohl Aufnahme von Lipidkomponenten

als auch *de novo* Lipogenese sind erhöht. Die Fettsäureoxidation erfolgt normalerweise als β-Oxidation in den Mitochondrien, wird jedoch durch die Überlastung des Prozesses bei erhöhten Konzentrationen zunehmend zur ω-Oxidation in Peroxisomen verschoben (29–31). Dies hat den Nebeneffekt, dass vermehrt reaktive Sauerstoffspezies im Cytosol entstehen, die Stress am endoplasmatischen Retikulum auslösen. Schließlich wird die Freisetzung von Cholesterin reduziert und die Anteile von *high-density*, *low-density* und *very-low-density* Cholesterin verschieben sich zu den weniger dicht gepackten Varianten (22,32). Insbesondere scheint die Sekretion von FFA über VLDL durch eine maximale Größe und minimale Dichte von VLDL limitiert zu sein, was zunächst zu einem Anstieg der VLDL-Sekretion bei NAFLD führt, aber durch die biologische Limitierung ein Plateau erreicht, über das hinaus kein erhöhter FFA-Export möglich ist (33,34).

Einen wesentlichen Anteil an der verschobenen Lipidhomöostase trägt die Insulin-Resistenz der Leberzellen. Durch die verminderte Wirkung von Insulin wird weniger Glukose aus der Zirkulation aufgenommen. Gleichzeitig wird aus den Glykogenreserven der Leber Glukose freigesetzt. Ein Nebeneffekt dieser permanent erhöhten Glukosekonzentration in der Zirkulation ist eine kontinuierliche Insulinproduktion und damit Überlastung der β-Zellen im Pankreas, deren dauerhafte Schädigung letztlich den Typ 2 Diabetes mellitus (T2DM) bedingt. Die zentralen Probleme für das Verständnis dieser Prozesse sind die starke Verflechtung und Wechselwirkungen von überhöhter Kalorienzufuhr, Adipositas, Insulinresistenz in Fettgewebe, Muskulatur und Leber sowie erhöhten Konzentrationen an Lipidkomponenten und Glukose im Blut aber auch von kognitiven Prozessen und der Regulation von Hunger- und Sättigungsgefühlen im Gehirn. Hier eine eindeutige und unzweifelhafte Ursache-Wirkung-Beziehung herzustellen ist für das humane System bei aktueller Datenlage kaum möglich. Außerdem ist bislang ungeklärt, ob auf Grund genetischer Unterschiede und der sehr hohen Variabilität humaner Biologie, der Verlauf und damit die Ursache-Wirkung-Kette individuell unterschiedlich ausgeprägt sein könnten.

Die klassische Sichtweise für die Pathogenese der NAFLD war die von Day und James vorgeschlagene *two-hit hypothesis* (35). Auf eine initiale Schädigung durch Lipidakkumulation und Insulinresistenz in der Leber erfolgte eine zusätzliche Schädigung, der "second hit", durch oxidativen Stress, Zytokinausschüttung von Immunzellen, erhöhte Endotoxine durch eine verschlechterte Darmbarriere sowie mitochondriale Schädigung. Grundsätzlich ist diese These nicht falsch, wurde jedoch durch die von Moschen und Tilg entwickelte *multiple-parallel-hits hypothesis* abgelöst (36). Die enorme Komplexität der Entstehung einer NAFLD legt nahe, dass nicht nur zwei Faktoren auslösend sind bzw. sein können. Vielmehr ergeben sich gleichzeitig, also parallel, mehrere Bedingungen, welche die Entstehung der NAFLD begünstigen bzw. verursachen. Zu diesen Bedingungen zählen alle vorgenannten Faktoren, wobei Insulinresistenz und veränderte Adipokin-Ausschüttung als zentrale Einflussfaktoren gelten. Hinzu kommen jedoch genetische und epigenetische Ursachen, der Einfluss des Darm-Mikrobioms und von diesem bereit gestellte

Metabolite und Abbauprodukte, sowie direkte ernährungsbedingte Ursachen. Genetische Ursachen, mit Beteiligung an der NAFLD-Entstehung oder zumindest dem Schweregrad der Ausprägung, sind Einzelnukleotidpolymorphismen (Englisch: *single nucleotide polymorphism*; SNP) in den Genen PNPLA3, TM6SF2 und Apolipoprotein CIII (37,38). Bei Vorhandensein des *minor* Allels oder Homozygotie für das seltenere Allel ist das Risiko eine NAFLD zu entwickeln oder zu einer NASH voranzuschreiten deutlich erhöht (siehe Tabelle 2). Weitere genetische Faktoren wurden ebenfalls als mögliche Kandidaten identifiziert, jedoch ist die Datenlage hierzu noch unzureichend, um eine sichere Aussage treffen zu können, ob eine ursächliche Beziehung dieser Polymorphismen zur NAFLD vorliegt. Für nähere Informationen zu den aktuell bekannten Polymorphismen und der entsprechenden Datenlage sei hier auf Übersichtsartikel zu diesem Thema verwiesen (38–40).

Tabelle 2: Bekannte Polymorphismen mit möglichem Einfluss auf NAFLD-Entwickung und/oder -Schweregrad.

Lage des Polymorphismus' / Genbezeichnung	**Auswirkung**
Adiponutrin (PNPLA3) rs378409 I148M	Verlust der Enzymaktivität, dadurch Akkumulation von Triglyzeriden und Estern in Hepatozyten und HSC (41,42) Assoziiert mit Schweregrad der NAFLD (43); Häufigkeit: 21 % allg. Bevölkerung / 33 % NAFLD.
TM6SF2 rs58542926 E167K	Verlust der Enzymaktivität für Bindung von Triglyzeriden an Apolipoprotein B100, dadurch erhöhte Leber Triglyzeride und verminderte Serum Lipoproteine; erhöhtes Risiko für Lebersteatose, geringeres Risiko für kardiovaskuläre Erkrankungen (44–46).
Apolipoprotein CIII rs2854116 T455C	Verminderte Insulin-Bindung, dadurch erhöhte APOC3 Expression und Wirkung als Lipaseinhibitor; verminderte Aufnahme triglyzerid-reicher Partikel, in Folge Hypertriglyceridämie (47–49).
Glucokinase regulator (GCKR) rs1260326 P446L	Verminderte Reaktion auf Fruktose-6-Phosphat, dadurch verstärkte hepatische Glukoseaufnahme, Reduktion von Blutglukose und Insulin, gleichzeitig intrahepatische Steigerung der Lipogenese und Inhibierung der Fettsäure β-oxidation (50,51).

Bei allen chronischen Lebererkrankungen, so auch bei der NAFLD, ist ein weiterer zellulärer Mechanismus involviert: der Zelltod der Hepatozyten. Der Tod von Zellen ist in vielen Fällen ein hochgradig regulierter Prozess und kann auf sehr unterschiedlichen Wegen ausgeführt werden (52). Derzeitig bekannte Varianten des Zelltodes von Säugetierzellen sind in Tabelle 3 zusammengefasst, wobei auf diesem Gebiet weiterhin intensive Forschungsbemühungen stattfinden.

Tabelle 3: Aktuell bekannte Zelltodmodalitäten.

Bezeichnung	**Zentrale Ursache / Auslösender Reiz**	**Morphologische Kennzeichen**	**Molekulare Kennzeichen**
Nekrose	Physikochemikalische Zerstörung der Zelle (53)	Vakuolenbildung, Karyolyse (52)	Abhängig von möglicher Regulation (54,55)
Apoptose	a) Bindung von Liganden an Zelltodrezeptoren b) Freisetzung von Calcium aus Mitochondrien	Auflösung des Kerns *Blebbing* der Zellmembran / Abschnürung apoptotischer Körperchen (56,57)	Permeabilisierung der äußeren Mitochondrinemembran; Aktivierung der Caspase-3, -6, -7; DNA-Fragmentierung (52)
Nekroptose	Wie Apoptose unter ATP-Mangel oder bei Inhibierung von Caspase 8 (58)	Zerstörung der Zellmembran	Aktivierung von RIPK1 und 3; Aktivierung von MLKL, hierdurch Perforation der Zellmembran (59,60)
Pyroptose	Bindung von *pathogen* oder *danger associated patterns* (PAMP / DAMP) an entsprechende Rezeptoren (61)		Bildung von Inflammasomen mit Nlrp-Proteinen (1 oder 3); Aktivierung von Caspase 1; Freisetzung von IL-1β und IL-18 (61–63)
Ferroptose	Unzureichende Antioxidative Kapazität und Überlastung peroxidativer Prozesse (64)	Degeneration der Mitochondrien bei intaktem Zellkern	Starker Anstieg reaktiver Sauerstoffspezies und Glutathiondepletion
Onkosis	Ischämische Schädigung (65)	Anschwellen der Mitochondrien und des Zellkerns; Vakuolisierung des Zytosols	
Mitotische Katastrophe	Fehlerhafte Mitose (hauptsächlich bei Tumorzellen unter Therapie) (66)		

Autophagie	Drastischer Nährstoffmangel oder starke Beschädigung von Zellorganellen (67)	Verstärkte Bildung von Autophagosomen	Komplexe Regulation in Interaktion mit Apoptose und in Abhängigkeit von Nährstoffsituation (68,69)

Tabelle nach: Cell death mechanisms in human chronic liver diseases: a far cry from clinical applicability. Mazzolini G, Sowa JP, Canbay A. Clin Sci (Lond). 2016 Dec 1;130(23):2121-2138. (70)

Einige der oben angeführten Zelltodmodi sind nur in bestimmten Zelltypen, teilweise nur unter experimentellen Bedingungen *in vitro*, beobachtet und beschrieben worden. Daher ist für viele dieser Zelltodarten eine klinische Relevanz unklar oder sogar zweifelhaft. In einem Übersichtsartikel haben wir vor einiger Zeit die damals aktuellen Erkenntnisse zu Zelltodmechanismen bei chronischen Lebererkrankungen und insbesondere der NAFLD zusammengestellt (70). Für die Einschätzung, ob und welche Zelltod-Mechanismen bei Erkrankungen des Menschen eine Rolle spielen, sind entsprechende diagnostische Möglichkeiten erforderlich. Diese liegen jedoch nicht oder nur mit deutlichen Limitierungen vor. Tatsächlich gibt es für den gesamten Zelltod und für die Apoptose von epithelialen Zellen, zu denen Hepatozyten zählen, gute Surrogatmarker. Im Serum vorhandenes Zytokeratin 18 (CK18, häufig mit der Antikörperkombination M65 bezeichnet) gibt ein objektives Maß für die Anzahl sterbender epithelialer Zellen, wobei vermutet wird, dass der überwiegende Anteil davon Hepatozyten sind (71,72). Durch Caspasen gespaltenes CK18 im Serum, detektiert durch den Antikörper M30, gilt als guter Surrogatmarker für epitheliale bzw. Hepatozyten-Apoptose. Es konnte in mehreren Studien gezeigt werden, dass beide Marker für unterschiedliche chronische Lebererkrankungen das Ausmaß der Schädigung abbilden können. Allerdings sind beide Marker nicht spezifisch für eine bestimmte Art der Schädigung, wie Alkoholtoxizität, virale Hepatitiden oder die NAFLD (71,73–75). Dennoch zeigen Studien mit diesen Zelltodmarkern klar, dass zunehmende Schädigung bei NAFLD mit höheren M65 und M30-Werten im Serum einhergehen. Eine translationale Studie weist darauf hin, dass der zentrale mechanistische Unterschied zwischen der NAFL und der NASH ein Wechsel von der Apoptose zur MLK3-abhängigen Nekroptose sein könnte (76). Leider gibt es bisher keine Studien an Menschen, die eine entsprechende Verschiebung von verschiedenen Surrogatmarkern oder entsprechende Prozesse in Leberbiopsien nachweisen konnten. Dies liegt unter anderem an der mangelnden Datenlage zu verlässlichen Markern verschiedener Zelltodformen. Die zentrale Limitierung ist jedoch, dass von den meisten Leberbiopsien aus NAFLD-Patienten keine Analysen zu darin ablaufendem Zelltod durchgeführt werden.

Trotz der offenen Fragen zu den Zelltodmechanismen insbesondere bei NAFLD und Progression zu NASH, ist unstrittig, dass hepatozellulärer Zelltod in allen chronischen Lebererkrankungen auftritt. Ebenso kann als gesichert gelten, dass der Zelltod von Hepatozyten einen zentralen Aktivierungs-Reiz für die hepatischen Sternzellen (*hepatic stellate cells*; HSC) darstellt (77). HSC liegen in der gesunden Leber in ruhender Form vor und fungieren unter anderem als Vitamin A Speicher. Wenn HSC durch

einen Reiz, wie z.B. Zytokine von Hepatozyten oder Kupfferzellen oder durch die direkte Aufnahme von apoptotischen Körperchen, aktiviert werden, lösen sie ihre Vitamin A Speicher auf und entwickeln einen kontraktilen, fibroblasten-ähnlichen Phänotyp (78). Aktivierte HSC produzieren einerseits Kollagen und erzeugen somit die extrazelluläre Matrix der Leber. In Situationen vermehrten Zellverlustes macht dies Sinn, um die Struktur der Leber aufrecht zu erhalten und kann als Wundheilungsprozess oder Narbenbildung gesehen werden (77,78). Weiterhin produzieren HSC auch *tissue inhitibors of matrix metalloproteinases* (TIMPs). Matrix Metalloproteinasen (MMPs) sind Enzyme, die Kollagen ab- und damit die extrazelluläre Matrix umbauen können (79,80). MMPs sind wesentlich für den Rückbau fibrotischer Veränderungen der Leber. Im aktivierten Zustand sekretieren HSC jedoch TIMPs, die die enzymatische Wirkung der MMP blockieren und somit eine Ansammlung des Kollagen ermöglichen. Weiterhin scheinen TIMPs dafür zu sorgen, dass aktivierte HSC resistent gegen Apoptose-Reize werden. Schließlich produzieren aktivierte HSC TGF-β, das umliegende, ggf. noch ruhende HSC, aktiviert (81). Bei kontinuierlichem Leberschaden und damit andauerndem Zelltod von Hepatozyten kommt es so zu einer positiven Feedbackschleife: aktivierte HSC produzieren Kollagen, sekretieren TIMPs, die einen Abbau des Kollagens verhindern, und parallel weitere HSC aktivieren. Hierdurch kommt es bei chronischem Leberschaden zu fortgeschrittener Fibrose und schließlich Zirrhose, bei der ein Großteil des Gewebes durch Kollagenmatrix ersetzt wurde und nur Inseln funktionaler Hepatozyten übrigbleiben (82). Die starke Reduktion der Anzahl funktionaler Hepatozyten stellt die Ursache für ein Versagen der Leber dar, wenn die kritische Menge an Zellen für eine Aufrechterhaltung der Organfunktion unterschritten wird.

Zusammengefasst lässt sich unser Wissensstand zur Pathogenese der NAFLD in drei Punkten darstellen:

- Entstehung der NAFLD basiert auf exzessiver Lipid-Akkumulation und Insulinresistenz in Kombination mit alterierter Adipokin-Signalgebung, initial verursacht durch hyperkalorische Lebensweise. Modulierend hierfür sind oxidativer Status der Leber, z.B. Produktion von Sauerstoffradikalen und Verschiebung der Fettsäureoxidation von den Mitochondrien in Peroxisomen, Freisetzung von entzündungsfördernden Zytokinen sowie genetische (und evtl. epigenetische) Faktoren.

- Progression zu oder Entstehung der NASH erfolgt durch eine Verschiebung zu stärker entzündlichen Prozessen in der Leber, möglicherweise durch Änderung von Zelltodmodi, verstärkter Belastung mit bakteriellen Substanzen aus dem Darm (PAMPs) oder auf Grund spezifischer Polymorphismen, die ein entsprechendes Risikoprofil fördern.

- Kontinuierliche Schädigung der Hepatozyten führt in beiden Varianten, verstärkt jedoch bei NASH, zu Fibrosierung der Leber ggf. mit Progression zur Zirrhose sowie zur Entstehung eines HCC, unabhängig von der Fibrosierung.

2.3. Epidemiologie der NAFLD

In einer großen Metaanalyse mit mehr als 8 Millionen eingeschlossenen Patienten (der Großteil stammte dabei aus den USA) wird die globale Prävalenz der NAFLD auf 25 % geschätzt (1). Allerdings ist dies eine konservative Rechnung und man muss von einer hohen Dunkelziffer, vor allem in medizinisch unterversorgten Gebieten, ausgehen. Innerhalb dieser Metaanalyse waren 60 % der mittels Biopsie überprüften Fälle als NASH kategorisiert, was einer globalen Rate von 15 % entsprechen würde. Dies beruht allerdings mit sehr hoher Wahrscheinlichkeit darauf, dass Leber-Biopsien in erster Linie bei Patienten erfolgen, bei denen entweder unklar ist, wie schwer der Leberschaden ist oder eine fortgeschrittene Lebererkrankung (Zirrhose, HCC) abgeklärt werden muss. Ältere epidemiologische Arbeiten legen nahe, dass nur etwa 10 bis 15 % der NAFLD Patienten tatsächlich eine NASH entwickeln, was ca. 2 bis 4 % der Gesamtbevölkerung entsprechen würde (5,29). Zu vermuten ist, dass der wahre Wert zwischen diesen Extremen liegt. Für Deutschland wird die Prävalenz der NAFLD aktuell auf ca. 25 % der Bevölkerung geschätzt. Bei nüchterner Betrachtung der bekannten Fakten und Daten ist dies jedoch vermutlich deutlich unterhalb der tatsächlichen Zahl. Dies beruht auf einer relativ hohen Dunkelziffer.

Nach aktuellen Zahlen der WHO sind ca. 40 % der erwachsenen, globalen Bevölkerung übergewichtig und ca. 15 % adipös (83). In Deutschland legen Zahlen der DEGS I Erhebung des Robert-Koch-Instituts nahe, dass ca. 65 % der Erwachsenen übergewichtig oder adipös ist, Adipositas liegt bei 23 % der deutschen Bevölkerung vor (84). Diese Erhebung wurde jedoch bereits 2008-2011 durchgeführt. In den vergangenen Jahren blieb zwar die Zahl der übergewichtigen oder adipösen Individuen stabil, allerdings gab es eine Verschiebung zu einem größeren Anteil an adipösen Personen und höheren BMIs. Aktueller sind die Zahlen der GEDA (Gesundheit in Deutschland aktuell) 2014/2015 mit einer Schätzung für Übergewicht bei 46 % der Frauen und 60 % der Männer sowie Adipositas bei ca. 13 % geschlechtsunabhängig (85). Hier ist die Limitierung, dass die Daten auf Eigenangaben der Befragten beruhen, was bekanntlich zu geringeren Gewichts- und erhöhten Körpergrößen-Angaben führt. Somit dürften diese Zahlen den tatsächlichen Stand unterschätzen. Ein weiteres Problem bei der Prävalenz-Abschätzung der Adipositas ist die Verwendung des BMI als Klassifikator, der die Verteilung der Körpermaße (Art und Lage) nicht beinhaltet. Tatsächlich liegt die sogenannte abdominelle Adipositas ab einem Taillenumfang von 80 cm bei europäischen Frauen und ab 94 cm bei Männern vor (86), wie beim BMI gelten für andere Populationen ggf. andere Grenzwerte. Der BMI-Grenzwert von 30 identifiziert mit sehr hoher Spezifität eine vorliegende abdominelle Adipositas. Dies bedeutet, dass es nur einen verschwindend geringen Anteil Individuen gibt, meist sehr groß und sehr muskulös, die einen BMI > 30 haben, aber nicht adipös sind. Umgekehrt hat der BMI aber nur eine moderate bis schlechte Sensitivität. Abhängig von der betrachteten Studie reichen die Anteile von 25 bis 75 % an Individuen mit abdomineller Adipositas, die nicht den BMI-Grenzwert von 30 überschreiten. Im

ungünstigsten Fall würde das bedeuten, dass drei Viertel der als übergewichtig klassifizierten Personen tatsächlich adipös sind, mit allen entsprechend höheren Gesundheitsrisiken. Die deutsche S3-Leitlinie zur "Prävention und Therapie der Adipositas" empfiehlt auch die Messung des Taillenumfangs ab einem BMI von 25 kg/m² (87), jedoch wird diese nicht immer durchgeführt. Sofern man also davon ausgeht, dass tatsächlich - konservativ betrachtet - 25 % der aktuell als Übergewichtig klassifizierten Bundesbürger abdominelle Adipositas aufweisen, steigt die tatsächliche Prävalenz in der Gesamtbevölkerung von 13 % auf 23 % nach DEGA und von 23 % auf 34 % nach DEGS I. Demnach wären dann 31 % bzw. 32 % der Deutschen "nur" übergewichtig. Für Populationen mit Risikofaktoren für NAFLD gibt es Schätzungen für die jeweilige spezifische Prävalenz, z.B. liegt die NAFLD-Prävalenz unter Diabetikern bei 44 % (88). Bei Übergewicht liegt die Prävalenz der NAFLD bei 75 - 80 %, wobei in vielen Studien hierzu nur eine einzige Kategorie für übergewichtig und adipös vorliegt. Somit könnte der Anteil für übergewichtige, aber nicht adipöse, Populationen niedriger sein. Unter adipösen Individuen wird eine Prävalenz von 90 - 100 % angenommen (89). Auf Grund der starken Überlappung an Risikofaktoren und Grundursachen von Adipositas, NAFLD und T2DM wird in der folgenden Schätzung der Anteil von Typ 2 Diabetikern in der Bevölkerung nicht berücksichtigt. Ausgehend von den oben genannten Daten der GEDA Erhebung wären 32 % der Bevölkerung (ca. 34,9 Mio. Menschen) von NAFLD betroffen. Eine Korrektur der vermuteten abdominellen Adipositas um 25 % falsch negative Aussagen nach BMI ergäbe sogar eine Prävalenz von 34 %. Legt man die Zahlen der DEGS I Studie zu Grunde, wären ohne BMI-Korrektur 52 %, mit Korrektur 54 % (zum Zeitpunkt der Erhebung 43,2 Mio. Menschen) von NAFLD in Deutschland betroffen. Die starke Diskrepanz zwischen der hier kalkulierten Prävalenz zwischen 32 und 54 % und der geschätzten Prävalenz aus Primärdaten von 14 - 27 % (90) ergibt sich durch ein diagnostisches Ungleichgewicht, welches im Abschnitt 2.5.1 (S. 15) erläutert wird. Aber selbst wenn die hier berechneten Prävalenzen überhöht und vielleicht sogar unrealistisch wirken mögen, steht außer Frage, dass wir uns selbst mit nur 20 % Betroffenen in der Bevölkerung einem enormen Gesundheitsproblem gegenüber sehen, dessen Auswirkungen für die Betroffenen und die Gesundheitsversorgung nur erahnt werden können (91).

2.4. Mögliche weitere Einflussfaktoren der NAFLD

Die in den bisherigen Kapiteln dargelegten Zusammenhänge von hyperkalorischem Lebensstil, Übergewicht oder Adipositas und der NAFLD sind weitestgehend unstrittig. Auch grundlegende zelluläre und molekulare Mechanismen, die eine Rolle bei der Entstehung der NAFLD und Progression zur NASH spielen, sind bekannt. Allerdings ist der Erkrankungskomplex um Adipositas, metabolisches Syndrom und NAFLD in seiner Gesamtheit nicht geklärt und viele Prozesse sowie Ursache-Wirkung-Beziehungen sind noch unverstanden. Daher gibt es weiterhin Untersuchungen, die den Einfluss anderer Komponenten und Mechanismen der menschlichen Biologie auf Entstehung der

NAFLD aufklären sollen. Mögliche Einflussfaktoren, die für ein individuelles Risiko oder die Ausprägung (NAFL oder NASH) eine Rolle spielen könnten, sind das Darm-Mikrobiom, die Ernährungszusammensetzung sowie die Epigenetik.

Das Darm-Mikrobiom steht in intensiver Wechselwirkung mit dem Verdauungssystem, in dem es beheimatet ist. Letztlich muss das Darm-Mikrobiom in Gemeinschaft mit dem jeweiligen Wirt als Ökosystem betrachtet werden (92). Mit hoher Wahrscheinlichkeit beeinflusst das Darm-Mikrobiom die menschliche Gesundheit, z.B. bei chronisch entzündlichen Darm-Erkrankungen oder Zöliakie (93–95). Unterschiedliche Bakterien-Spezies bevorzugen unterschiedliche Nährstoffquellen und schließen die vom Wirt aufgenommene Nahrung auf verschiedene Art auf (96,97). Dies resultiert in deutlichen Unterschieden in der Resorbierbarkeit von Nahrungskalorien aber auch in den Substanzen und Metaboliten, die über die Darm-Schleimhaut aufgenommen werden können. Umgekehrt beeinflusst die aufgenommene Nahrung die Zusammensetzung des Mikrobioms, was auf einfache ökologische und evolutionäre Prinzipien zurückzuführen ist (92,98). Die Leber beeinflusst dieses Ökosystem ebenfalls, durch Sekretion von Gallensäuren, die einerseits als Emulgatoren für Lipide und Fettsäuren dienen (99), andererseits von spezifischen Bakterienspezies genutzt und metabolisiert werden (100). Bakterien-Bestandteile, PAMPs, werden ebenfalls über die Darmbarriere aufgenommen (101). Durch die zu 75 % über die Portalvene erfolgende Blutversorgung der Leber gelangen letztlich alle Substanzen, die aus dem Darm in den Blutkreislauf übertreten, in die Leber (102). Die aktuelle Datenlage zeigt, dass bei Adipositas und auch bei NAFLD gegenüber normgewichtigen Individuen eine Verschiebung des Darm-Mikrobioms auftritt. Einerseits wird in der Regel eine verminderte Artenvielfalt vorgefunden anderseits erfolgt meist eine Verschiebung des Artenspektrums zu weniger Arten des Stammes Firmicutes und größeren Zahlen an Bakterien des Stammes Proteobacteria (103–105). Bisher kann auf Basis der Datenlage keine verlässliche Aussage über die Ursache-Wirkung-Beziehung zwischen Darm-Mikrobiom und z.B. Adipositas getroffen werden. Für die NAFLD ist die Datenlage letztlich noch vager. Tatsächlich stellt die Forschung am Darm-Mikrobiom mit Hilfe der Sequenzierung der 16 S rRNA noch ein relativ junges Thema dar. Die enormen Datenmengen, die bei einer solchen Untersuchung entstehen müssen in Bezug zu den demographischen und klinischen Daten gesetzt werden. Die großangelegte populationsbasierte und auf Crowdsourcing basierende Studie American Gut Project zielt unter anderem darauf ab, zunächst Mikrobiome von möglichst vielen Individuen aus unterschiedlichen Populationen zu untersuchen und zu kategorisieren (106). Erste Auswertungen lassen vermuten, dass die Varianz so beträchtlich ist, dass individuelle Mikrobiom-Zusammensetzungen betrachtet werden müssen, auf der Ebene der vertretenen Spezies, und im Verlauf des Lebens sowie bei Gesundheit und Krankheit verglichen werden müssen, um relevante Aussagen tätigen zu können (107,108). Ohne Zweifel befinden wir uns hier noch in einem Stadium, das viele Fragen offenlässt. Daher soll dieser kurze Ausblick in einen möglichen, zusätzlichen Einflussfaktor für Entstehung und Ausprägung der NAFLD hiermit abgeschlossen werden, dass zum jetzigen Zeitpunkt keine klare Aussage getroffen werden kann.

Ähnlich offen ist die Datenlage zur Ernährungszusammensetzung als Einflussfaktor für die NAFLD. Diverse Nährstoffkomponenten und Metabolite wurden schon als Ursache für die NAFLD, für die entzündliche Komponente, für die Fibrosierung und für die Progression zu NASH identifiziert und vorgeschlagen. In vielen Fällen basieren diese Arbeiten jedoch auf Tiermodellen, in erster Linie murinen Diät-Modellen oder auf retrospektiven humanen Studien mit dem Versuch die Ernährungsgewohnheiten über Fragebögen zu erheben. Derartige Ansätze sind intrinsisch fehlerbehaftet (109,110). Vor allem übergewichtige und adipöse Patienten berichten tendenziell weniger aufgenommene Kalorien, als tatsächlich konsumiert wird (111). Insofern sind Aussagen zur Ernährungszusammensetzung als Risikofaktor für die NAFLD nicht valide. Umgekehrt gibt es natürlich prospektive Studien mit Lebensstil-Intervention zur Gewichtsreduktion. Hier wurden bereits häufig unterschiedliche Diätzusammensetzungen getestet. Dabei konnte jedoch keine spezifische Ernährungsform, die mit determinierten Anteilen an Kohlenhydraten, Proteinen und Fetten arbeitet, als besser gegenüber anderen identifiziert werden (112). Lediglich das Ausmaß der Kalorienreduktion bzw. des erzeugten Kaloriendefizits korreliert konsistent über alle Ernährungsformen mit Gewichtsabnahme und, soweit getestet, einer Verbesserung der NAFLD (113–115). Möglicherweise erzielen Ernährungsmodelle mit reduzierter Kohlenhydratzufuhr allerdings über eine Reduktion des Leberfettgehaltes zusätzlich eine Verbesserung der Insulinresistenz (113,115). Insgesamt stellt die Zusammensetzung unserer Ernährung auf Grund der großen Bandbreite an enthaltenen Substanzklassen und Einzelkomponenten ein extrem komplexes Gemisch dar. Die einzigen Einzelkomponenten, die in randomisierten Studien eine signifikant größere Reduktion der Lebersteatose zeigen konnten sind mehrfach ungesättigte Fettsäuren (116,117). Weitere gut kontrollierte Studien sind hier notwendig, um individuell angepasste Ernährungsempfehlungen zu entwickeln. Letztlich ist wesentlich, dass die Patienten eine Ernährungsform finden, die sie über einen langen Zeitraum aufrecht erhalten können (118).

Ebenso wie die Analyse des menschlichen Mikrobioms ist die Epigenetik ein relativ junges Forschungsfeld. Es gehört sicherlich zu den spannendsten Entdeckungen der Biologie in den vergangenen Jahrzehnten, dass das Genom keine fixierte Bauanleitung für Organismen darstellt, sondern durch biochemische Veränderung der zu Grunde liegenden Basen moduliert werden kann (119). Beispielsweise wird über Methylierung gesteuert, welche Gene bei der Embryonalentwicklung vom mütterlichen und welche vom väterlichen Allel abgelesen werden (120). Für die Adipositas und speziell für die NAFLD gibt es Hinweise, dass epigenetische Effekte zur „Erblichkeit“ von Adipositas beitragen könnten (121). In Primaten und Menschen konnte gezeigt werden, dass einerseits Adipositas, aber auch eine besonders fettreiche Ernährung der Mutter während der Gravität zu Veränderungen im Stoffwechsel des Nachwuchses führen (122–124). Diese Veränderungen im Stoffwechsel, partielle Insulinresistenz, höhere Lipidkonzentrationen im Serum, blieben bestehen, wenn die Jungtiere normal ernährt wurden und persistierten bis in die Adoleszenz, zum Teil sogar in adulten Tieren (122). Diese lange Auswirkungsdauer auf Basis eines kurz wirkenden Einflussfaktors

(Ernährung während der Schwangerschaft), weist darauf hin, dass epigenetische Effekte zu Grunde liegen. Die Datenlage insgesamt zeigt sehr deutlich, dass bestehende Adipositas der Mutter oder eine zu kalorienreiche, zu zucker- und fettlastige Ernährung während der Schwangerschaft beim Menschen sich negativ auf den Metabolismus der Kinder auswirkt (124–128). Die Vermutung liegt nahe, dass hier epigenetische Veränderungen Insulinresistenz, Sättigungsgefühl und bevorzugte Nährstoffklassen für die Energiegewinnung steuern und die Grundlage für Adipositas und NAFLD schaffen (129). Es bleibt zu bedenken, dass beim menschlichen Embryo Fettgewebe erst zum dritten Trimester angelegt wird (130). Bis dahin findet die Speicherung überschüssiger Nährstoffe in der Leber statt. Wie oben geschildert bewirkt ein andauernder Kalorienüberschuss bereits bei Erwachsenen fundamentale Veränderungen wie Insulinresistenz, erhöhte *de novo* Lipogenese und vermehrte Glykogenolyse. Es wäre denkbar, dass eine bereits im Mutterleib überlastete embryonale Leber dauerhafte Veränderungen an der Steuerung von Stoffwechselprozessen vornimmt, um ein Überangebot zu kompensieren. Insofern könnte in diesen Individuen die Basis für Adipositas und metabolisches Syndrom eine vorgeburtliche NAFLD sein.

Zusammengefasst ist relativ wahrscheinlich, dass die Mikrobiom-Zusammensetzung und die Epigenetik sowie mit geringerer Wahrscheinlichkeit die Ernährungszusammensetzung auf die Entwicklung und Ausprägung einer NAFLD Einfluss nehmen. In welcher Weise das geschieht, welche spezifischen Faktoren sich positiv oder negativ auswirken und ob hierunter ernstzunehmende Kandidaten für therapeutische Ansätze für NAFLD und insbesondere NASH zu finden sind, bleibt in künftigen Untersuchungen und Studien zu klären.

2.5. Klinische und wissenschaftliche Problemstellungen durch die NAFLD

Die NAFLD ist für die klinische Einschätzung mit einer Reihe an Problemen verbunden. Das erste Problem ist die aktuell etablierte Diagnostik, deren Limitierungen sich vermutlich auf eine realistische Einschätzung der Dimension dieser Gesundheitsproblematik auswirken. Zu befürchten ist eine insgesamt inadäquate diagnostische Abdeckung der NAFLD und hierdurch einerseits die Gefahr Betroffene nicht korrekt zu identifizieren, andererseits in größerer Dimension die Prävalenz zu unterschätzen. Eine weitere Problematik ist das Risikoprofil von Patienten mit NAFLD, das sich negativ auf ein breites Spektrum von Ko- und Folgeerkrankungen auswirkt. Das dritte Problem, das hier nur kurz angerissen werden kann, ist die Therapie der NAFLD. Konkret existiert für NAFLD generell und insbesondere für NASH keine Pharmakotherapie, so dass indirekte Behandlung über Gewichtsreduktion die konservative Standardtherapie ist. Schließlich gibt es eine große Diskrepanz zwischen Daten, die in Tiermodellen der NAFLD erhoben werden und der tatsächlichen humanen Situation. Hierdurch wird die Identifikation potentieller therapeutischer Ziele in Modellsystemen erschwert und verzögert.

2.5.1. Diagnostische Probleme der NAFLD

Die NAFLD verursacht, wie alle chronischen Lebererkrankungen, über einen sehr langen Zeitraum keine oder nur geringfügige und unspezifische Symptome. Schmerzen treten in der Leber generell so gut wie nicht auf (mit Ausnahme von Gallensteinen) und sind bei einer Steatose der Leber nicht zu erwarten. Symptome wie Abgeschlagenheit und Müdigkeit können bei fortschreitender Erkrankung auftauchen, sind aber meist auf Grund der dann bereits deutlicher ausgeprägten kardiovaskulären Symptome, erhöhtem Blutdruck und Dyslipidämie in betroffenen Patienten nachrangig für Diagnostik und Therapie. Somit werden die meisten Fälle der NAFLD entweder als Zufallsbefund erhoben z.B. als „Begleiterkrankung“ bei Adipositas oder kardiovaskulären Erkrankungen oder erst sehr spät entdeckt, wenn bereits eine fortgeschrittene Erkrankung mit Leber- oder kardiovaskulärer Symptomatik vorliegen. Kürzlich wurde eine eindrucksvolle Studie publiziert, die vermuten lässt, dass ein Anteil von 28 % sogenannter Gesundkontrollen bei klinischen Studien, gerade bei Fragestellungen zu metabolischem Syndrom und NAFLD, tatsächlich eine NAFLD aufweisen könnte (131).

Während eine Lebersteatose per Ultraschall erst erkannt werden kann, wenn ca. 20 – 30 % der Leber betroffen sind, ist durch HE-Färbung im Lebergewebe bereits eine Steatose von 5 % durch Pathologen erkennbar, was für die Erstdiagnose NAFLD ausreichend wäre (132,133). Da eine Leberbiopsie immer ein gewisses Risiko für eine Blutung mit sich bringt und die Durchführung zeit- und kostenaufwändig ist, eignet sich die Leberbiopsie nicht als Screening-Methode, um die betroffenen Kohorten bzw. die Patienten mit erhöhtem Risiko für NAFLD zu untersuchen. Gleiches gilt für Magnetresonanz-Spektroskopie (MRS) und Magnetresonanz-Imaging (MRI), die beide mittels Protonendichte Fettfraktion (PDFF) den Fettgehalt der Leber sehr exakt bestimmen können (134). Tatsächlich sind moderne Protokolle für MRI-PDFF in 3 Minuten Scanning-Zeit aufzunehmen und können mit allen gängigen MRI-Geräten durchgeführt werden (134,135). Insbesondere für die MRS-PDFF, aber in geringerem Ausmaß für die MRI-PDFF sind Limitierungen der Zeit- und Kostenaufwand, sowie die Verfügbarkeit entsprechender Messgeräte und qualifizierten Personals für die Aufnahmen (in erster Linie MRS). Ultraschall-basierte Messungen des Leberfettgehaltes sind zu ungenau und erfordern, wie oben beschrieben, einen nicht unerheblichen Fettanteil. Ein derartiges Ausmaß an Leberverfettung impliziert, dass bei NAFLD-Patienten, die durch Ultraschall identifiziert werden können, die Erkrankung bereits über einen gewissen Zeitraum vorliegt. Dies bedeutet einen Zeitverlust für ein mögliches therapeutisches Eingreifen, stellt aber auch eine zentrale Limitierung vieler klinischer Studien dar. Wenn NAFLD mittels Ultraschall-Diagnose festgestellt wird, geschieht dies einerseits in einem Stadium der Persistenz der NAFLD, andererseits werden viele Patienten mit geringerem Anteil der Leberverfettung schlichtweg nicht korrekt diagnostiziert. Auch Studien auf denen die im Abschnitt Epidemiologie (2.3, S.10) dargestellten Prävalenzen beruhen, verwendeten diese Methode, um NAFLD zu detektieren. Dies wäre eine mögliche Erklärung für die Diskrepanz zwischen kalkulierter

und gemessener Prävalenz: die gängigste Screening-Methode ist nicht sensitiv genug, um alle Betroffenen zu identifizieren. Mittels transienter Elastographie, einer Ultraschall-basierten Messung der Gewebe- und Lebersteifigkeit (136,137), können z.B. fibrotische Prozesse detektiert werden. Durch diese Messung kann auch der sogenannte CAP-Wert (*controlled attenuation parameter*) ermittelt werden, der eine genauere Aussage über den Fettgehalt der Leber geben soll, als gewöhnliche Ultraschall-Untersuchungen (138). Diese Messung scheint eine vielversprechende Möglichkeit für ein Screening zu sein, wurde jedoch nicht in größeren Kohorten mit Validierung durch Leberbiopsie unabhängig geprüft. Die größte Limitierung der transienten Elastographie ist, dass selbst mit speziellen Sonden für übergewichtige Personen verlässliche Messungen nur bis zu einem BMI-Bereich von ca. 30 möglich sind. Auf Grund der relativ geringen Eindringtiefe der Ultraschallwellen in das Gewebe, verhindert die Dicke der subkutanen Fettschicht bei stark übergewichtigen und adipösen Personen valide Messungen.

Ein wesentlicher Nachteil von allen nicht-invasiven Methoden (Ultraschall, transiente Elastographie, MRT) ist, dass lediglich der Fettgehalt gemessen wird. Die Entzündungskomponenten und die Schädigung der Hepatozyten, die bei histologischer Bewertung des Lebergewebes durch Pathologen fundamental sind, werden nicht detektiert. Daher ist die histologische Bewertung einer Leberbiopsie bislang auch die einzige validierte und generell anerkannte Methode, eine NASH zu diagnostizieren. Allerdings ist die histologische Bewertung des Gewebes selbst wiederum mit Problemen behaftet. Zum einen umfasst die Biopsie ca. 1/50.000 des Lebergewebes (139). Da gerade in frühen Stadien von chronischen Lebererkrankungen fibrotische Prozesse und bei NAFLD die Verteilung der Fettakkumulation nicht homogen über die gesamte Leber sind, ist die Aussagekraft der Histologie auf einen sehr kleinen Ausschnitt beschränkt. Zum anderen ist die Bewertung der einzelnen Kriterien, mit denen der NAFLD-Schweregrad eingeschätzt wird, Steatose, lobuläre Entzündung und Ballonierung der Hepatozyten, durch Pathologen bis zu einem gewissen Grad subjektiv (139,140). Daher wird für Studien die unabhängige Bewertung durch mindestens zwei Pathologen empfohlen.

Für die Diagnostik der NAFLD liegen also zwei fundamentale Probleme vor: 1. der Goldstandard erfordert eine invasive Probenentnahme, erfasst dabei einen sehr kleinen Teil des Gewebes, ist partiell subjektiv in der Bewertung und ist durch den Aufwand ungeeignet als Screeningverfahren; 2. Nicht-invasive diagnostische Verfahren sind entweder extrem zeit- und kostenaufwändig, nicht sensitiv genug oder nicht in Risikopopulationen durchführbar und daher ebenso wenig für ein Screening geeignet. Diese Limitierungen der verschiedenen Verfahren lassen vermuten, dass die NAFLD in relevanten Anteilen von Risikopopulationen unentdeckt bleibt, wodurch die starke Diskrepanz zwischen gemessener NAFLD-Prävalenz in der Bevölkerung und aus Populationsdaten kalkulierter Prävalenz erklärt werden könnte.

2.5.2. Risikoprofil und Folgeerkrankungen der NAFLD

Lange wurde angenommen, dass die reine Steatose der Leber, die NAFL, benigne sei und nur die NASH ein ernstzunehmendes Gesundheitsrisiko darstellen würde. In den letzten Jahren verschob sich der Fokus auf das Ausmaß der Fibrosierung und ggf. Zirrhose, die als zentrale Determinanten für leberbezogene Morbidität und Mortalität gelten (141). Dies ist grundsätzlich korrekt, sofern die Limitierung auf leberbezogenes Überleben beachtet wird. Zu den wesentlichen Risiken der NAFLD zählt jedoch auch eine Progression zu kardiovaskulären Erkrankungen, metabolischem Syndrom und Diabetes (142,143). In der Tat stellt die NAFLD einen von anderen Faktoren unabhängigen Risikofaktor für kardiovaskuläre Erkrankungen und kardiovaskuläre Mortalität dar (29,144,145), so dass Mortalität durch kardiovaskuläre Ereignisse die Haupttodesursache bei NAFLD darstellen (1).

Unabhängig von den Risiken metabolischer Erkrankungen erhöhen NAFLD und insbesondere NASH das Risiko ein HCC zu entwickeln. Tatsächlich ist die jährliche kumulative Inzidenz von 2,6 % bei NAFLD nicht so hoch, wie z.B. bei viralen Lebererkrankungen mit 4 % (146,147). Durch die enorme Anzahl an Betroffenen ist jedoch die sogenannte *population attributable fraction* (also die Populations-basierte Menge) von NAFLD für die HCC-Entwicklung relativ hoch und wird in den USA auf 36,6 % geschätzt (148). In der Tat war in den vergangenen Jahren NASH die am stärksten zunehmende Indikation für Lebertumore und Listungen für Lebertransplantationen (149). Eine klinische Besonderheit in diesem Bereich ist zudem, dass HCC bei NAFLD oder NASH nicht mit so großer Häufigkeit in zirrhotisch veränderten Lebern auftreten (150–153). Da fortgeschrittene Fibrose und vor allem Zirrhose als Risikofaktor für HCC bei anderen chronischen Lebererkrankungen gilt, fällt dieses Warnzeichen bzw. Kriterium für eine engmaschige Überwachung für HCC weg. Ob sich die Mechanismen der HCC-Entstehung bei zirrhotischer und nicht-zirrhotischer NAFLD unterscheiden und welche Faktoren dabei eine Rolle spielen ist bislang nicht geklärt. Generell gilt T2DM als Risikofaktor für die HCC-Entstehung und Mortalität bei NAFLD-assoziiertem HCC, jedoch unabhängig von Fibrose oder Zirrhose. Auf Grund der diagnostischen Problematik (2.5.1) werden auch HCCs bei Patienten mit NAFLD meist in späten Stadien diagnostiziert. Hierdurch sind die therapeutischen Optionen in vielen Fällen auf eine Lebertransplantation reduziert. Das 5-Jahres überleben bei HCC insgesamt liegt bei 15 % und scheint für NAFLD-induziertes HCC generell nicht geringer zu sein (154,155). Allerdings ist bei NAFLD-assoziiertem HCC die Prognose stark von begleitenden Faktoren wie fortgeschrittener Fibrose und Adipositas abhängig (155). Obwohl also die Problemkonstellation für NAFLD (Übergewicht, Adipositas, Anzeichen des metabolischen Syndroms) weit verbreitet sind, ist die Aufmerksamkeit für diese darauf basierende Tumor-Erkrankung sehr gering. Damit droht ein bislang wenig beachtetes, schwerwiegendes Gesundheitsproblem, mit einem voraussichtlich deutlichen Anstieg in der absoluten Prävalenz in der nahen Zukunft und dadurch enormer Auswirkung auf Morbidität und Mortalität der Betroffenen sowie direkten und indirekten ökonomischen Konsequenzen (91).

Weiterhin erhöht NAFLD das Risiko für die Entwicklung eines T2DM (3,156), einer chronischen Nephropathie (unabhängig von T2DM) (157) und ist bei Frauen mit dem polycystischen Ovarialsyndrom (PCOS) assoziiert (158,159). NAFLD ist gegenüber der Normalbevölkerung mit erhöhter gesamt-, leberbezogener und kardiovaskulärer Mortalität verbunden (1).

Die NAFLD und auch die NAFL basieren auf grundlegenden Veränderungen im hepatischen Metabolismus verschiedener Substanzklassen. NAFLD an sich, unabhängig vom vorliegenden Schweregrad muss als Vorstufe zu weiteren metabolischen Veränderungen im gesamten Organismus angesehen werden und ist Risikofaktor für metabolisches Syndrom, Diabetes, kardiovaskuläre Erkrankungen sowie das HCC. Auf Grund der hohen Anzahl an Betroffenen sind dringend Maßnahmen für eine adäquate Diagnose, Screening für Patienten mit erhöhtem Risiko für kardiovaskuläre Erkrankungen und HCC sowie präventive Maßnahmen notwendig, da Therapie-Optionen weitgehend fehlen oder mit erheblichen Schwierigkeiten verbunden sind.

2.5.3. Aktuelle Therapie der NAFLD

Eine direkte auf NAFLD oder auf NASH ausgerichtete Therapie ist aktuell nicht verfügbar. Um eine NAFLD zu behandeln, können drei unterschiedliche Strategien verfolgt werden. Die erste Möglichkeit, ist eine Gewichtsreduktion. Studien mit gepaarten Leberbiospien vor und nach Behandlung zeigen, dass ein Gewichtsverlust von 7 - 10 % des Ausgangsgewichtes bei adipösen Patienten erforderlich ist, um eine sichere Auswirkung auf den Fettgehalt und die Entzündung in der Leber zu erzielen (160). Eigene, bislang nicht publizierte Daten weisen darauf hin, dass ein absoluter Gewichtsverlust von mehr als 20 kg zu metabolischen Verbesserungen führt. Um Gewichtsverlust zu erzielen sind wiederum unterschiedliche Verfahren denkbar. Als Erstlinien-Therapie und zentraler Baustein des konservativen Managements von Patienten mit NAFLD, Adipositas und / oder metabolischem Syndrom gilt eine intensive Lebensstilveränderung (160). Hierbei wird einerseits die Ernährung umgestellt, so dass geringere Kalorienmengen aufgenommen und eine gesündere Ernährung insgesamt angestrebt wird (Vermeidung von Fertigprodukt, „Fastfood“ und gesüßten Getränken). Andererseits wird das Bewegungspensum erhöht, sowohl im Alltag als auch durch sportliche Aktivität. Für die Beeinflussung der NAFLD ist vor allem die Komponente Bewegung und Sport wesentlich, da mehrere Studien belegen konnten, dass der Fettgehalt der Leber sich bei ausreichend intensiver sportlicher Aktivität auch ohne Gewichtsverlust bessert (161–163). Dies beruht vermutlich auf einer Verbesserung der peripheren Insulinresistenz (164). Eine aktuelle Maßgabe des britischen Gesundheitsministeriums empfiehlt auch eindeutig zusätzlich zu der Steigerung alttäglicher Bewegung mindestens zwei sportliche Aktivitäten für Beweglichkeit und Kraft pro Woche.

Es werden weiterhin Studien durchgeführt, um möglichst effektive und wirksame Varianten der Ernährungsumstellung und Erhöhung der körperlichen Aktivität zu identifizieren. Ein abschließendes

Fazit zu der idealen Art (Ernährungszusammensetzung, Art und Umfang der Bewegungssteigerung) der Lebensstilveränderung kann auf Basis der aktuellen Datenlage nicht getroffen werden (118). Ein wesentlicher Aspekt von intensiven Lebensstiländerungen ist jedoch die Motivationslage der Patienten (165,166). Es ist nicht leicht, dauerhaft – also für den Rest des Lebens – massive Veränderungen in Ernährung, Bewegung und Sport einzuleiten aber noch weitaus schwerer, dies nach erreichen initialer Erfolge beizubehalten. Gerade wenn Plateaus im Gewicht oder Trainingszustand erreicht werden, wenn sich Lebensbedingungen verändern, kann die Motivation einen Lebensstil zur Gewichtsreduktion weiterzuführen nachlassen und komplett verloren gehen. Die Gefahr eines Relapse ist also jederzeit gegeben. In den vergangenen Jahren konnten Programme mit fortgesetzter Beobachtung (*monitoring*), telemedizinischen Ansätzen und psychosomatischer Unterstützung sowie Einbindung des familiären Umfeldes erfolgreich zeigen, dass derartige Maßnahmen die Motivationslage bessern und stabilisieren (165–169). Trotzdem ist die Gewichtsreduktion über intensive Lebensstilveränderung keine triviale Behandlung, die jeder Patient einfach umsetzen kann. In diesem Bereich sind ohne Frage große Anstrengungen erforderlich, um die Versorgungslage zu verbessern. Als Alternative in Fällen vergeblicher Versuche, Gewicht zu reduzieren, und bei Vorliegen metabolischer Ko-Erkrankungen zur Adipositas kommt eine bariatrisch chirurgische Behandlung in Frage (170). Abhängig von Gewicht, Morbidität und Verteilung der viszeralen Fettmasse können rein restriktive Verfahren (Magenband, Schlauchmagen) oder restriktiv-malabsorptive Verfahren (Roux-en-Y-Magenbypass) eingesetzt werden. Das Prinzip bei restriktiven Verfahren ist eine Reduktion des Magenvolumens und somit eine erzwungene Verminderung der aufgenommenen Kalorien. Das Magenband stellt hier eine Anpassungsfähige und potentiell reversible Option dar, während der Schlauchmagen dauerhaft eine Reduktion des Volumens erzielt. Durch einen Magenbypass werden Teile des Verdauungssystems (Großteil des Magens und Ileum) ausgespart und eine kleine verbleibende Magentasche direkt mit dem Dünndarm verbunden. Hierdurch wird einerseits das Volumen drastisch reduziert, das bei einer Mahlzeit aufgenommen werden kann, gleichzeitig werden Verweildauer der Nahrung und Kontakt mit Magen- und Darmschleimhaut reduziert, so dass eine geringere Menge Nährstoffe aus der Nahrung resorbiert werden kann. Derartige Verfahren erfordern immer auch eine intensive Ernährungsberatung und Supplementation von Vitaminen und Spurenelementen, um Mangelerscheinungen vorzubeugen (171,172). Limitierungen dieses Verfahrens sind die unbekannten langfristigen Konsequenzen, einem generellen Risiko für eine umfangreiche abdominelle Operation, postoperative Depression und die relativ hohen Kosten für eine Behandlung (172,173). Analog zu diesen chirurgischen Verfahren existieren endoskopisch einsetzbare Magenballons (restriktiv) und die sogenannte EndoBarrier (malabsorptiv), die durch Trennung von Magenschleimhaut und Nahrungsbrei mittels eines Kunststoffschlauches die Kalorienresorption blockiert (174,175). Diese Methoden erzielen eine Gewichtsreduktion, jedoch gibt es dazu bislang keine Langzeitstudien und keine konkreten Daten zur Behandlung der NAFLD. Rückfälle sind bei Entfernen von Magenballon oder Endobarrier allerdings sehr wahrscheinlich.

Die zweite Strategie zur Behandlung der NAFLD zielt auf indirekte Pharmakotherapie der zu Grunde liegenden Problematik oder von Komorbiditäten ab (176). Ein wesentliches Problem dieser Behandlung ist, dass die Ursache des Problems, das Übergewicht bzw. die Adipositas nicht beseitigt wird. Einige der eingesetzten Wirkstoffe haben als Nebenwirkung sogar Gewichtssteigerung. Um den Effekt einer Verbesserung der NAFLD und einer Normalisierung des Stoffwechsels aufrecht zu erhalten, muss diese Behandlung dauerhaft weitergeführt werden. Langfristige Auswirkungen vieler Wirkstoffe sind jedoch noch unbekannt und einige Substanzen stehen im Verdacht Karzinogenese in der Leber oder im Gastrointestinaltrakt zu fördern.

Als dritte Option würde die direkte Behandlung entzündlicher Komponenten der NASH oder eine Widerherstellung normaler Stoffwechseltätigkeit der Leber in Frage kommen. Durch eine Normalisierung der lebereigenen Prozesse und Fließgleichgewichtige von Substanzen könnte eine Reduktion der Verfettung und in der Folge auch eine Verbesserung der Entzündung erreicht werden. Hierfür liegt eine Reihe möglicher Wirkstoffe vor, die in klinischen Studien getestet werden (177). Aus Gründen der Übersichtlichkeit sind nur Substanzen in Studien der Phase III in Tabelle 4 aufgelistet.

Tabelle 4: Wirkstoffe zur Behandlung der NAFLD oder NASH in klinischen Studien der Phase II oder III.

Wirkstoff / Bezeichnung	**Wirkungsweise**
Obeticholsäure	FXR-Agonist
Elafibranor	PPARγ-Agonist
Cenicriviroc	CCR2/5-Antagonist
Selonsertib	ASK1-Inhibitor

ASK1: *apoptosis signaling kinase 1*; CCR2/5: *cysteine–cysteine motif chemokine receptor* 2 oder 5; FXR: *farnesoid X receptor*; PPAR: *peroxisome proliferator-activator receptor γ*.

Die große Bandbreite an Substanzen, die aktuell in klinischen Studien erprobt werden, zeigt, dass NAFLD eine extrem komplexe Erkrankung ist. Viele Mechanismen sind beteiligt und pathophysiologisch verändert. Die Substanzen in Phase III Studien adressieren Gallensäure- und Cholesterinhaushalt (Obeticholsäure), Lipidmetabolismus und Fettsäureoxidation (Elafibranor), Entzündungsprozesse (Cenicriviroc) und Apoptose (Selonsertib). Es muss hier betont werden, dass keiner der hier genannten Wirkstoffe bislang explizit für die Behandlung der NAFLD oder der NASH zugelassen wurde, weder durch die FDA noch durch europäische oder deutsche Behörden. Sicher gibt es mehrere vielversprechende Kandidaten, von denen aber kein Wirkstoff alle beim metabolischen Syndrom und der NAFLD betroffenen Prozesse adressiert und korrigiert. Daher wäre es durchaus denkbar, dass zur rein pharmakologischen Behandlung von NAFLD und allen Komorbiditäten die Einnahme mehrerer Wirkstoffe erforderlich wird. Dies steigert durch Interaktion jedoch ggf. die

Nebenwirkungen oder kann zu neuen Nebenwirkungen führen. Weiterhin ist zu erwarten, dass alle neuen Wirkstoffe zunächst sehr teuer sind und somit die Gesundheitssysteme deutlich belasten werden. In den kommenden Jahren wird sich durch weitere klinische Studien hierzu erst klären, ob eine pharmakologische Therapie der NAFLD oder der NASH in naher Zukunft möglich ist.

Eine direkte Therapie für NAFLD und NASH ist aktuell nicht verfügbar. Durch Gewichtsverlust mittels intensiver Lebensstilveränderung, bariatrischer Chirurgie oder spezialisierten, endoskopischen Verfahren analog zur Chirurgie, kann indirekt eine Verbesserung der NAFLD erzielt werden. Hierzu ist ein erheblicher Gewichtsverlust erforderlich, der in der Regel nicht dauerhaft aufrechterhalten wird. Pharmakologische Optionen beruhen auf indirekter Einwirkung über bekannte Prozesse, wie Insulinresistenz, oder gezielte Beeinflussung von Prozessen in der Leber. Bislang ist jedoch keine pharmakologische Therapie zugelassen.

2.5.4. Übertragbarkeit von Modellsystemen auf den Menschen und Entwicklung von Therapien

Ein zentrales Problem für das Verständnis der molekularen Pathogenese der NAFLD und die Entwicklung von neuen therapeutischen Optionen sind die für die Grundlagenforschung eingesetzten Modelle. Ein erheblicher Teil von Daten aus murinen Modellen mit Diät-induzierter NAFLD bzw. NASH basiert auf der lange etablierten Methionin-Cholin-defizienten Diät (MCD). Durch den Mangel an Methionin und Cholin entwickelt sich bei Mäusen eine sehr robuste Lebersteatose mit deutlicher Entzündung. Allerdings entwickeln Tiere unter MCD kein Übergewicht und keine Insulinresistenz, sondern verlieren in der Tat Gewicht. Am ehesten entspricht diese Situation beim Menschen der Zöliakie (Glutenunverträglichkeit), die meist bei sehr schlanken Individuen auftritt und bei nicht-angepasster Ernährung mit Lebersteatose und Entzündung im Lebergewebe verbunden ist. Andere ernährungsbedingte Modelle mit hohem Kalorienanteil und/oder hohem Fettanteil führen bei Mäusen zu weniger robuster Ausprägung einer NAFLD, seltener noch zu NASH. Vor allem werden viele dieser Ernährungsmodelle nur über lange Zeiträume wirksam, was sie für Experimente unattraktiv macht. Eine Arbeit von Teufel et al. untersuchte die Expressionsmuster verschiedener muriner Modelle und verglich diese mit menschlichen Leberproben von NAFL- und NASH-Patienten (178). Tatsächlich fand sich die stärkste Übereinstimmung an in gleicher Weise regulierter Gene zwischen humanen NASH-Lebergewebe und muriner Leber unter MCD. Allerdings beinhalten diese Gene ausschließlich entzündliche Faktoren und kaum Gene, die mit Lipid- oder Glukosemetabolismus assoziiert wären. Insgesamt war die Überlappung gleichgerichteter Expressionsveränderung zwischen murinen Modellen und humaner Situation sehr gering. Schließlich ist die Auswirkung von Diät-basierten Modellen der NAFLD stark vom verwendeten Maus-Stamm abhängig, da es durchaus Stämme gibt, die resistent gegenüber Adipositas und NAFLD sind, selbst wenn hohe Kalorienmengen gefüttert werden (179,180). Die Unterschiede in der Ernährungsphysiologie, Mäuse sind immerhin

Koprophagen mit einem auf die Körperlänge bezogen 5 mal größerem Caecum als beim Menschen, sind möglicherweise zu groß, um verlässliche Aussagen zur humanen Situation bei NAFLD treffen zu können (181). Neben Diät-basierten Tiermodellen, existiert eine Reihe monogener Modelle der NAFLD (182). Diese Spiegeln durchaus die Situation bei genetisch ausgelöster Adipositas und daraus resultierender NAFLD beim Menschen wieder (180,182). Allerdings machen monogene Ursachen der Adipositas beim Menschen einen extrem geringen Anteil an der Gesamtproblematik aus. Insofern ist fraglich, ob die Daten aus den monogenen Tiermodellen der NAFLD auf die in erster Linie ernährungs- und lebensstilbedingte NAFLD der meisten Betroffenen übertragbar sind.

2.6. Zentrale offene Fragen in Bezug auf die NAFLD

Aus den in Abschnitt 2.5 dargestellten Problemen in Bezug auf NAFLD ergeben sich die aus unserer Sicht leider noch sehr weit gefassten Fragestellungen, die dringend geklärt werden müssen. Die erste Fragestellung ist, welchen Anteil verschiedene zelluläre und molekulare Mechanismen an der Pathogenese der NAFLD haben. Insbesondere ist offen, wodurch genau die NAFLD ein erhöhtes Risiko für andere metabolische Komorbiditäten verursacht und es ist unklar, wodurch die Progression von der reinen Steatose zur NASH und dann zu Fibrose oder zum hepatozellulären Karzinom führt. Sind diese Fragen hinreichend beantwortet, würden die entsprechenden Ergebnisse zusätzlich sinnvolle molekulare Angriffspunkte für spezifische Therapie gegen NASH, NASH-assoziierte Fibrose sowie NAFLD-basiertes hepatozelluläres Karzinom aufzeigen.

Die zweite Fragestellung ergibt sich aus dem diagnostischen Dilemma, durch das vermutlich einerseits eine erhebliche Dunkelziffer an Betroffenen vorliegt und andererseits eine Überwachung der Erkrankungsprogression oder Regression nur mit mehrschrittigen diagnostischen Verfahren (Ultraschall, Bluttests, ggf. Leberbiospie) und erheblichem Aufwand möglich ist. Einfach zu bestimmende und auswertbare nicht-invasive Marker für die NAFLD oder für den Schweregrad der Erkrankung sind dringend gefordert, um die Situation für Patienten zu verbessern.

Die dritte Fragestellung für die vorliegende Arbeit betrifft das Risikoprofil von NAFLD-Patienten. Es ist zwar klar, dass NAFLD ein Risiko für bestimmte Ko- und Folgeerkrankungen darstellt. Allerdings ist weiterhin offen, inwiefern unterschiedliche Schweregrade der NAFLD das Risiko z.B. für kardiovaskuläre Erkrankungen oder Tumorentstehung beeinflussen. Weiterhin ist zu vermuten, dass die NAFLD an sich bereits ein Grundrisiko für metabolische Veränderungen mit sich bringt, die häufig unterschätzt werden, hierzu liegen jedoch bislang nur wenige Daten vor.

Die hier behandelten Fragestellungen lassen sich demnach in molekulare Mechanismen und Pathogenese der NAFLD, nicht-invasive Diagnostik der NAFLD und Risikobewertung der NAFLD zusammenfassen. Die in dieser Arbeit dargestellten Ergebnisse sind daher in diese Kategorien gegliedert.

3. Ergebnisse

Die im Folgenden zusammengestellten Arbeiten haben darauf abgezielt, spezifische Aspekte der NAFLD in Bezug auf molekulare Mechanismen, Pathophysiologie, Epidemiologie und Diagnostik zu untersuchen. Zum Zeitpunkt der jeweiligen Publikation waren die bearbeiteten Fragestellungen noch weitgehend offen und sind dies zum Teil bis heute. Die Ergebnisse sind strukturiert in molekulare Mechanismen der NAFLD-Entstehung und -Progression, epidemiologische und translationale Untersuchungen sowie klinisch-diagnostische Arbeiten.

3.1. Molekulare Mechanismen der NAFLD

In unseren Arbeiten haben wir verschiedene mögliche Faktoren untersucht, die auf zellulärer bzw. molekularer Ebene an Entstehung oder Progression der NAFLD beteiligt sein könnten. Mit translationalen Ansätzen wurde gezeigt, dass CD36 und Fetuin A in der Leber bei NASH gegenüber reiner Steatose erhöht sind und mit Zelltod sowie anderen Surrogatmarkern der Leberschädigung assoziiert sind. Weiterhin konnten wir *in vitro* bestätigen, dass Vitamin D Signalgebung als Antagonist des TGF-β Signalweges antifibrotisch wirkt und einerseits unabhängig vom Vitamin D Rezeptor wirken kann, die Wirkung aber andererseits durch Polymorphismen im VDR-Gen beeinflusst wird. Schließlich konnten wir basierend auf einem murinen Modell des Niemann-Pick Syndroms (Funktionsverlust der aziden Sphingomyelinase) zeigen, dass Morphologie und Genregulation im Fettgewebe sowie Signalgebung über den mTORC2-Komplex in der Leber mit protektiven Effekten gegenüber einer Lebersteatose verbunden sind.

3.1.1. Mechanismen der Erkrankungsprogression bei NAFLD

Bechmann, Gieseler, Sowa, Kahraman, Erhard, Wedemeyer, Emons, Jochum, Feldkamp, Gerken, Canbay. Apoptosis is associated with CD36/fatty acid translocase upregulation in non-alcoholic steatohepatitis. Liver International 2010;30(6):850-859.

Kahraman, Sowa, Schlattjan, Sydor, Pronadl, Wree, Beilfuss, Kilicarslan, Altinbaş, Bechmann, Syn, Gerken, Canbay. Fetuin-A mRNA expression is elevated in NASH compared with NAFL patients. Clinical Science 2013; 125: 391-400.

Ausgehend von der Fragestellung, welche Mechanismen zu verstärktem Zelltod der Hepatozyten bei NAFLD führen könnten, untersuchten wir in Patienten mit bariatrisch-chirurgischem Eingriff die Expression von Zelltod-assoziierten Genen und Fettsäuretransportern (183). Die Hypothese war, dass erhöhte Aufnahme von FFA über Fettsäuretransporter zu vermehrter Zellschädigung und letztlich Apoptose der Hepatozyten führen würde. Tatsächlich fanden wir stark erhöhte mRNA- und Protein-

Expression des Scavenger und Fettsäuretransporters CD36/FAT (*fatty acid translocase*) assoziiert mit erhöhten Serumkonzentrationen an FFA. Parallel hierzu war die Expression des FAS-Liganden und des FAS-Rezeptors (ein auslösender Stimulus für extrinsische Apoptose) sowie von mehreren Faktoren, die wesentlich für intrinsisch ausgelöste Apoptose sind, erhöht. Dies war mit, gegenüber gesunden Kontrollen, erhöhten M30-Serumkonzentrationen verbunden. Schließlich konnten wir zeigen, dass die M30 Konzentration im Serum, als Surrogatmarker für Apoptose, in Patienten mit NASH (NAS > 4) höher war als bei NAFL. Dabei war die Rate an sterbenden Zellen, bestimmt mittels TUNEL-Färbung im Lebergewebe nicht unterschiedlich. Weiterhin korrelierten die M30-Serumkonzentration mit dem NAS. In dieser Studie konnten wir demonstrieren, dass Fettsäuretransporter-Expression, Apoptose der Hepatozyten und der Schweregrad der NAFLD assoziiert zu sein scheinen. Die Apoptose-Rate, anscheinend jedoch nicht die Gesamtzelltodrate, scheint ein Surrogatmarker für das Ausmaß der Leberschädigung bei NAFLD zu sein.

In einem translationalen Ansatz gingen wir Hinweisen nach, dass Fetuin-A als Hepatokin einen möglichen Surrogatmarker für die Leberschädigung in NAFLD darstellen könnte und möglicherweise an der Erkrankungsprogression beteiligt sein könnte. In einem Patientenkollektiv von 108 NAFLD-Patienten, denen im Rahmen bariatrischer Chirurgie Lebergewebe entnommen wurde, wurden Serumkonzentrationen von FFA, Zelltodmarkern und Fetuin-A vor und nach der Operation gemessen. Parallel zum Gewichtsverlust durch die bariatrische Operation waren 4 Wochen postoperativ niedrigere Serumkonzentrationen der Zelltodmarker M30 und M65 sowie höhere Konzentrationen an Adiponektin und Fetuin-A messbar. Der postoperative Anstieg an Serum Fetuin-A war jedoch nur für Patienten mit NASH signifikant. Die FFA-Konzentration im Serum ging 4 Wochen nach dem Eingriff nur marginal zurück (kein signifikanter Unterschied zum Zeitpunkt vor Operation). Insgesamt bestätigt dies einen positiven Effekt einer bariatrischen Operation, vermutlich zumindest partiell durch den Gewichtsverlust, auf den Status einer NAFLD. Weiterhin wurde untersucht, ob sich die Serumkonzentration sowie mRNA und Proteinexpression von Fetuin-A im Lebergewebe zwischen NASH und NAFL unterscheiden. In der Tat waren bei NASH höhere Expressionsraten auf mRNA- und Proteinebene, sowohl mittels Western Blot als auch immunhistochemisch detektiert, von Fetuin-A zu verzeichnen. Dies steht in gewisser Weise im Wiederspruch zu dem Anstieg der Fetuin-A Serumkonzentration bei Patienten mit NASH nach dem Eingriff, da dieser mit einer Minderung der Leberschädigung einherging. Um genauer zu eruieren, wie die Fetuin-A Expression in Hepatozyten durch FFA reguliert wird, wurden primäre menschliche Hepatozyten mit diesen behandelt. Die Gabe von FFA führte zu erhöhter mRNA Expression von Fetuin-A in humanen Hepatozyten, parallel zur Lipidakkumulation. Tatsächlich scheint Lipidakkumulation in Hepatozyten die Fetuin-A Expression zu erhöhen (184), was mit den von uns beobachteten Daten übereinstimmt. Ferner scheint die Fetuin-A Expression und Serumkonzentration mit dem Schwergrad der NAFLD bzw. mit NASH anzusteigen. Möglicherweise basiert der zusätzliche Anstieg des Serum-Fetuin-A auf einer kurzfristig erhöhten Freisetzung von Lipiden aus dem Fettgewebe nach bariatrischer Chirurgie (185). Diese Arbeit konnte

Daten bestätigen, die Fetuin A als möglichen Marker der Lebersteatose identifiziert hatten (184) und weiterhin zeigen, dass die Serumkonzentration an Fetuin-A mit dem Schweregrad der NAFLD assoziiert ist.

Durch diese grundlegenden Arbeiten konnten wir bestätigen, dass hepatozellulärer Zelltod, insbesondere die Apoptose, mit dem Schweregrad der Leberschädigung bei NAFLD assoziiert ist. Weiterhin sind Fettsäuretransporter und Hepatokine einerseits mögliche zusätzliche Marker für den Schweregrad der NAFLD, andererseits könnten diese sinnvolle Ziele für therapeutische Ansätze darstellen.

3.1.2. Interaktion von Vitamin D und dem Vitamin D Rezeptor mit Fibrosierung bei NAFLD

Beilfuss, Sowa, Sydor, Beste, Bechmann, Schlattjan, Syn, Wedemeyer, Mathé, Jochum, Gerken, Gieseler, Canbay. Vitamin D counteracts fibrogenic TGF- signalling in human hepatic stellate cells, both receptor-dependently and independently. Gut 2015;64(5):791-799.

Die Ausprägung einer Fibrose gilt als zentraler Faktor für leberbezogene Morbidität und Mortalität in der NAFLD (siehe 2.5.2, S.17). Bei anderen chronischen Lebererkrankungen ist weitgehend unstrittig, dass der hepatozelluläre Zelltod zur Aktivierung von HSC und damit einer vermehrten Kollagen-Produktion im Lebergewebe führt. Dies gilt als Wundheilungsreaktion des Fibroblasten-artigen Zelltyps der HSC. HSC werden durch verschiedene Reize aktiviert, wie Zytokine sekretiert von residenten Immunzellen oder gestressten Hepatozyten, allerdings auch durch direkte Aufnahme apoptotischer Körperchen (77,186). In dieser Arbeit war die initiale Fragestellung, ob die Belastung der Hepatozyten durch Lipide, insbesondere durch freie Fettsäuren, einen Einfluss auf die Stimulus-Stärke der apoptotischen Körperchen auf die HSC hat. In der Tat war dies nicht der Fall, apoptotische Körperchen von Hepatozyten mit Konditionierung durch erhöhte FFA-Konzentrationen führten nicht zu einer verstärkten HSC-Aktivierung (187). Wir stellten uns die Frage, ob es andere Faktoren geben könnte, durch die überhaupt eine Fibrosierung auf Basis der NAFLD entstehen könnte, da die Zelltod-Rate in dieser Erkrankung zwar erhöht, aber nicht so hoch ist, wie in anderen chronischen Lebererkrankungen. Wir betrachteten Patienten nach bariatrisch-chirurgischem Eingriff, denen vor der Operation Blut abgenommen wurde und bei denen intraoperativ eine Leberbiopsie erfolgte. Hierbei bestätigten sich Daten anderer Gruppen, dass extreme Adipositas mit erniedrigten Vitamin D Spiegeln einhergeht (188). Weiterhin war im Lebergewebe unserer Patienten die Expression des Vitamin D Rezeptors (VDR) erniedrigt. Vitamin D und der VDR wiederum sind als zentrale Antagonisten des TGF-β Signalweg bekannt, der wesentlich für die Aktivierung von HSC ist. Als Modell für unsere Fragestellung verwendeten wir primäre humane HSC (phHSC), isoliert aus nativem Lebergewebe, das als Überhangmaterial von partiellen Hepatektomien stammte. Die phHSC wurden *in vitro* mit FFA

behandelt und / oder mit Vitamin D. Die FFA-Gabe führte jedoch nicht zu einer Aktivierung der HSC, sondern zu deren Zelltod. Um die Wirkung einer Vitamin D Supplementation *in vitro* demonstrieren zu können, wurde der Stimulus auf TGF-β gewechselt, was zu einer deutlichen Aktivierung und fibrogenen Reaktion der HSC führte. Ein transienter Knockdown der VDR-Expression mittels siRNA steigerte nicht nur die Wirkung von TGF-β sondern erhöhte bereits die Expression von α-SMA, Kollagen1α und TGF-β, ohne TGF-β als Stimulus. Die Gabe von Vitamin D führte unter diesen Bedingungen zu mehreren Effekten und Erkenntnissen. Zum einen wirkte Vitamin D klassisch über den VDR, resultierte in vermehrter SMAD2 Phosphorylierung und führte auch zu vermehrter Expression des VDR. Innerhalb eines Zeitfensters von maximal 30 min. nach Vitamin D-Gabe war die Wirkung von Vitamin D unabhängig vom VDR, erfolgte also auch dann, wenn VDR durch siRNA supprimiert war. Die Phosphorylierung von SMAD2 konnte dennoch beobachtet werden, was auf einen zusätzlichen antifibrotischen Wirkmechanismus des Vitamin D hinweist. Vitamin D war bereits vorher in Studien als mögliches antifibrotisches Therapeutikum getestet worden, jedoch mit geringem Erfolg. Wir untersuchten daher einerseits in den *in vitro* behandelten Zellen andererseits in der Patientenkohorte nach bariatrischer Chirurgie Zusammenhänge von bekannten Polymorphismen des VDR mit dem Ausmaß der TGF-β induzierten Aktivität bzw. dem Fibrosegrad. Tatsächlich waren sowohl *in vitro* als auch in den Patienten mehrere Polymorphismen mit einer verminderten Wirkung des Vitamin D oder einer stärkeren Reaktion der HSC auf TGF-β bzw. mit höherem Fibrosegrad assoziiert. Hierbei führte Homozygotie des GG Allels im A1012G-Polymorphismus (rs4516035) zur Unwirksamkeit von Vitamin D *in vitro*, bei Patienten mit homozygot GG für A1012G lag eine geringere mRNA Expression des VDR in der Leber als bei Gesundkontrollen vor und bereits bei Anwesenheit eines G-Alles war die pro-fibrogene mRNA Expression erhöht.

Zusammengefasst könnte eine reduzierte Verfügbarkeit von Vitamin D bei Adipositas die Entstehung der Leberfibrose bei NAFLD fördern. Eine Supplementation mit Vitamin D gegen Fibrose oder Fibrogenese kann durch vorhandene Polymorphismen im VDR vermindert oder sogar unwirksam werden. Dies sollte bei künftigen Studien an Vitamin D als antifibrotischer Therapie, unabhängig von der zu Grunde liegenden Lebererkrankung, bedacht werden.

3.1.3. Identifikation möglicher pharmakologischer Ansatzpunkte über ein Knock-Out Modell der aziden Sphingomyelinase mit partieller Resistenz gegen Diät-induzierte hepatische Steatose

Sydor, Sowa, Megger, Schlattjan, Jafoui, Wingerter, Carpinteiro, Baba, Bechmann, Sitek, Gerken, Gulbins, Canbay. Acid sphingomyelinase deficiency in Western diet-fed mice protects against adipocyte hypertrophy and diet-induced liver steatosis. Molecular Metabolism 2017;6(5):416-427.

Wie im Abschnitt "Pathophysiologie der NAFLD und der NASH" (2.2) beschrieben sowie bestätigt durch eigene Daten (3.1.1, Mechanismen der Erkrankungsprogression bei NAFLD) trägt der Zelltod der Hepatozyten zur NAFLD Progression bei. Daten anderer Gruppen (189,190) und eigene vorläufige Experimente hatten gezeigt, dass eine alterierte Ceramid-Synthese in Zellmembranen an diesem Prozess in der NAFLD beteiligt sein könnte. Für die extrinsisch aktivierte Apoptose über TRAIL und den TRAIL-Rezeptor fungieren sogenannte *lipid rafts* aus Ceramiden, Bereiche der Zellmembran die ausschließlich aus dieser Lipidkomponente bestehen, als verstärkender Faktor (191). In den *lipid rafts* können TRAIL-Rezeptoren mit gebundenem Liganden einfacher zu Multimeren akkumulieren und so ein verstärktes Apoptose-Signal an die Zelle vermitteln. Die azide Sphingomyelinase (ASM, codiert durch das Smpd1-Gen) katalysiert die Umwandlung von in der Zellmembran eingebautem Sphingomyelin zu Ceramid. Bei NAFLD finden sich erhöhte Ceramid-Konzentrationen im Lebergewebe. In einem murinen Modell mit Smpd1-Knockout haben wir untersucht, inwiefern eine durch Diät erzeugte NAFLD anders verläuft als in Wildtyp-Mäusen (192). Tatsächlich sind Smpd1-KO Mäuse vor einer Diät-induzierten Lebersteatose partiell geschützt. Interessanterweise trat bei Smpd1-KO Mäusen unter einer *western diet* (hochkalorisch, hoher Fettanteil), wie von uns zur Induktion einer Lebersteatose eingesetzt, keine Hypertrophie der Adipozyten auf. Auch eine Hyperleptinämie blieb aus. Dies war verbunden mit einer höheren Expression von Genen, die mit einer "Bräunung" des Fettgewebes assoziiert sind, im Fettgewebe der Smpd1-KO Mäuse. Diese Gene wurden im Fettgewebe von adipösen Patienten nicht exprimiert. Um mögliche Ziele im Lebergewebe, die durch diese veränderte Situation im Fettgewebe beeinflusst werden, zu identifizieren, wurde eine Proteomanalyse mittels Massenspektrometrie durchgeführt. Hierbei wurde entdeckt, dass Rictor bzw. der mTORC2-Komplex als zentraler Regulator verschiedener Prozesse, z.B. Insulinresistenz und -sensitivität, herab reguliert war. Dies deckte sich mit einer verminderten Phosphorylierung von Akt und einer entsprechend veränderten mRNA Expression einiger Zielgene.

In dieser Arbeit konnten wir zeigen, dass der protektive Effekt eines Smpd1-KO gegenüber Diät-induzierter Steatose möglicherweise auf veränderte Adipozytenmorphologie und reduzierte Leptin-Sekretion zurückzuführen sein könnte. In der Leber wurde eine reduzierte Aktivierung des mTORC2-Komplexes mit der verminderten Steatose unter *western diet* beobachtet und stellt damit ein interessantes Ziel für weitere Untersuchungen dar.

3.2. Vermutete Verbreitung der NAFLD im Ruhrgebiet und klinisches Risikoprofil der NAFLD

Zu den offenen Fragen (2.6) bezüglich NAFLD zählt weiterhin, wie hoch tatsächlich die exakte Prävalenz in verschiedenen Populationen ist. Gerade für Deutschland liegen nur sehr grobe Schätzungen vor und ein großer Teil der Zahlen zur NAFLD-Epidemiologie ist bereits mehrere Jahre

alt. Weiterhin ist unklar, wie groß tatsächlich das Risiko bei NAFLD ist, kardiovaskuläre Erkrankungen und HCC zu entwickeln bzw. wie diese Erkrankungen miteinander in Verbindung stehen. In mehreren klinisch-epidemiologisch angelegten Studien haben wir versucht, in dieses Feld mehr Klarheit zu bringen.

3.2.1. Serumkonzentrationen klassischer Leberschädigungsmarker korrelieren mit dem BMI unabhängig von klinischen Grenzwerten

Kälsch, Bechmann, Heider, Best, Manka, Kälsch, Sowa, Moebus, Slomiany, Jöckel, Erbel, Gerken, Canbay. Normal liver enzymes are correlated with severity of metabolic syndrome in a large population based cohort. Scientific Reports 2015;5:13058.

Im Ruhrgebiet wurde von 2000 bis 2013 die Heinz-Nixdorf-Recall Studie durchgeführt (193). Hierbei handelt es sich um eine populationsbasierte Studie, deren Teilnehmer zufällig aus der Bevölkerung des Ruhrgebietes ausgewählt wurden. Die Teilnehmerinnen und Teilnehmer im Alter von 45 bis 75 wurden bei einem Aufnahmetermin ärztlich untersucht, wobei der Fokus auf unerkannten kardiovaskulären Erkrankungen und T2DM lag, und erhielten mehrere Fragebögen. Wir betrachteten Daten von 4814 Teilnehmern mit zwei Fragestellungen (194): 1. wie hoch ist in dieser Kohorte die Prävalenz der NAFLD, basierend auf Surrogatmarkern? 2. Kann anhand der verfügbaren Serumparameter eine Aussage über den Zustand der Leber gemacht werden? Da in dieser Kohorte weder gezielte Ultraschalluntersuchungen an der Leber noch Leberbiopsien durchgeführt wurden, sind die Ergebnisse unserer Analysen nicht durch diese unzweifelhaften Daten bestätigt. Dennoch konnten wir mehrere interessante Erkenntnisse gewinnen. Zum einen lag die Prävalenz der Adipositas dieser Kohorte bei über 50 %, was sicherlich auf das Altersspektrum zurückzuführen ist. Zum anderen versuchten wir, über die klassischen Serum-Parameter der Leberschädigung (AST, ALT, GGT) zu eruieren, welche Individuen ggf. eine NAFLD haben könnten. Hierbei entdeckten wir, dass die überwiegende Mehrheit eines relativ alten, nahezu vollständig übergewichtigen und Großteils adipösen Patientenkollektivs keine Erhöhung klassischer Leberschädigungsmarker aufwies. Da eine ganze Reihe an Probanden bereits T2DM hatten (13,7 %) und Teilnehmer mit Grad drei Adipositas (BMI > 40) vertreten waren (n = 85), war ausgeschlossen, dass keine NAFLD in dieser Kohorte vorlag. Tatsächlich korrelierten die Serum-Marker für Leberschädigung deutlich mit dem BMI, auch wenn sie unterhalb der gängigen Grenzwerte lagen. Die tatsächliche Prävalenz der NAFLD kann auf Grund der verfügbaren Daten nicht sicher angegeben werden, allerdings ist unzweifelhaft, dass die klassischen Serum-Parameter für Leberschädigung mit den gängigen Grenzwerten nicht zuverlässig eine NAFLD detektieren können.

Eine weitere Beobachtung in dieser Kohorte war eine große Dunkelziffer an T2DM, der im Rahmen der Erstuntersuchung diagnostiziert wurde (bekannte Diabetiker 8 %, durch Erstuntersuchung

identifiziert 5,4 %). Da diese Diagnose unzweifelhaft war, versuchten wir, auf Basis der verfügbaren Serum-Parameter ein einfaches Vorhersagemodell zu entwickeln. Anhand von HbA1c und dem BMI oder HbA1c und Adiponektin konnte mit einer Genauigkeit um 85 % ein Diabetes vorhergesagt werden. Somit könnte Adiponektin einen objektivierbaren Ersatz für den BMI sein, um den Status des Fettgewebes einzuschätzen. Adiponektin könnte sich also als objektiv quantifizierbarer Prädiktor für das metabolische Syndrom und damit assoziierter Risiken eignen. Der BMI ist bekanntermaßen mit Limitierungen behaftet, was sehr große, sehr kleine sowie gut trainierte bzw. muskulöse Individuen anlangt und kann darüber hinaus keine Angaben zur Fettverteilung machen (195,196).

In einer großen populationsbasierten Kohorte konnten wir zeigen, dass die Prävalenz der Adipositas, des T2DM sowie vermutlich der NAFLD höher als erwartet ist. Weiterhin eignen sich klassische Leberserumparameter nur bedingt für eine Einschätzung der NAFLD. Zumindest sind die aktuellen Grenzwerte zu hoch gewählt, um eine NAFLD-bedingte Leberschädigung detektieren zu können. Schließlich kann mit einfachen nicht-invasiven Verfahren eine Vorhersage von Erkrankungen, wie T2DM, mit hoher Genauigkeit vorgenommen werden, wobei Adiponektin als möglicher Marker für den metabolischen Status des Fettgewebes fungieren kann.

3.2.2. In einer *real-life* Kohorte mit gastrointestinalen Fragestellungen ist NAFLD die häufigste Grunderkrankung und mit einem erhöhten Risikoprofil für kardiovaskuläre Erkrankungen verbunden

Kälsch, Keskin, Schütte, Baars, Baba, Bechmann, Canbay, Sowa. Patients with ultrasound diagnosis of hepatic steatosis are at high metabolic risk. Zeitschrift für Gastroenterologie 2016;54(12):1312-1319.

Als Folgearbeit zu den Untersuchungen in der populationsbasierten Heinz-Nixdorf-Recall Studie, analysierten wir Daten aus unserer gastroenterologischen Ambulanz (197). Diese über ein Jahr konsekutiv rekrutierte Kohorte spiegelt das gesamte Spektrum gastroenterologischer Erkrankungen in einem Zentrum tertiärer Versorgung wieder. Eine Limitierung der Studie ist, dass Lebersteatose ausschließlich mittels Ultraschall ermittelt wurde, mit entsprechend geringer Sensitivität (siehe 2.5.1). Tatsächlich wiesen 76 von 106 Patienten (72 %) eine Steatose auf, hiervon entfielen 60 auf die Diagnose NAFLD. Wurden die NAFLD-Patienten mit allen anderen Patienten verglichen, zeigte sich ein deutlich schlechteres metabolisches Risikoprofil, wobei auch hier keine Erhöhung der klassischen Leberserumwerte zu beobachten war. Eine Eingruppierung der Patienten nur abhängig von einer mittels Ultraschall diagnostizierten Steatose, unabhängig von der Diagnose NAFLD, verstärkte die Unterschiede im metabolischen Risiko. Patienten mit Steatose wiesen höheren HbA1c, niedrigeres Serum-Adiponektin und -HDL auf. Außerdem waren T2DM, kardiovaskuläre Erkrankungen und Bluthochdruck deutlich häufiger bei Patienten mit Steatose (Signifikant für Bluthochdruck).

Patienten mit per Ultraschall diagnostizierter Steatose haben, unabhängig von der vorliegenden Grunderkrankung, ein schlechteres metabolisches Serumprofil und häufiger Erkrankungen, die mit dem metabolischen Syndrom assoziiert sind. Basierend auf der schlechten Sensitivität von Ultraschallverfahren zur Detektion einer Lebersteatose sollte ein positiver Befund ein Warnzeichen für metabolische Erkrankungen sein. Aus unserer Sicht ist hier bereits eine umgehende weiterführende Diagnostik zu den bekannten Ko- und Folgeerkrankungen sowie intensive Lebensstilveränderung zu empfehlen.

3.2.3. Adipozytengröße und alterierte Adipokin- und Fettsäurefreisetzung als Einflussfaktoren für die NAFLD-Progression

Wree, Schlattjan, Bechmann, Claudel, Sowa, Stojakovic, Scharnagl, Köfeler, Baba, Gerken, Feldstein, Trauner, Canbay. Adipocyte cell size, free fatty acids and apolipoproteins in severely obese patients. Metabolism 2014;63(12):1542-1552.

Ein aus unserer Sicht lange Zeit vernachlässigter Faktor bei der Pathogenese der NAFLD und somit bei Diagnostik und der Suche nach Therapieoptionen ist der Einfluss des Fettgewebes. Es gab einige kleine, klinische Studien, die darauf hinwiesen, dass das Ausmaß des viszeralen Fettgewebes einen größeren Einfluss auf den Schweregrad der NAFLD hat, als subkutanes Fettgewebe (11,12,198,199). Allerdings war unklar, warum das so ist und was sich im Fettgewebe bei der Einlagerung extremer Mengen an Lipiden abspielt. Um die Interaktion von Fettgewebe und Leber bei NAFLD näher zu untersuchen, analysierten wir Daten von 93 adipösen Patienten nach bariatrisch-chirurgischem Eingriff, denen Fett- und Lebergewebe während der Operation entnommen wurde und von denen wir Serum zum Zeitpunkt des Eingriffs zur Verfügung hatten (200). Für die Untersuchung wurden die Patienten nach dem histologisch beurteilten Schweregrad der Leberschädigung nach dem NAS (≤ 4 = NAFL oder > 4 = NASH) aufgeteilt. Eine zentrale Erkenntnis dieser Arbeit war, dass die Adipozytengröße mit dem NAS korrelierte. Somit scheint die Erhöhung der Speicherkapazität des Fettgewebes mit dem Schweregrad der NAFLD zusammenzuhängen. Wird das Fettgewebe durch Hyperplasie erweitert (Vermehrung der Zellen), fällt die Leberschädigung schwächer aus als bei Hypertrophie der Adipozyten. Außerdem war das Lipidprofil im Serum unterschiedlich zwischen NAFL und NASH. Vor allem langkettige Fettsäuren wie die Stearinsäure oder die Dihomogammalinolensäure (DGLS) waren in höheren Konzentrationen im Serum von NASH-Patienten zu finden. Das Apolipoprotein ApoCIII war ebenfalls mit signifikant höheren Konzentrationen bei NASH-Patienten vorhanden. Wie auch in anderen Arbeiten (3.1.1, 3.2.2, 3.3.2), war die Konzentration des Adiponektin bei stärkerer Leberschädigung geringer. Eine weitere interessante Beobachtung war, dass nach der bariatrischen Operation die Stearinsäure und DGLS signifikant reduziert gegenüber dem Status vor Operation waren. Dies weist darauf hin, dass bariatrische Eingriffe möglicherweise über den reinen Gewichtverlust hinaus durch eine Veränderung

der von Adipozyten sekretierten Lipidkomponenten und Adipokine einen positiven Einfluss auf die NAFLD haben könnten.

Die vorliegende Arbeit bestätigt, dass für die Entstehung und Progression der NAFLD auch die Situation im Fettgewebe wesentlich ist. Die Größe der Adipozyten, eine vermehrte Ausschüttung langkettiger Fettsäuren sowie ein verändertes Adipokinprofil sind Faktoren, die für prognostische Verfahren herangezogen werden könnten. Weiterhin scheinen Therapieansätze vielversprechend, die auf eine Normalisierung der Adipozytengröße und der von den Adipozyten sekretierten Substanzen abzielen.

3.2.4. NAFLD als häufigste Ursache des hepatozellulären Karzinoms bei Abwesenheit einer Leberzirrhose

Ertle, Dechêne, Sowa, Penndorf, Herzer, Kaiser, Schlaak, Gerken, Syn, Canbay. Non-alcoholic fatty liver disease progresses to hepatocellular carcinoma in the absence of apparent cirrhosis. International Journal of Cancer 2011;128(10):2436-2443.

Eine der schwerwiegenden Folgen chronischer Lebererkrankungen ist die Entstehung eines HCC. Die vorliegende Studie wurde ursprünglich begonnen, um mögliche Biomarker für HCC zu identifizieren und die aktuellen Hauptursachen des HCC in einer deutschen Kohorte zu identifizieren. Im Laufe der Studie wurden zwei für uns überraschende Beobachtungen gemacht. Zum einen war die häufigste Ursache für HCC in der rekrutierten Kohorte (Zeitraum 02.2007-03.2008) mit 24 % NASH (151). Zu diesem Zeitpunkt waren in den meisten Studien, die allerdings häufig aus dem asiatischen Raum stammten, virale Ätiologien am häufigsten vertreten (201–204). Zum anderen konnten wir erstmalig in einer Gruppe von 36 NASH-induzierten HCC Fällen bestätigen, dass HCC in dieser Ätiologie unabhängig von einer Zirrhose auftreten kann, was bis dato nur in Fallberichten dokumentiert war. Im Vergleich zu der Gesamtkohorte, in der HCC zu 77 % mit Zirrhose verbunden war, trat Zirrhose nur in 53 % der NASH-induzierten HCC auf. HCC bei viralen Ursachen oder alkoholischer Lebererkrankung war zu über 90 % mit Zirrhose verbunden. Lediglich bei Fällen mit kryptogener Ursache, war Zirrhose mit 39 % der Fälle noch seltener als bei NASH. Da vermutet wurde, dass kryptogene Fälle häufig unerkannte NASH-bedingte HCCs darstellen, wurden auch klinische Daten und Faktoren des metabolischen Syndroms zwischen den verschiedenen Ätiologien verglichen. Zwar ähnelten sich die Datenprofile kryptogener und NASH-bedingter Fälle, allerdings waren die Komponenten des metabolischen Syndroms ähnlich häufig bei Alkohol-bedingten und NASH-induzierten HCCs. Somit konnte die These, dass ein Großteil kryptogener HCCs möglicherweise tatsächlich NASH-induziert sind nicht bestätigt werden.

In dieser Arbeit haben wir gezeigt, dass die NASH als Ursache für das HCC in industrialisierten Populationen die häufigste Ursache sein kann. Außerdem haben wir den Grundstein für die inzwischen

etablierte Ansicht gelegt, dass NASH-induziertes HCC auch unabhängig von einer Leberzirrhose auftreten kann. Dies impliziert einerseits, dass bei der Entstehung andere Mechanismen beteiligt sind, als bei anderen chronischen Lebererkrankungen. Andererseits bedeutet dies, dass eine engmaschige Überwachung ausschließlich von Patienten mit fortgeschrittener Fibrose oder Zirrhose einen Teil der NASH-bedingten HCC-Fälle zwangsläufig übersehen muss. Hier sind nach wie vor bessere Möglichkeiten und Richtlinien für eine individuelle Risikoabschätzung und eine Anpassung von Verlaufskontrollen gefordert.

3.3. Nicht-invasive Diagnostik der NAFLD und des Schweregrades der Ausprägung

Im Abschnitt "Diagnostische Probleme der NAFLD" (S.15) wurde bereits dargelegt, dass einerseits die Diagnose einer NAFLD, aber insbesondere die Überwachung von Progression oder Regression sowie die Einschätzung des Schweregrades mit aktuellen Verfahren nicht oder nur mit erheblichem finanziellem und zeitlichen Aufwand zu bewerkstelligen ist. Einfache, objektive, nicht-invasive Testverfahren wären hier von großem Nutzen, um Patienten mit besonderen Risiken für Erkrankungsprogression zu identifizieren oder die Erkrankungsschwere, also z.B. NAFL vs. NASH unterscheiden zu können. Für diesen Aspekt haben wir mehrere Studien mit unterschiedlichem Fokus anhand von Daten aus den Kollektiven mit bariatrischer Chirurgie durchgeführt.

3.3.1. Separation milder Fibrosegrade bei NAFLD

Sowa, Heider, Bechmann, Gerken, Hoffmann, Canbay. Novel Algorithm for Non-Invasive Assessment of Fibrosis in NAFLD. PLOS One 2013;8(4):e62439.

Wie oben geschildert gilt aktuell für leberbezogene Morbidität und Mortalität der Fibrosierungsgrad als wesentlicher prädiktiver Faktor (141). Insbesondere bei Patienten mit morbider Adipositas tritt nur selten fortgeschrittene Fibrose und Zirrhose auf (139,205). Daher haben wir bei adipösen Patienten, die sich einer bariatrischen Operation unterzogen getestet, ob mittels nicht-invasiver Verfahren eine Unterscheidung milder Fibrosestadien und damit die Überwachung einer Erkrankungsprogression grundsätzlich möglich wäre (206). Aus den vorhandenen klinischen und demographischen Variablen sollte ein Vorhersagesystem für die histologisch bewertete Fibrose (Grad 1 oder Grad 2) entwickelt werden. Zum Einsatz kamen hierfür maschinelle Lernsysteme, also Programme, die auf Basis der verfügbaren Daten das bestmögliche Vorhersagesystem erzeugen. Zunächst konnten wir zeigen, dass kein einzelner nicht-invasiv erhobener Parameter hinreichende Genauigkeit erzielt, um eine Unterscheidung der Fibrosegrade zu ermöglichen. Verschiedene maschinelle Lernverfahren konnten jedoch Modelle entwickeln, die eine Vorhersage mit akzeptabler Performanz erlauben. Ein einzelner

Entscheidungsbaum erreichte eine Genauigkeit von 79 %, ein sogenannter *random forest* (Mehrheitsvotum aus 500 zufällig generierten und wiederholt selektierten Entscheidungsbäumen) konnte nur eine *area under the receiver operator characteristics curve*[1] (AUC) von 0,67 erzielen. Obwohl grundsätzlich eine Unterscheidung selbst milder Fibrosegrade möglich ist, zeigen die erreichten Genauigkeiten bzw. AUCs, dass dies keine triviale Aufgabe ist. Ein Vorteil maschineller Lernverfahren ist, dass diese ermitteln können, welche Parameter für die Klassifikation am wichtigsten sind. In dieser Arbeit erreichten die Zelltod- bzw. Apoptosemarker M65 und M30 die höchsten Werte für Wichtigkeit. Dies deckt sich mit anderen klinischen Studien, in denen Zelltodmarker mit dem Schweregrad chronischer Lebererkrankungen und der Fibrosierung korrelierten (207–209). Der Zelltod der Hepatozyten gilt als zentraler Stimulus für eine Fibrosierung des Lebergewebes (siehe auch 2.2 und 3.1.2), daher interpretieren wir das durch maschinelles Lernen erzeugte Modell auch als biologisch relevant.

Maschinelle Lernverfahren können Modelle für die Vorhersage von Erkrankungsschwere unterstützen und mittels Identifikation wichtiger Parameter Hinweise auf zugrunde liegende Mechanismen geben.

3.3.2. Separation alkoholischer und nicht-alkoholischer Steatohepatitis

Sowa, Atmaca, Kahraman, Schlattjan, Lindner, Sydor, Scherbaum, Lackner, Gerken, Heider, Arteel, Erim, Canbay. Non-invasive Separation of Alcoholic and Non-Alcoholic Liver Disease with Predictive Modeling. PLOS One 2014;9(7):e1011444.

Ein wesentlicher Faktor bei der Beurteilung, ob eine NAFLD vorliegt, ist die Abklärung relevanten Alkoholkonsums, um Alkohol bedingte Leberschädigung als Ursache für die Lebersteatose auszuschließen. Hierbei gibt es mehrere Probleme: die Grenzwerte für "relevanten Alkoholkonsum" (in Deutschland >20g/Tag für Männer, >10g/Tag für Frauen) weichen zwischen verschiedenen Gesundheitssystemen ab, sind somit oft nicht vergleichbar; Patienten unterschätzen häufig ihren Alkoholkonsum oder geben bewusst geringere Mengen an; da Menschen extrem variabel in ihren Gewohnheiten und ihrer Ernährung sind, existieren nicht wenige Fälle, in denen durchaus eine NAFLD und eine alkoholische Schädigung gleichzeitig vorliegen kann, wobei der jeweilige Anteil an der Gesamtproblematik "Lebererkrankung" ebenfalls variabel ist. Aus diesen Gründen wäre es für klinisch tätige Ärzte wünschenswert, eine objektive Unterscheidung von (hauptsächlich) alkoholischer und (hauptsächlich) nicht-alkoholischer Lebererkrankung treffen zu können. Hierzu haben wir in einem bioinformatischen Ansatz versucht, ein Vorhersagemodell zur Separation von alkoholischer

[1] Wird für ein Vorhersagemodell die Sensitivität gegen (1-Spezifität) bei jedem möglichen Wert, den das Modell annehmen kann, aufgetragen, erhält man eine sogenannte *receiver operator characteristics curve* (ROC). Der Flächeninhalt (*area*, AUROC oder AUC) unter diese Kurve wird als Maß für die Gesamtperformanz des Modells gewertet, entspricht entgegen häufiger Verwendung des Begriffs jedoch nicht exakt der Genauigkeit (*accuracy*) eines Modells.

Steatohepatitis (ASH) und NASH zu entwickeln (210). Es wurden insgesamt drei Patientenkohorten für diese Untersuchung eingesetzt, 31 "schlanke" Patienten mit NASH (mittlerer BMI 25,6 kg/m²), 51 Patienten mit ASH ohne Zirrhose sowie 51 Patienten mit zirrhotischer ASH. Zwei Untersuchungen wurden anhand dieser Daten durchgeführt. Mittels der verfügbaren nicht-invasiven Parameter wurde versucht, entweder zwischen alkoholischer (ohne Zirrhose) und nicht-alkoholischer Schädigung zu unterscheiden oder zwischen ASH mit und ohne Zirrhose. Die Auswahl idealer Klassifikatoren wurde wieder maschinellen Lernsystemen überlassen. Für die Unterscheidung zwischen NASH und ASH wurde der De-Ritis-Quotient (GOT/GPT Verhältnis), die Serum Konzentration von TNF-α sowie von GPT als wichtigste Parameter ausgewählt und in einem Entscheidungsbaum zusammengestellt. Dieser Entscheidungsbaum erreichte eine Genauigkeit von über 89 %, ein *random forest* konnte anhand ähnlicher Parameterkonstellationen eine AUC von 0,8932 erreichen. Für die Unterscheidung zwischen zirrhotischer und nicht-zirrhotischer ASH war der zentrale Parameter die transiente Elastographie (siehe 2.5.1), der in einer Sub-Analyse entfernt wurde, um einen möglichen Bestätigungsfehler zu vermeiden. Bei einem Teil dieser Patienten war das Vorliegen einer Zirrhose mittels transienter Elastographie detektiert worden. Unter Berücksichtigung dieser Limitierung waren die Parameter M65, TNF-α und Alter am wichtigsten für die Unterscheidung von ASH mit und ohne Zirrhose. Ein einzelner Entscheidungsbaum erreichte eine Genauigkeit von 95 %, ein *random forest* erzielte eine AUC von 0,8971. Klassische Modelle mittels logistischer Regression erzielten Genauigkeiten von jeweils 0,88 für die Unterscheidung von NASH / ASH bzw. von ASH mit und ohne Fibrose. Dies unterschied sich jedoch nicht signifikant von der Performanz der Modelle, die durch maschinelles Lernen generiert wurden.

Mit dieser Arbeit konnten wir zeigen, dass einerseits eine Objektive Unterscheidung einer hauptsächlich nicht-alkoholischen vs. einer hauptsächlich alkoholischen Schädigung möglich ist. Die hierfür gewählten Parameter maschinell lernender System bestätigte die lange geltende Ansicht, dass der De-Ritis-Quotient hierfür gut geeignet ist. Auch eine Separation von nicht-zirrhotischer und zirrhotischer ASH war auf Basis der verwendeten Daten möglich. Schließlich konnten wir zeigen, dass für den Bereich der inneren Medizin selbstlernende Programme interessante Modelle liefern können. Insbesondere die Möglichkeit aus einer Vielzahl von Parametern diejenigen auszuwählen, die für eine Unterscheidung wichtig sind, ist ein Vorteil gegenüber klassischen Regressionsmodellen. Die vom maschinellen Lernverfahren angenommene Wichtigkeit von Parametern kann Hinweise auf mechanistische Zusammenhänge geben oder bislang als unwichtig angenommene Faktoren in den Fokus rücken. Eine wesentliche Limitierung der vorliegenden Arbeit ist, dass keine Validierung der generierten Systeme in unabhängigen Kollektiven erfolgt ist.

4. Diskussion

4.1. Zusammenfassung der eigenen Ergebnisse

Die in der vorliegenden Arbeit präsentierten Ergebnisse haben Erkenntnisse zu den molekularen, pathogenetischen Mechanismen der NAFLD, zur Epidemiologie und spezifischen Risiken sowie zu nicht-invasiven diagnostischen Verfahren erbracht. Es konnte gezeigt werden, dass an der Progression der NAFLD zur NASH möglicherweise verstärkte Aufnahme von Fettsäuren in die Hepatozyten über Fettsäuretransporter, hierbei insbesondere CD36, beteiligt ist. Parallel zu der vermehrten Expression von CD36 stieg die Apoptoserate der Hepatozyten an, was für eine verstärkte Schädigung des Lebergewebes spricht. Weiterhin war die Expression des Hepatokins Fetuin-A bei NASH höher als bei NAFL und wurde *in vitro* durch Gabe von FFA in Hepatozyten verstärkt exprimiert. Somit könnte Fetuin-A als therapeutisches Ziel zur pharmakologischen Behandlung der NASH in Frage kommen, aber vor allem als Marker für eine Erkrankungsprogression dienen, da es von den Hepatozyten sekretiert wird. An einer Progression der NAFLD zur Fibrose könnten verminderte Vitamin D Konzentrationen bei adipösen Individuen beteiligt sein, wodurch eine Stimulation des TGF-β Signalweges in HSC verstärkt werden könnte. Polymorphismen des Vitamin D Rezeptors haben vermutlich Einfluss auf den Effekt einer Vitamin D-Gabe als Therapie der Fibrose. In einem murinen, diätbasierten NAFLD-Modell konnten anhand des protektiven Effekts eines Knockout der aziden Sphingomyelinase Mechanismen identifiziert werden, die als therapeutische Ziele näher untersucht werden sollten. Dies ist einerseits eine Resistenz der Adipozyten gegen Hypertrophie und eine tendenziell „braune“ Genexpression der Adipozyten. Andererseits ist dies in der Leber eine verminderte Aktivierung von Rictor bzw. dem mTORC2-Komplex. Basierend auf Daten einer großen populationsbasierten Kohorte und auf eigenen Daten aus der Ambulanz eines tertiären Versorgungszentrums haben wir demonstriert, dass Adipositas, Lebersteatose und vermutlich auch die NAFLD häufiger sind, als aktuell angenommen. Metabolische Erkrankungen, wie Diabetes und insbesondere die NAFLD scheinen in einem relevanten Anteil der Betroffenen nicht diagnostiziert zu werden, stellen aber ein metabolisches Risiko dar, auch bei Patienten die mit anderen Krankheitsbildern vorstellig werden. Weiterhin sind klassische Marker der Leberschädigung ungeeignet, die NAFLD zu detektieren oder zu überwachen. Bei Patienten, die sich einem bariatrisch-chirurgischen Eingriff unterziehen, konnten wir bestätigen, dass Hypertrophie der Adipozyten im viszeralen Fettgewebe mit dem Ausmaß der Leberschädigung bei NAFLD korreliert. Parallel hierzu fanden sich Korrelationen mit dem Schweregrad der NAFLD mit den Konzentrationen von Adiponektin und von langkettigen Fettsäuren im Blut. Nach dem bariatrisch-chirurgischen Eingriff erhöhten sich die Serum-Adiponektin-Konzentration und die Konzentration von zwei langkettigen Fettsäuren, assoziiert mit dem Schweregrad der NAFLD, ging zurück. In einer Kohorte von HCC-Patienten konnten wir für eine europäische Population demonstrieren, dass NASH-induziertes HCC als häufigste Ätiologie auftreten kann. Insbesondere kann HCC bei NASH unabhängig von Zirrhose

entstehen, die bei anderen chronischen Lebererkrankungen als größter Risikofaktor gilt. Schließlich war es möglich bei adipösen Patienten aus nicht-invasiv gewonnenen Parametern Vorhersagemodelle für milde Fibrosegrade zu generieren sowie ein Modell zur Unterscheidung primär alkoholischer und primär metabolischer Schädigung der Leber (ASH *vs.* NASH). Dank der eingesetzten maschinellen Lernverfahren war darüber hinaus eine Identifikation der wichtigsten Parameter für die Vorhersage möglich. Diese schlossen Zelltodmarker und Adipokine ein, jedoch nur nachrangig die klassischen Leberschädigungsmarker.

Zentrale zusammengefasste Erkenntnisse der Arbeit:

- Adipositas und NAFLD stellen in Deutschland ein erhebliches Gesundheitsproblem dar und werden sowohl in Dimension als auch Konsequenzen unterschätzt;
- Zusätzlich zu den komplexen molekularen und zellulären Mechanismen in der Leber sollte für eine Einschätzung der NAFLD auch der Status des viszeralen Fettgewebes und die von Adipozyten sekretierten Substanzen analysiert werden. Möglicherweise bietet das Fettgewebe oder davon ausgeschüttete Hormone und Lipidkomponenten Ansatzpunkte für eine spezifische NAFLD-Therapie;
- Um der großen diagnostischen Lücke für Screening und Überwachung der NAFLD zu begegnen sind nicht-invasive Marker eine mögliche Option. Breit gefächerte Datensätze mit vielen Parametern können mittels maschineller Lernverfahren auf ideale Markerkombinationen untersucht werden.

Diese Daten und Ergebnisse bringen uns dem Verständnis und einer effektiven Bekämpfung der NAFLD und deren Folgeerkrankungen einen kleinen Schritt näher, erfordern jedoch noch weitere Studien und Untersuchungen mit spezifischem Design für die noch offenen Fragen.

4.2. Bezug zu aktuellem Kenntnisstand

Unsere Arbeiten konnten dazu beitragen, die Rolle der Apoptose und des Fettgewebes bei der Entstehung und Progression der NAFLD zu bestätigen und zu erweitern (211–213) sowie epidemiologische Daten für Deutschland ergänzen. Insbesondere für NASH-induziertes Zirrhose-unabhängiges HCC haben wir dazu beigetragen, dass ein zentrales Paradigma chronischer Lebererkrankungen bei dieser Ätiologie aufgeweicht wurde (214,215).

Die hier vorgestellten Arbeiten decken jedoch nur einen kleinen Teil der Probleme, Fragen und Mechanismen ab, die sich zur NAFLD stellen und daran beteiligt sind. Neben den von uns untersuchten Mechanismen sind Gallensäuren und deren Regulatoren (z.B. FXR-Rezeptor) (216,217), regulatorische Elemente des Lipidmetabolismus wie PPARs und SREBP (218,219), hormonelle Regulation (220), oxidativer und endoplasmatischer Retikulum Stress (221,222) und neurologische

Aspekte der Adipositas (223) nennenswerte Einflussfaktoren auf die NAFLD. Umgekehrt wirken in der Leber produzierte Faktoren und Zytokine auf den gesamten Organismus, vor allem die Auswirkungen der veränderten Lipoprotein-Sekretion der Leber z.B. auf kardiovaskuläre Erkrankungen ist unstrittig. Die Details zu den in der Leber beteiligten Prozessen und den Wirkmechanismen an Endothelzellen der Blutgefäße und Herzmuskelzellen sind weniger klar. Vor kurzen wurde zudem die These vorgeschlagen, dass es grundsätzlich wenigstens zwei Varianten der NAFLD gibt (224): eine rein metabolische Variante, die primär auf Überernährung / Bewegungsmangel / Adipositas beruht; eine primär genetische Variante mit Polymorphismen von PNPLA3, TM6SF2 und ggf. weiteren Faktoren, die eine initiale Leberschädigung determinieren, welche durch Übergewicht und Adipositas verstärkt wird. Diese These beinhaltet auch ein unterschiedliches Risikoprofil mit hauptsächlich kardiovaskulären Endpunkten oder Diabetes für die „Metabolische NAFLD“ und hauptsächlich leberbezogenen Endpunkten bei der „Genetischen NAFLD“ (224). Eine Basis für diese These sind aktuelle Arbeiten, die mittels modernster Detektionsmethoden und Auswertungssoftware Genexpression in der Leber und Metabolite in der Zirkulation untersucht haben (225,226). Hierbei wurde eine reduzierte metabolische Flexibilität bei NAFLD festgestellt, was impliziert, dass die Hepatozyten für bestimmte Prozesse keinen Wechsel der Substrate durchführen können. Gut geplante Studien in großen Kollektiven sind gefordert, um diese Daten zu bestätigen.

4.3. Ausblick

Die NAFLD basiert grundsätzlich auf einem einfachen Problem: ein Überangebot an unterschiedlichen Nährstoffen gegenüber dem Verbrauch dieser Substanzen im Organismus. Trotz dieser einfachen Ursache ist die Entstehung der NAFLD auf zellulärer und molekularer Ebene sowie die Progression zu NASH, Fibrose, HCC und Zirrhose extrem komplex. Wir beginnen erst, die Interaktion von Leber und Fettgewebe, Darm-Mikrobiom, neurologischen Prozessen (Hunger und Sättigung), der Nahrungszusammensetzung und der im Organismus zugrunde liegenden Genetik zu verstehen. Mit hoher Wahrscheinlichkeit wird die Pathogenese aufgrund der Varianz menschlicher Biologie, Kultur und Lebensweise individuell unterschiedlich sein. Dennoch erkennen wir gemeinsame Prozesse und Prinzipien, die zumindest Hoffnung auf spezifische Therapie fortgeschrittener Lebererkrankung bei NAFLD machen. Da die gesundheitliche Problematik epidemische Proportionen angenommen hat und möglicherweise noch zunehmen wird, sind dringend Maßnahmen erforderlich, die eine größere Aufmerksamkeit erzeugen. Folgeerkrankungen für betroffene Patienten und hohe Kosten für das Gesundheitssystem können nach aktueller Datenlage nur durch frühzeitige Prävention verhindert werden. Hierzu ist ein größeres Bewusstsein für eine gesunde Ernährung, mit adäquater Kalorienzufuhr, und insbesondere für Bewegung und Sport als integralen Bestandteilen des Lebensstils notwendig. Entsprechende Informations- und Bildungsprogramme sind wichtiger denn je.

Parallel hierzu muss weiter an einer Verbesserung der diagnostischen Abdeckung für NAFLD-Screening-Verfahren gearbeitet werden. Zusätzlich müssen diagnostische Methoden etabliert werden, die Patienten mit besonders hohem Risiko für z.B. Diabetes oder kardiovaskulären Erkrankungen identifizieren und mit denen diese Überwacht werden können. Eine der dringendsten Aufgaben hier ist, Patienten mit spezifischem Risiko für HCC frühzeitig zu identifizieren und die speziellen bei NAFLD-induziertem HCC beteiligten Mechanismen aufzuklären. In den vergangenen Jahren wurden viele neue Erkenntnisse zur NAFLD und damit zu unserem Verständnis metabolischer Prozesse im Organismus gewonnen, viele Fragen sind weiterhin offen. Es bleibt eine große Aufgabe, das Gesundheitsproblem NAFLD in allen Facetten zu verstehen und zu bekämpfen.

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CLINICAL STUDIES

Apoptosis is associated with CD36/fatty acid translocase upregulation in non-alcoholic steatohepatitis

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Keywords
apoptosis – fatty acid transporter – NASH – obesity

Abbreviations
CD36/FAT, fatty acid translocase; CD95/Fas, an apoptosis-inducing cell surface receptor (advanced nomenclature: TGF-β: transforming growth factor β; TNF superfamily receptor 6); DR4/5, death receptor 4 or 5; FABP-1, fatty acid binding protein-1; FATP, fatty acid transport protein; FFA, free (non-esterified) fatty acids; NAFLD, non-alcoholic fatty liver disease; NAS, non-alcoholic fatty liver disease histological score.

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Abstract

Background & aims: Hepatocyte apoptosis is a key event in non-alcoholic steatohepatitis (NASH). We studied the effect of obesity on free fatty acid (FFA) levels, fatty acid transport proteins (FATPs) and on extrinsic and intrinsic activation of apoptosis in the liver. *Methods:* Liver biopsies were harvested from 52 morbidly obese patients [body mass index (BMI): 53.82 ± 1.41; age: 45 ± 10.50; 15 males/37 females] undergoing bariatric surgery, and were scored for NASH, evaluated for fibrosis, and investigated for intrahepatic expression of FATPs, death receptors and cytosolic apoptosis-related molecules. Findings were correlated with serum FFA levels and the degrees of intrahepatic (terminal dUTP nick end labelling) and systemic (M30) apoptosis. *Results:* In patients' liver sections, FATPs as well as select parameters of extrinsic and intrinsic apoptosis were found to be upregulated (CD36/FAT: $\times 11.56$; FATP-5: $\times 1.33$; CD95/Fas: $\times 3.18$; NOXA: $\times 2.79$). These findings correlated with significantly elevated serum FFAs (control: 14.72 ± 2.32 mg/dl vs. patients: 23.03 ± 1.24 mg/dl) and M30 levels (control: 83.12 ± 7.46 U/L vs. patients: 212.61 ± 22.16 U/L). We found correlations between FATPs and apoptosis mediators as well as with histological criteria of NASH and fibrosis. *Conclusions:* Increased FFA and FATPs are associated with extrinsically and intrinsically induced apoptosis, liver damage and fibrosis in obese patients. Thus, FATPs may offer an interesting new approach to understand and potentially intervene NASH pathogenesis.

With a rising prevalence of the metabolic syndrome in societies with western(ized) dietary habits, the diagnosis of non-alcoholic fatty liver disease (NAFLD) has greatly increased in clinical practice. NAFLD is the most common cause of elevated liver enzymes and probably the most common liver disease in the western European and North American countries, with a prevalence of up to 30% (1–3). In the face of global epidemic obesity and westernized lifestyle habits in developing and emerging countries, we must therefore expect a worldwide increase in the prevalence of NAFLD (4).

Hepatic steatosis is believed to be chiefly induced by insulin resistance, a key feature of the metabolic syndrome. A 'first hit' in the development of NAFLD is caused by insulin resistance and excess amounts of free fatty acids (FFAs). Subsequently, a 'second hit' by additional inflammation, hepatocellular damage by oxidative stress or even destruction by apoptosis brings about non-alcoholic steatohepatitis (NASH) (5).

The case that elevations of CD95/Fas expression and hepatocyte apoptosis are more prominent in NASH than in alcoholic hepatitis (6, 7) may explain the higher rate of cirrhosis in NASH patients (6). This is because of several interacting mechanisms, i.e. (i) in HepG2 hepatoma cells, FFAs promote the upregulation of CD95/Fas *in vitro* (8); (ii) activation of caspases-3 and -7 in NASH liver samples correlates positively with the severity of disease and fibrosis as well as with results in HepG2 cells (6, 7, 9) and (iii) mitochondrial dysfunction upregulates the generation of reactive oxygen species that are known to induce apoptosis and thereby liver injury and inflammation.

Obesity (particularly its central form) as well as insulin resistance upregulate FFA release from adipocytes. Specifically, the increase in adipocyte mass and triacylglycerol hydrolysis via activated hormone-sensitive lipase lead to elevated levels of FFAs (10), which once translocated into hepatocytes, are esterified and stored as TAGs. It is thought that, in this process, FFAs transit hepatocyte plasma membranes passively as well as actively by membrane-associated fatty acid transport proteins (FATPs) (11) whose key function is to deliver long-chain fatty acids (12, 13). Candidate FATPs in hepatocytes include fatty acid translocase (CD36/FAT), fatty acid binding protein-1 (FABP-1), fatty acid transport proteins-2 and -5 (FATP-2, FATP-5), and caveolin-1. For example, decreased hepatic FFA uptake and TAG storage in FATP-5 knock-out mice suggest an important role of FATP-5 (10, 14).

While FFA abundance leads to death receptor upregulation and hepatocyte apoptosis (12), the concrete link between both remains poorly understood (13). In NAFLD, however, an enhanced expression of FATPs, FABPs and death receptors suggests a link between increased FFAs and upregulation of apoptosis (15). Here, caveolin-1 is an interesting example as this FATP may either act pro- or anti-apoptotically and/or promotes hepatocyte regeneration or liver fibrosis respectively (16–18). However, for substantiating the actual role of FATPs in NAFLD, we clearly need more data on their action in FFA-rich environments in obesity, as well as on their effects on death-receptor expression and hepatocyte apoptosis.

Apoptosis is alternatively activated via intrinsic and extrinsic pathways that eventually converge by caspase activation (19, 20). The extrinsic pathway is initiated by the binding of pro-apoptotic ligands – e.g. CD95 L/FasL or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) – to pro-apoptotic receptors such as CD95/Fas, DR4 or DR5 (21). In contrast, the intrinsic pathway is triggered intracellularly by severe stress (such as DNA damage or hypoxia) (22), which leads to the upregulation of p53 with consecutive transcriptional activation of cytosolic proteins like the p53-upregulated modulator of apoptosis (PUMA) and a pro-apoptotic member of the BCL2 protein family termed NOXA (23). Current evidence supports the concept of extrinsic activation of excess hepatocyte apoptosis in patients with NASH. Still, the pathogenic processes in NASH also include the possibility of (additional) intrinsic activation, which has not yet been studied. This study thus aimed at determining the influence of excess FFAs on select FATPs and death receptors, as well as on the two pathways of apoptosis in the livers of NASH patients.

Patients, material and methods

Patients

Fifty-two morbidly obese patients [body mass index (BMI): 53.82 ± 1.41; age: 45 ± 10.50; 15 males/37 females] who underwent bariatric surgery at a renowned Centre for Bariatric Surgery were included (Table 1). Indication for bariatric surgery was made according to NIH guidelines (BMI $\geq 40\,kg/m^2$ or $\geq 35\,kg/m^2$, plus co-morbidities). Subjects reporting excessive alcohol consumption ($> 20\,g$/day in males or $> 10\,g$/day in females) indicating alcoholic liver disease were excluded. A control group of 10 healthy volunteers (seven males; three females/median age: 26 ± 7.6 years) had an average BMI of $22.4 \pm 0.82\,kg/m^2$ (Table 1).

Table 1. Demographical and clinical characteristics of patients and healthy controls

	Patients ($n = 52$)	Healthy controls ($n = 10$)
Gender ratio	F: $n = 37$; M: $n = 15$	F: $n = 3$; M: $n = 7$
Age (years)	45 ± 10.50	26.0 ± 7.6
Weight (kg)	163.02 ± 28.18	69.70 ± 16.05
Height (cm)	166.8 ± 26.72	174.70 ± 11.52
BMI (kg/m^2)	53.82 ± 9.27	22.40 ± 2.46
AST (mg/dl)	37.55 ± 22.84	15.20 ± 1.74
ALT (mg/dl)	43.79 ± 30.17	18.96 ± 3.77
Bilirubin (mg/dl)	0.55 ± 0.24	0.52 ± 0.28
Serum cholesterol (mg/dl)	201.77 ± 38.44	141.31 ± 17.32
HbA1c (mg/dl)	5.86 ± 1.13	3.10 ± 0.78

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index.

Ethics

This study was approved by the Ethics Committee (Institutional Review Board) of the University Hospital Essen. Patients volunteering were informed about intra-operative risks and benefits of wedge liver biopsy and provided informed consent.

Bariatric surgery

The surgeon's choice – i.e. adjustable gastric band; Roux-Y; or gastric bypass surgery – was based on the current guidelines as adapted to the patients' clinical conditions and co-morbidities as well as on clinical experience. Wedge liver biopsies were taken during the procedure.

Liver biopsies and interim storage

Individual specimens were split evenly, with portion 1/2 stored in 4% formalin solution (Roth, Karlsruhe, Germany) for histological examination, and portion 2/2 stored in the RNA-preserving agent, RNALater (Ambion, Applied Biosystems, Darmstadt, Germany), for subsequent determination of gene expressions.

Histology

The degree of NAFLD was quantified according to the NASH Scoring System (NAS) (24). Additionally, steatosis (0–3), hepatocellular ballooning (0–2) and lobular inflammation (0–2) were determined. NAS scores of ≥ 5 or ≥ 4 when associated with a score of at least one for

ballooning were defined as NASH. The grade of liver fibrosis was assessed using the modified METAVIR criteria. Tissue-specific apoptosis was detected by terminal dUTP nick end labelling (TUNEL) assay as described previously (25). TUNEL index was calculated as TUNEL-positive cells per total cell number in one high power field ($\times$ 20 magnification) in percent.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Preparation

Liver tissue was homogenized using a blade homogenizer (IKA, Staufen, Germany) in TRIZOL (Invitrogen, Karlsruhe, Germany) and total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). Purity and concentration of mRNA were determined photometrically, each 2 µg RNA sample was dissolved in 100 µl RNAse-free water, and reverse transcription was performed using the QuantiTect RT Kit (Qiagen).

mRNA expression

mRNA levels of FATPs (FATP-5, FABP-1, CD36/FAT and caveolin-1), death receptors of the extrinsic pathway of apoptosis (CD95/Fas; FasL; Caspase-8, TNFR1) and pro-apoptotic molecules of the intrinsic apoptosis pathway (PUMA, NOXA, Bax, PTEN and Foxo3A) were assessed by qRT-PCR using hypoxanthine phosphoribosyltransferase 1 and/or succinate dehydrogenase as housekeeping genes (see Table 2: primers). qRT-PCRs of the cDNAs were performed using an iCycler iQ thermal cycler (Biorad, Hercules, CA, USA) with real-time detection system software 3.0a and GENEX software (Biorad) in 30 µl reactions containing 15 µl QuantiTect SYBR Green master mix (Qiagen), 2 µl cDNA, 1 µl forward primer, 1 µl reverse primer (at 10 pmol/µl each) and 11 µl distilled water. Amplification was performed for 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. Melting-curve data were collected from 95 °C to 55 °C, at -0.5 °C steps for 10 s, each. Relative gene expressions vs. untreated controls or healthy donors were calculated from the threshold cycles in relation to the housekeeping gene.

Western blotting

Tissue lysates were prepared by homogenizing in lysis buffer [50 mM Tris; 150 mM NaCl; 0.1% Igepal; 1% desoxicholic acid; containing complete mini, EDTA-free protease inhibitor cocktail tablets (Roche, Mannheim, Germany)]. 20–30 µg of total protein were loaded on 4–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels (Invitrogen), separated by electrophoresis and transferred to PVDF membranes as verified by staining with 0.2% Ponceau Red (Sigma). Membranes were blocked and exposed to primary and secondary antibodies for approx. 12 h at 4 °C. Primary antibodies for actin (SC-11, polyclonal rabbit; Santa Cruz Biotechnology), FABP-1 (monoclonal mouse; R&D, Minneapolis, MN, USA), caveolin-1 (polyclonal rabbit; Abcam, Cambridge, MA, USA), FATP-5 (SLC27A5, polyclonal mouse; Abnova, Taipei City, Taiwan), CD36/FAT (polyclonal rabbit; Abcam), Foxo3a (polyclonal rabbit; Cell Signaling) and pFoxo3a (polyclonal rabbit; Cell Signaling) were used. Bands were visualized using the ECL-Development Kit (GE Healthcare, Freiburg, Germany).

Table 2. Primers employed for quantitative real-time polymerase chain reaction

Gene	Primer sequence
HPRT1	
F	5′-gaccagtcaacaggggacat-3′
R	5′-cttgcgaccttgaccatctt-3′
SDHA	
F	5′-acacagacctggtggagacc-3′
R	5′-caaagggcttcttctgttgc-3′
FATP-5	
F	5′-gccctgccctcttcatctat-3′
R	5′-ccccagataaggacagcatc-3′
FABP-1	
F	5′-gcagagccaggaaaactttg-3′
R	5′-caccccctgatatccttcc-3′
CD36(FAT)	
F	5′-aacagaggctgacaacttcaca-3′
R	5′-aacagttctctcaaagttctgacttg-3′
Caveolin-1	
F	5′-tctgggggcaaatacgtaga-3′
R	5′-caggtcgatctccttggtgt-3′
CD95(Fas)	
F	5′-caaagcccatttttcttcca-3′
R	5′-tttggtttacatctgcacttgg-3′
CD120a/(TNFR1)	
F	5′-ccttcaccgcttcagaaaac-3′
R	5′-cggtccactgtgcaagaag-3′
DR4	
F	5′-gagcgatggtcaaggtcaag-3′
R	5′-agcaacggaacaaccaaagt-3′
DR5	
F	5′-cactggaatgacctccttttc-3′
R	5′-cttccggcacatctcagg-3′
PUMA	
F	5′-gacgacctcaacgcacagta-3′
R	5′-aggagtcccatgatgagattgt-3′
NOXA	
F	5′-gagatgcctgggaagaagg-3′
R	5′-ttctgccggaagttcagttt-3′
Bax	
F	5′-tctgacggcaacttcaactg-3′
R	5′-ggaggaagtccaatgtccag-3′
FasL	
F	5′-gggggcagtgttcaatctta-3′
R	5′-tggaaagaatcccaaagtgc-3′
PTEN	
F	5′-cataacgatggctgtggttg-3′
R	5′-gaactggcaggtagaaggca-3′
FoxO3a	
F	5′-ggaggaggaatgtggaaggt-3′
R	5′-ctcgcttccttcccttcag-3′

Enzyme-linked immunosorbent assay

The apoptosis marker M30, cell death marker M65, caspase-8 and adiponectin were assessed in the sera of patients and healthy controls using the M30-Apoptosense (Peviva, Bromma, Sweden), M65 (Peviva), human caspase-8/FLICE (Bender Medsystems, Vienna, Austria) and competitive human adiponectin (Biovendor, Modrice, Czech Republic) ELISA kits. All procedures were conducted according to the manufacturers' instructions. M30 is a neo-epitope exposed upon caspase-3-dependent cleavage of cytokeratin-18 (CK18).

Free fatty acids

Free fatty acids concentrations were measured enzymatically in 20 µl samples of patient and control sera (26). Using the NEFA-C kit (WAKO Chemicals, Neuss, Germany), FFAs were converted into acyl-CoA esters using acyl-CoA synthetase, followed by oxidation via acyl-CoA oxidase. The resultant hydrogen peroxide was determined colorimetrically at $\lambda = 550$ nm.

Statistics

All data shown are mean ± SEM, if not stated otherwise. Differences between FFA concentrations, death receptor expression rates and M30 neo-epitope concentrations were evaluated by one-way ANOVA, repeated-measure ANOVA or paired Student's *t*-test. ANOVA was also applied for comparing FATP and death receptor expressions as well as M30 concentrations in patients vs. controls. For categorical variables, frequencies and percentages were estimated. χ^2 or Fisher's exact tests were used for categorical factors. Putative correlations between serum M30 levels with the NASH score or the stage of fibrosis, respectively, were assessed by Spearman's correlation coefficient. Variables that were found to be associated with M30 levels by univariable analysis or parameters known to be associated with NASH severity (aspartate aminotransferase/alanine aminotransferase ratio, diabetes, hyperlipidaemia and BMI) were assessed and correlated. A $P \leq 0.05$ was considered statistically significant. Analyses were performed using SPSS 15.0.1, version 2006 (SPSS Inc., Chicago, IL, USA).

Results

Clinical findings

Basic parameters and characteristics of all patients enrolled are given in Table 1.

Non-alcoholic steatohepatitis: serum free fatty acids and liver fatty acid transport protein mRNAs and proteins

Systemic FFAs were significantly elevated in NASH patients (FFA: 23.03 ± 1.24 mg/dl, $P < 0.05$) when compared with controls (FFA: 14.72 ± 2.327 mg/dl) (Fig. 1a). In obese patients, expression rates of intrahepatic FABP mRNAs were upregulated (Fig. 1b). However, the expression of CD36/FAT (11.59 ± 1.86-fold increase vs. healthy controls; $P < 0.05$) greatly exceeded the expression rates of all other FATPs, with FATP-5 at 1.33 ± 0.12-fold upregulation vs. control ($P < 0.05$). The increases in caveolin-1 (1.65 ± 0.62-fold) and FABP-1 (1.33 ± 0.48-fold) failed to attain statistical significance. Immunoblot analyses verified an over-expression of CD36/FAT and FATP-5 in patients with NAFLD (Fig. 1c).

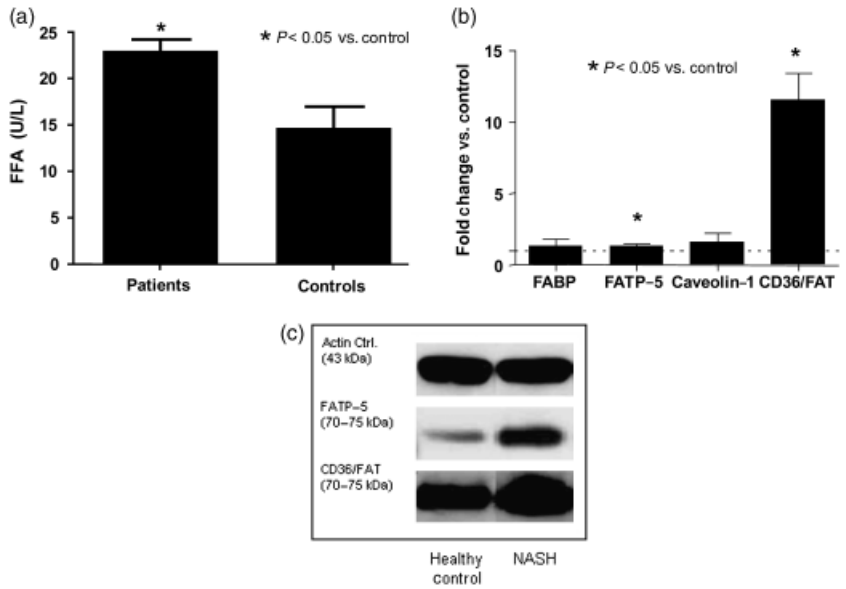

Fig. 1. Free fatty acids (FFAs) and fatty acid transport proteins in non-alcoholic steatohepatitis patients. FFA serum concentrations were significantly increased (a). While fatty acid transport protein mRNAs were generally upregulated, CD36/FAT mRNA stood out impressively (b). These results were confirmed by Western blotting for respective translation products of significantly altered genes (c).

Non-alcoholic steatohepatitis: extrinsic and intrinsic pathways of apoptosis

Apoptosis was detected in patients and control sera by measuring M30 and caspase-8. The serum level of M30 was significantly elevated in patients (212.61 ± 22.16 U/L, $P < 0.05$) when compared with healthy controls (83.12 ± 7.48 U/L) (Fig. 2d). While other studies demonstrate increased serum M30 in various diseases, the concentration in control individuals vary between 120 and 200 U/L, suggesting the values of our controls to be rather low (27–29). Caspase-8 release into the serum was also increased in patients (control: 2.7 ± 0.2 U/ml, $n = 14$; NAFLD patients: 8.02 ± 0.8 U/ml, $n = 32$; $P < 0.001$; Fig. 2e). Compared with controls, the serum levels of the counter-regulatory and anti-apoptotic adipocytokine, adiponectin, were significantly decreased in patients (20.60 ± 2.55 U/L, controls: 100.63 ± 11.44 U/L; $P < 0.05$; Fig. 2d). In addition, we performed TUNEL assays to quantify cell death rates in corresponding liver biopsies. The amount of TUNEL-positive cells was higher in patients than in control subjects (TUNEL index control: $0.63 \pm 0.48\%$; NAFLD patients: $2.99 \pm 0.53\%$; Figs 3c–f and 4a).

As to the extrinsic pathway of apoptosis in NASH patients, CD95/Fas mRNA expression significantly increased relative to the controls by 3.18 ± 0.4-fold (Fig. 2a). A similar pattern was found for FasL (8.37 ± 1.1-fold). Interestingly, we also found a strong increase within the intrinsic pathway of apoptosis where the mRNAs of pro-apoptotic effector molecules such as NOXA, a known early transcriptional target of p53 (2.78 ± 0.8-fold $P < 0.05$; Fig. 2b) and Bax (7.78 ± 4.09-fold; $P = 0.106$) were upregulated (30). Expression of the regulative component PTEN (2.95 ± 0.5-fold, $P < 0.05$) and the transcription factor FoxO3A (2.38 ± 0.52-fold; $P < 0.05$) was elevated. In contrast to these findings, the mRNA level of PUMA was in the control range. Collectively increased serum levels of M30 and caspase-8, elevated mRNA expression of NOXA, CD95/Fas and FasL in the liver tissue as well as a high numbers of TUNEL positive cells in liver sections clearly show ongoing apoptosis within the liver.

Activation of FoxO3a was additionally analysed in a phospho-FoxO3a Western Blot with protein samples taken directly from the liver tissue of six patients with high levels of apoptosis, as evaluated by their M30/M65 ratios. Inactive phosphorylated FoxO3a was increased in three of the six patients (Fig. 2c). Although no significant differences between these patients were found, some parameters of apoptosis and fatty acid transport were increased in patients without detectable pFoxO3a (S1).

Non-alcoholic steatohepatitis: correlations between fatty acid transport proteins, apoptosis and severity of disease

A potentially outstanding role of CD36/FAT as link between FATP and death receptor levels had become

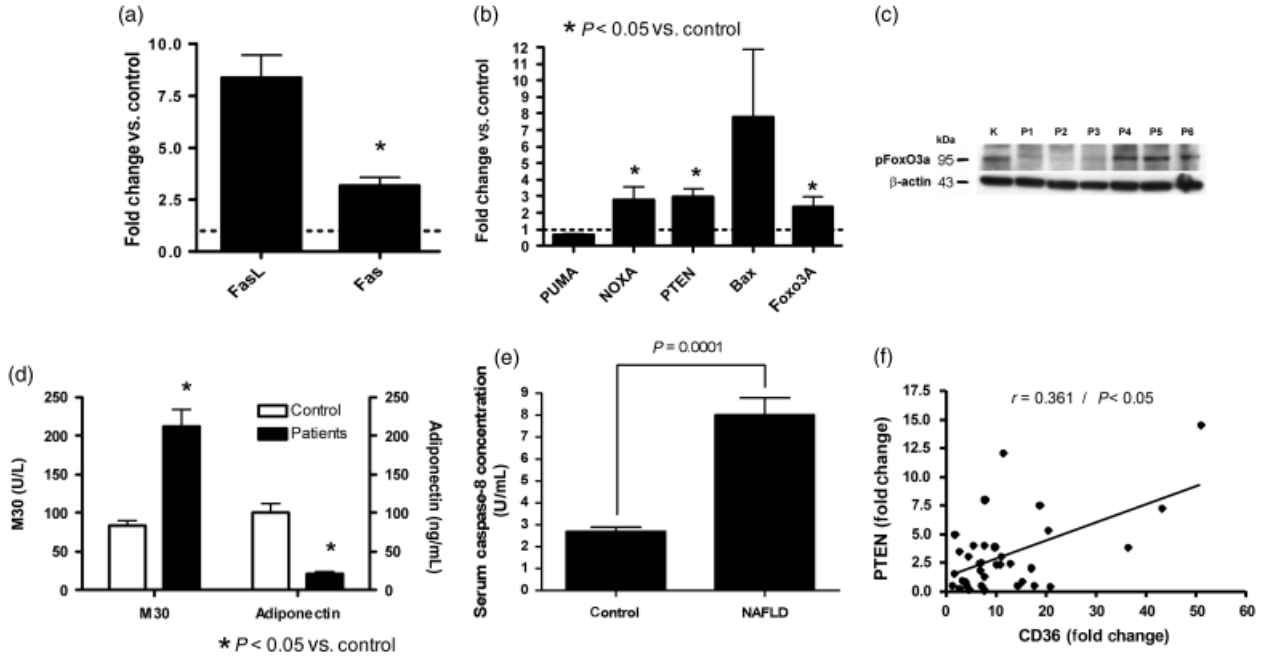

Fig. 2. Apoptosis in non-alcoholic steatohepatitis: Expression of death receptor mRNAs (a) and factors involved in intrinsic apoptosis (b). CD95/Fas and NOXA (early transcriptional target of p53) were the most upregulated. FoxO3A was also found to be significantly upregulated in patients, both at the mRNA (b) and protein levels (c). Results of six representative patients (P1 through 6) with medium to high apoptosis are presented. We also found significantly elevated levels of CK-18-derived M30 (d) and caspase-8 (e) in patients' sera while levels of the counter-regulatory adipocytokine, adiponectin, were significantly decreased. (d). Expressions of PTEN and CD36 (f) correlated positively. *P vs. control < 0.05.

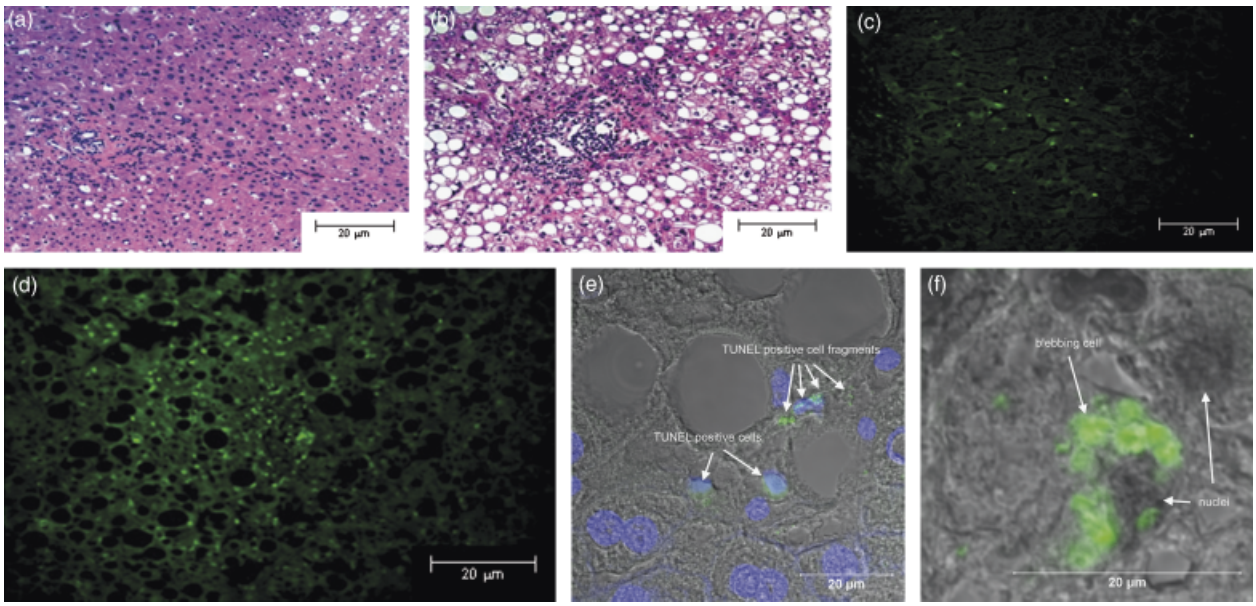

Fig. 3. Intracellular lipid accumulation and hepatocyte apoptosis in liver tissue samples. An increase in the NAS score coincided with the enlargement of lipid vacuoles and with a higher number of terminal dUTP nick end labelling (TUNEL)-positive cells as visible in representative liver sections [(a) and (c): NAS $\leq$ 4; (b) and (d): NAS $\geq$ 5). High-power magnification clearly revealed apoptotic cell morphology in patients with NASH. Representative images of two patients demonstrating blebbing and fragmentation of the nucleus are given in (e) and (f). Shown are overlays from photomicrographs of three different channels: diaminodino-2-phenidole and TUNEL fluorescence as well as haematoxylin staining in white light/greyscale. NAS, NASH Scoring System.

apparent both by our *in-vitro* (unpublished data) and *in-vivo* results. This observation could be supported by a statistical correlation of CD36/FAT expression rates with other parameters tested. Specifically, we found two highly specific levels of evidence when correlating CD36/FAT with NOXA ($r = 0.697$; $P < 0.001$) and with the number of TUNEL-positive cells ($r = 0.377$; $P < 0.05$).

The NAS score is a well-established scoring system for NASH as the progressive form of NAFLD (24). Steatosis, ballooning and inflammation are important features of NASH, which is associated with liver fibrosis. Because hepatocyte apoptosis can induce liver inflammation and fibrosis, we investigated a potential correlation of hepatocyte apoptosis with the NAS score. In 35 of all 52 patients, although levels of liver enzymes mostly were in the reference ranges or only slightly elevated, the NAS score was $\geq$5. Forty-eight of the 52 patients revealed a METAVIR fibrosis stage of 2. These results demonstrate that progressed stages of liver damage may well occur on a background of reference or close-to-normal levels of serum liver enzymes. In addition, different NAS score levels positively correlated with apoptosis rates both within the liver (via TUNEL) (Fig. 4a) and systemically (via CK-18 M30; Spearman's ρ: 0.436; $P < 0.005$; Fig. 4b).

Fibrosis: involvement of free fatty acids and apoptosis

As only four patients had a METAVIR score of 1, while all others had stage-2 fibrosis, we further investigated fibrosis-related genes. Serum FFA concentrations correlated positively with TGF-β as the marker for stellate cell activation ($r = 0.381$; $P < 0.05$) (Fig. 4d). Also, we found a positive correlation between the number of TUNEL-positive cells and TGF-β expression ($r = 0.685$; $P < 0.001$). As to the METAVIR score, FFA levels were significantly higher in stage-2 fibrosis (23.86 ± 0.7 mg/dl; Fig. 4c) compared with stage-1 fibrosis (18.54 ± 1.55 mg/dl; $P < 0.05$), and the numbers of TUNEL-positive cells per field of view also increased with advanced fibrosis (stage-2: 7.20 ± 0.56 vs. stage-1: 3.92 ± 1.13; $P < 0.05$). Furthermore FasL was significantly higher in liver tissue of patients with stage-2 fibrosis, compared with stage-1 (10.6 ± 1.4-fold; $P < 0.001$). We found no significant differences between fibrosis stages regarding the expression of fatty acid transport proteins (data not shown).

Discussion

This study reveals a tentative chain of events that connects the FFA-dependent sequential upregulation of FATPs, death receptors and hepatocyte apoptosis. This sequence of events thus appears decisive in the development of NAFLD-induced liver injury, including fibrosis. Our cohort of morbidly obese patients with NASH revealed significantly elevated FFA levels, increased FATP mRNA and protein expression and increased death receptor expression. Moreover, here we firstly show that both the extrinsic and intrinsic pathways of apoptosis are implicated in the

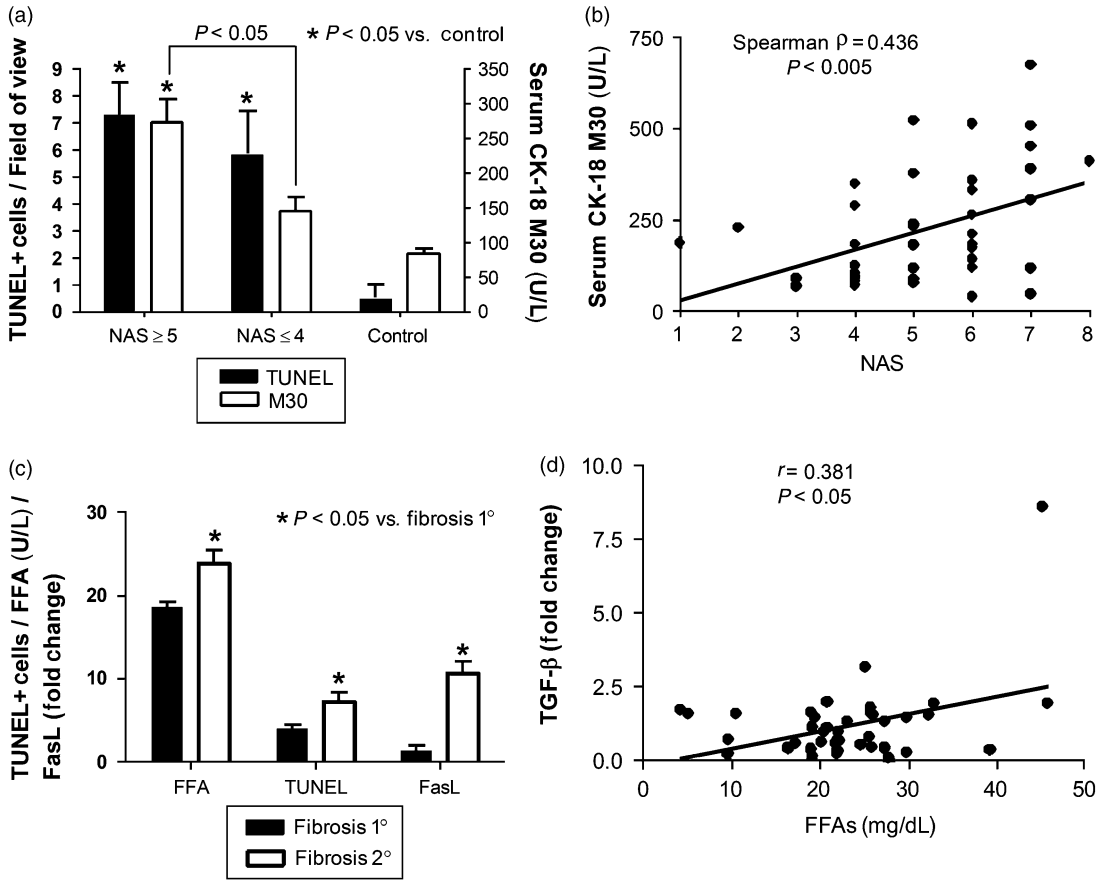

Fig. 4. Correlation between NAS score and hepatocyte apoptosis in NASH patients. (a): Different NAS score levels were associated with different rates of apoptosis as indicated by terminal dUTP nick end labelling (TUNEL) (cell death in liver tissue) and CK-18 M30 (systemic level) respectively. (b): The latter relationship was elaborated in greater detail, whereas non-alcoholic steatohepatitis (NAS) scores of 3 did not become apparent at the systemic level, higher NAS scores coincided with progressively higher CK-18 M30 concentrations. (c): Correlations between serum free fatty acids (FFAs) and the fibrosis stage as well as hepatocyte apoptosis (by TUNEL), especially the extrinsic pathway (FasL) and the METAVIR stage. (d): Similar correlations were found between the HSC activation factor, transforming growth factor-β and FFA levels. NAS, NASH Scoring System.

induction of excess apoptosis in these patients as evidenced both intrahepatically and systemically.

As had been shown before (31, 32), our patients' sera revealed high FFA levels as a common feature of the metabolic syndrome. While earlier studies found FATPs to be associated with weight development and leptin levels (33), our cohort did not reveal such correlations (data not shown). Also, the expression of CD36/FAT and FABP-1 associates with hepatic steatosis in overfed mice (34), and FATP-5 has been shown to play an important role in the correlation of FFA uptake with hepatic lipid metabolism (13, 35). Still, most aspects concerning the role and relevance of FATPs in the course of NAFLD are still largely unknown.

One recent study identified differences in the patterns of FATP and CD36/FAT expression between obese and lean Zucker rats' following metabolic stimulation (36). These findings complemented results published previously in a murine model of NASH that showed an over-expression of CD36/FAT in such animals (37). Such results suggested that FATPs may activate death receptors. Besides their anti-oxidative effects (38), several studies indicated a role for FATPs in the induction of apoptosis (39, 40). Moreover, it has been shown that CD36/FAT acts pro-apoptotically (41).

A significant increase in CD36/FAT, as found in this study, strongly supports a major role of this protein in the events leading to liver damage in NASH. CD36/FAT is a ubiquitously expressed membrane glycoprotein that acts as a fatty acid translocase, and a receptor for collagen type-I and thrombospondin (42). Importantly, oxidative stress is involved in the pathogenesis of chronic liver injury and fibrogenesis by provoking lipid peroxidation and protein modification. Upon oxidative stress, cultured hepatic stellate cells (HSCs) generate oxidized low-density lipoproteins (oxLDL) that stimulate the synthesis of collagen types-I/III and fibronectin. Inhibition of oxLDL uptake by HSC-expressed CD36 reduces

oxLDL-stimulated collagen type-I synthesis (43). The CD36 gene is regulated by the peroxisome proliferated-activated receptor α (PPARα), with some evidence on a potential key role of PPARα in the phenotypic transformation of HSCs (44). In atherosclerosis, increased oxLDL uptake by arterial-wall macrophages activates PPARα-dependent transcription through a novel pathway involving particle uptake by scavenger receptors such as CD36. Finally, peroxisome proliferators not only influence the metabolism of intracellular fatty acids but also their cellular uptake, e.g. by CD36/FAT, which may be an important step in lipid homeostasis (45). Indeed, our finding of NOXA upregulation in hepatocytes of patients with NASH indicates a role of cellular stress in the context of NAFLD. Among other stress factors, FFAs (46) and TGF-β (47) can activate FoxO3a, which again promotes both extrinsic and intrinsic apoptosis (48, 49), including FasL, TRAIL and NOXA. While our FoxO3a data pool is somewhat ambiguous, it either suggests the activation of diverging pathways leading to similar tissue damage or may relate to different stages within one single pathway. This open question can be addressed by future experimental designs that could not be implemented in our current setting.

Taken together, we found a correlation between excess serum FFAs, hepatocyte apoptosis and fibrosis in patients with NAFLD and, based thereupon, the present evidence for three hitherto unrecognized elements in the progression towards liver damage and failure in NASH, i.e., (i) a general involvement of FATPs; (ii) a special role for CD36/FAT and (iii) first evidence of intrinsically elicited apoptosis as an exacerbating factor. These findings may serve as a starting point from where to scrutinize the exact role and relevance of FATPs – and, especially, CD36 – in the pathogenesis of NASH, which may ultimately lead to urgently needed novel treatment options.

Acknowledgements

The expert experimental contributions by Mr. Martin Schlattjan, MS and Ms. Nina Wiesemann, MD are gratefully acknowledged.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Select parameters are presented by activation of FoxO3a. Expression of CD36/FAT, FABP and NOXA (as are related to the intrinsic pathway of apoptosis) are clearly elevated in pFoxO3a-low patients. Conversely, the extrinsic apoptosis pathway, as indicated by caspase-8 and FasL expression, is downregulated in pFoxO3a-low patients when compared to patients with

high levels of phosphorylated FoxO3a. The same was found for adiponectin with its antiapoptotic, insulin resistance-promoting and regulatory properties. Due to the small sample size, these differences did not reach statistical significance.

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Original Paper

Fetuin-A mRNA expression is elevated in NASH compared with NAFL patients

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Abstract

Fetuin-A is a pro-inflammatory protein expressed by hepatocytes. Its course in morbidly obese patients with NAFLD (non-alcoholic fatty liver disease) following weight loss by BAS (bariatric surgery) has not been fully elucidated yet. In the present study, we prospectively examined the effects of weight loss on various metabolic factors at 4 weeks and 6 months after surgery. Blood and liver tissues were retrieved from 108 morbidly obese NAFLD patients before/during BAS, and 50 of these individuals met the criteria for NASH (non-alcoholic steatohepatitis). Fetuin-A expression was measured by qPCR (quantitative real-time PCR), Western blotting and immunohistochemistry. Hepatocyte apoptosis was quantified via M30 (caspase-cleaved cytokeratin-18 fragments). Plasma concentrations of adiponectin and fetuin-A were determined by ELISA. Serum-derived parameters were additionally taken at 4 weeks and 6 months post-operatively. In addition, primary human hepatocytes were treated with NEFA (non-esterified fatty acid) to investigate changes in fetuin-A. BMI (body mass index) decreased significantly from 53.0 ± 1.1 to 36.4 ± 1.9 kg/m^2 in the NAFL group and from 53.3 ± 1.1 to 37.6 ± 1.2 kg/m^2 in the NASH group ($P < 0.0001$) at 6 months post-surgery. This was associated with diminishing M30 and M65 (total cytokeratin-18) levels over 6 months after surgery. Adiponectin levels increased continuously in NASH patients, whereas NAFL patients plateaued at 4 weeks post-operatively. Hepatic fetuin-A mRNA and protein expression was elevated before surgery-induced weight loss. However, plasma concentrations of fetuin-A increased signficantly in NASH patients 4 weeks post-operatively. Treatment of hepatocytes with NEFA led to up-regulation of fetuin-A expression. BAS probably has a beneficial effect on NAFLD, as indicated by reduced hepatocyte apoptosis and improved adipokine profiles. In addition, fetuin-A expression is more prominent in NASH.

Key words: adiponectin, bariatric surgery, fetuin-A, non-alcoholic fatty liver disease (NAFLD)

INTRODUCTION

Obesity is a worldwide pandemic with excessive growth that causes a multitude of co-morbid conditions [1]. Much interest is now focused on treating not only obesity itself, but also the deleterious metabolic sequelae accompanying this entity. This includes insulin resistance, diabetes, hypertension, dyslipidaemia, and, as liver-related manifestation, NAFLD [NAFL (non-alcoholic fatty liver) disease] [2]. With the increasing number of bariatric surgical procedures being performed over the past few years, the available data suggest that after BAS (bariatric surgery) clinical improvement or even resolution may be achieved in 64–100% of patients with diabetes, 62–69% of patients with hypertension, 60–100% of patients with dyslipidaemia and up to 90% of patients with NAFLD [3].

NAFLD is currently the most common liver disease in Western countries [4] and encompasses a spectrum that is initiated with simple steatosis (NAFL); it can progress to NASH

Abbreviations: ALT, alanine aminotransferase; BAS, bariatric surgery; BMI, body mass index; DAPI, 4′,6-diamidino-2-phenylindole; HBSS, Hanks balanced salt solution; HCC, hepatocellular carcinoma; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LAGB, laparoscopic adjustable gastric banding; NEFA, non-esterified fatty acid; M30, caspase-cleaved cytokeratin-18; M65, total cytokeratin-18; NAFL, non-alcoholic fatty liver; NAFLD, NAFL disease; NAS, NAFLD activity score; NAFL NASH, non-alcoholic steatohepatitis; qPCR, quantitative real-time PCR; SG, sleeve gastrectomy; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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Table 1 Demographic and biochemical characteristics of the patients with NAFLD following BAS for morbid obesity and the healthy controls enrolled in the present study

*Significant difference between NAFL and NASH group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyltransferase.

Parameter	Control	NAFLD (all patients)	NAFL	NASH
n	10	108	58	50
Gender (female/male) (*n*)	5/5	83/25	46/12	37/13
Age (years)	32.5 ± 5.5	41.9 ± 0.9	42.0 ± 1.3	41.7 ± 1.2
Height (cm)	170.7 ± 9.7	172.0 ± 0.9	172.2 ± 1.3	171.8 ± 1.2
Weight (kg)	69.7 ± 11.8	157.6 ± 2.73	157.4 ± 3.7	157.8 ± 4.0
ALT (units/l)	24.5 ± 4.9	35.0 ± 2.1	29.2 ± 2.3	41.8 ± 3.6*
AST (units/l)	23.2 ± 2.4	28.7 ± 1.4	26.2 ± 1.7	31.6 ± 2.3
γ-GT (units/l)	31.6 ± 12.8	38.9 ± 2.8	33.8 ± 3.6	44.98 ± 4.2*

(non-alcoholic steatohepatitis), cirrhosis, end-stage liver failure and HCC (hepatocellular carcinoma) [5]. Although the prevalence of NAFLD in the general population is estimated to range from 20 to 40%, approximately 85–95% of morbidly obese patients seem to have NAFLD and up to 33% even suffer from NASH [6,7]. Given that life-style modification and oral medication are relatively ineffective strategies for achieving significant and sustained weight loss among morbidly obese patients, BAS has become an increasingly popular treatment option in this cohort [8]. This procedure is not only effective in achieving weight loss, but also allows an understanding of the pathophysiological mechanism involved in NASH and its progression. Indeed, it has been reported recently that fetuin-A, a pro-inflammatory protein expressed by hepatocytes, may contribute to liver injury [9]. Furthermore, it seems that fetuin-A counteracts adiponectin, which is known as a protective adipokine [10].

Therefore the present study investigated the impact of laparoscopic-guided weight loss procedures on cytokine profiles in morbidly obese patients. In particular, the role of fetuin-A to differentiate between patients with NAFL and NASH following weight loss was investigated further.

MATERIALS AND METHODS

Patients

The study population consisted of 108 Caucasian patients undergoing BAS at the Department II of Surgery, Alfried-Krupp Hospital Essen, Germany. All patients met the following criteria for surgical weight loss therapy established by the NIH consensus conference in 1991 [11]: age >18 years, severe obesity with a BMI (body mass index) $\geqslant$40 or $\geqslant$35 kg/m^2 with co-morbidities, failure of medical weight loss, absence of medical or psychological contra-indications for BAS, and evaluation by a multi-disciplinary team of medical, nutrition, psychiatry and surgical specialists. Demographic and clinical data included age, gender, BMI, liver enzymes and metabolic sequelae (Table 1). Patients aged <18 or >65 years with liver pathologies other than NAFLD, history of organ transplantation, history of malignancy within the previous 5 years, alcohol abuse defined as an average daily consumption of >20 g/day for women and >30 g/day for men, drug abuse within the previous year, autoimmunity or genetic disorders, and therapy with immunosuppressive or hepatotoxic agents were excluded. A total of ten subjects were used as healthy controls (Table 1).

Using the area under the ROC (receiver operator characteristic) curve approach, Feldstein et al. [12] calculated the potential cut-off value to separate patients with definitive NASH from those with simple steatosis (NAFL) by determining M30 (caspase-cleaved cytokeratin-18). The M30 value with the best combination of sensitivity and specificity was 275 units/l [12]. This independent predictor of NASH was applied in our experiments. In detail, patients were grouped according to Feldstein et al. [12] as NAFL, when serum M30 was below 275 units/l or as NASH, when serum M30 was 275 units/l or above, respectively.

Surgical intervention

BAS was carried out by laparoscopic approach in all patients. Operations were either performed as RYGB (Roux-en-Y gastric bypass), SG (sleeve gastrectomy) or gastric banding. Briefly, gastric bypass was conducted to create a 15 ml reservoir and either a 75 cm (short limb) or 150 cm (long limb) Roux segment of the small intestine [13]. SG was achieved by a longitudinal resection of the stomach parallel to a F48 bogie advanced along the lesser curvature [14]. The type of surgical technique was based on the current guidelines adjusted to the patient's preference, clinical status and co-morbid conditions, as well as on the surgeon's expertise.

All patients were informed about the additional risks of a wedge liver biopsy during the bariatric procedure. Liver specimens were split and stored in either 4% (v/v) formalin solution (Roth) for subsequent histological examination or in RNAlater (Ambion Applied Biosystems) for RNA isolation. The study was conducted in accordance with the ethical guidelines of the 2008 Helsinki Declaration and the protocol was approved by the ethics and research committees of the University Hospital of Essen. All patients provided written informed consent before enrolment.

Histology

H&E (haematoxylin and eosin) staining was performed according to standard techniques [15].

Apoptosis

The cell death markers M30 (for apoptosis) and M65 (total cytokeratin-18) (for overall cell death) were assessed in patients'

sera using the M30 (Apoptosense) and M65 ELISA® kits (both from PEVIVA, Alexis) following the manufacturer's instructions. Both kits detect epitopes of cytokeratin-18, with M65 binding to cytokeratin-18 in any form. M30 only binds to an epitope exposed by caspase cleavage of cytokeratin-18 and is thus deemed as surrogate marker of apoptosis.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining

TUNEL assay was performed as described previously [16].

qPCR (quantitative real-time PCR)

Liver tissue was homogenized with a blade homogenizer (IKA) according to the standard laboratory procedures [16]. Total RNA was isolated with the RNeasy mini kit (Qiagen) following the manufacturers' instructions. PCR of complementary DNA was performed using the iCycler iQ thermal cycler (Bio-Rad Laboratories). Relative gene expressions were calculated from the threshold cycles in relation to a reference gene [HPRT (hypoxanthine-guanine phosphoribosyltransferase)], controls or healthy donors. Oligonucleotides utilized for the PCR (all obtained from Eurofins MWG Operon) were as follows: HPRT: 5′-GACCAGTCAACAGGGGACAT-3′ (forward), 5′-CTTGCGACCTTGACCATCTT-3′ (reverse); fetuin-A: 5′-CCAGTCCAGACTCAGCCCAGGA-3′ (forward), 5′-CTGAGCGTTGAAGGCGGCCA-3′ (reverse).

Immunohistochemistry

Tissue sections were pre-incubated with Block-ace (Dako Cytomotion) for 10 min at 37 °C to block non-specific binding of the primary antibody. Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 and 0.1 % sodium nitrite in distilled water for 10 min at room temperature (20 °C). Sections were then incubated overnight with 1:500-diluted fetuin-A-specific monoclonal antibody (AbD Serotec), rinsed three times with PBS and incubated with avidin–biotin peroxidase complexes (Vector Laboratories). Histochemical development was achieved by employing a DAB (3,3′-diaminobenzidine) substrate kit (Vector Laboratories). Finally, sections were counterstained for 3 min with haematoxylin and cover-slipped with mounting medium for light microscopy.

ELISA

Adiponectin and fetuin-A concentrations (both as Quantikine ELISA® kits; R&D Systems) were determined by sandwich ELISA. Plates pre-coated with human monoclonal antibodies were blocked by adding 15 % (w/v) BSA, washed and incubated with sera from the patients. Absorbance was measured at 450 nm.

Western blot analysis

Liver tissue was directly lysed for 30 min on ice with lysis buffer [16]. After centrifugation at 13 000 ***g*** for 15 min at 4 °C, protein concentration in the supernatant was measured using Bradford's reagent (Bio-Rad Laboratories). After denaturing for 10 min, proteins were resolved by SDS/PAGE on a gradient gel and then transferred on to nitrocellulose membranes. Blocking was carried out using 5 % (w/v) non-fat dried skimmed milk in Tris-buffered saline with 0.1 % Tween 20 for 1 h at room temperature. Primary antibodies were diluted 1:1000 in blocking solution and incubated overnight at 4 °C. The following antibodies were used: anti-fetuin-A (R&D Systems) and anti-β-actin (Cell Signaling Technology). To detect the specific antigen–antibody complexes, peroxidase-conjugated secondary antibodies (Biosource International) were diluted 1:3000 in blocking solution and incubated for 45 min at room temperature. Immune complexes were visualized using chemiluminescent substrate (ECL) and Kodak X-OMAT film (Eastman Kodak) according to the manufacturers' instructions.

Cell culture

For isolation of human primary hepatocytes, liver samples were perfused with HBSS (Hanks balanced salt solution) and EGTA (PAA) to wash out remaining blood, followed by HBSS with 240–250 units/ml collagenase type IV (Sigma–Aldrich) to digest connective tissue. The obtained cell suspension was filtered through a 4 μm mesh and washed 3 times in HBSS. The cell pellet was resuspended in cell culture medium (PAA) and seeded at a density of approximately 10^6 cells/cm^2. For mimicking a steatosis-like state, cells were incubated with 0.5 and 1 mM mixed long-chain NEFA (non-esterified fatty acid; 2:1, oleate/palmitate; Sigma–Aldrich). Controls were kept without NEFA. Total RNA from cultured cells was isolated with the RNeasy mini kit (Qiagen). Gene expression levels were measured by qPCR using SdhA (succinate dehydrogenase complex subunit A) as housekeeping gene.

Oil-Red-O staining

Cells were fixed in 4.5 % Histofix (Sigma–Aldrich) for 24 h and washed with 60 % (v/v) isopropanol. After complete drying, cells were incubated with Oil-Red-O solution (Sigma–Aldrich) for 10 min, washed with water and mounted with ProLong® Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen).

Statistics

All data are expressed as means $\pm$ S.E.M. Statistical analysis was performed using GraphPad Prism. Differences between the groups were evaluated by one-way ANOVA. Differences between NAFL and NASH at one timepoint were calculated with Student's *t* test or Mann–Whitney test (in case of non-parametric variables). Differences between time points within one patient group were calculated with paired Student's *t* test or Wilcoxon matched-pairs signed ranks test (in case of non-parametric variables). Statistical significance was assumed at a $P < 0.05$.

RESULTS

BAS diminishes co-morbid conditions of the metabolic syndrome

Demographic and biochemical characteristics of the study population are presented in Table 1. Overall, 108 morbidly obese patients with NAFLD underwent BAS, whereas ten consented liver transplantation donators served as healthy controls.

Table 2 Histological evaluation of available liver samples ($n = 104$) in NAFLD patients and the subgroups (NAFL and NASH)
Values are number of patients (percentage). *Steatosis grades differed significantly between NAFL and NASH groups. NAS, NAFLD activity score.

Score	NAFLD (all patients)	NAFL ($n = 55$)	NASH ($n = 49$)
NAS 0–4	79 (76.0%)	45 (81.8%)	34 (69.4%)
NAS 5–8 (histological NASH)	25 (24.0%)	10 (18.2%)	15 (30.6%)
Steatosis			
0	12 (11.5%)	8 (14.5%)	4 (8.2%)*
1	64 (61.5%)	39 (70.9%)	25 (51.0%)*
2	22 (21.2%)	8 (14.5%)	14 (28.6%)*
3	6 (5.8%)	0 (0%)	6 (12.2%)*
Lobular inflammation			
0	0 (0%)	0 (0%)	0 (0%)
1	74 (71.1%)	41 (74.5%)	33 (67.3%)
2	22 (21.2%)	9 (16.4%)	13 (26.5%)
3	8 (7.7%)	5 (9.1%)	3 (6.1%)
Ballooning			
0	28 (26.9%)	17 (30.9%)	11 (22.4%)
1	68 (65.4%)	32 (58.2%)	36 (73.5%)
2	8 (7.7%)	6 (10.9%)	2 (4.1%)
Fibrosis			
0	0 (0%)	0 (0%)	0 (0%)
1	13 (12.5%)	7 (12.7%)	6 (12.2%)
2	89 (85.6%)	46 (83.6%)	43 (87.8%)
3	2 (1.9%)	2 (3.6%)	0 (0%)

As demonstrated, liver enzymes at baseline were within normal ranges – a typical feature in patients with NAFLD [17].

NAFLD patients were grouped as NAFL or NASH according to Feldstein et al. [12], with an M30 level below 275 units/l as NAFL and an M30 level above 275 units/l as NASH. Histological features of NAFLD and fibrosis for all patients as well as the subgroups (NAFL and NASH) are given in Table 2.

Parameters of blood glucose and conditions of the metabolic syndrome are shown in Supplementary Figures S1(A) and S1(B) (at http://www.clinsci.org/cs/125/cs1250391add.htm). Usage of insulin fell significantly from 13% before BAS to approximately 2% at 4 weeks after surgery in the NAFL group (Supplementary Figure S1C). No differences were found in insulin usage for NASH patients.

A fall in BMI occurs early following BAS

As demonstrated in Figure 1(A), BMI decreased significantly over the observed period in both patient groups. No differences between the groups were detected. Although NEFA concentrations were lower in the NASH group after 4 week post-operatively, significance was not reached (Figure 1B).

Surgery-induced weight loss is paralleled by reduced apoptosis and general cell death

Increased hepatocyte apoptosis plays a key role in liver injury and disease progression in NAFLD [18]. Systemic apoptosis can be detected by the M30 antibody, while overall cell death is determined by M65. As established previously by Feldstein et al. [12], M30 is also a well-validated serum marker of NAFLD progression [19,20]. Before surgical intervention, M30 in the NAFLD patients was significantly raised compared with controls (99.2 ± 16.4 units/l compared with 319.8 ± 23.4 units/l, $P < 0.0001$). Separating NAFL and NASH patients according to the M30 cut-off at 275 units/l led to an artificially significant difference in M30 between the groups. Although in the NAFL group no changes were observable at 4 weeks after BAS, NASH patients exhibited a significant reduction in M30 (Figure 2A) as well as of M65 as a marker of overall cell death (Figure 2B). At 6 months post-operatively both groups had significantly reduced serum M30 and M65 concentrations compared with the pre-operative status. M65 also differed significantly between NAFL and NASH before BAS and after 6 months post-operatively.

Adiponectin concentration is elevated after BAS

Adiponectin, an adipocyte-derived cytokine (adipokine), has been demonstrated to alleviate hepatic steatosis and inflammation [7]. Serum concentrations of adiponectin were significantly reduced in morbidly obese patients with NAFL or NASH compared with healthy controls before BAS (controls compared with NAFL or compared with NASH: $P \leqslant 0.0001$; Figure 3A). Following surgery-induced weight loss after 4 weeks, adiponectin concentrations increased significantly in the NAFL as well as in the NASH groups. Overall 6 months after surgery, no further increase in adiponectin was observed in NAFL patients. In contrast, adiponectin concentrations raised continuously in NASH exceeding the values of controls, although significance was not reached.

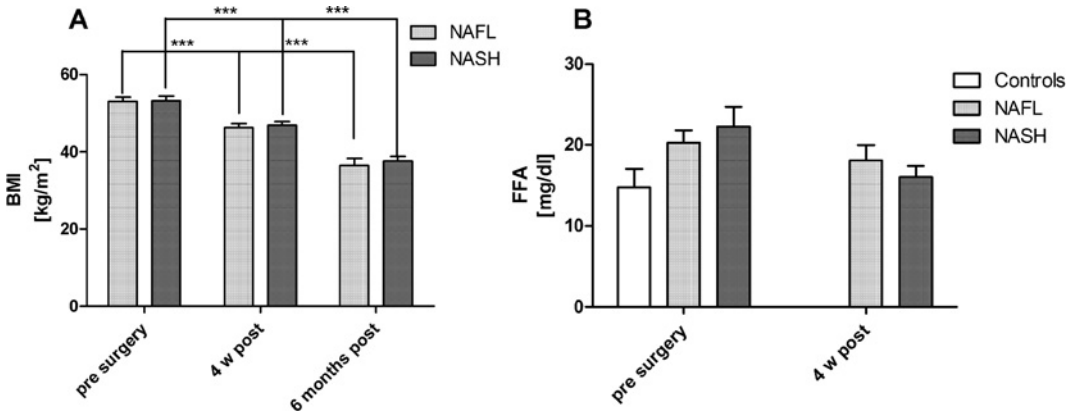

Figure 1 Effective weight reduction by BAS in NAFL and NASH patients

BMI was significantly reduced 4 weeks (4w post) and 6 months (6m post) after surgery in both groups (**A**). Distribution into groups was done using a M30 cut-off (NAFL<275 units/l; NASH⩾275 units/l serum concentration). (**B**) NEFA were slightly reduced at 4 weeks post-operatively, although no significance was reached. $^{***}P < 0.0001$ compared with pre-surgery within the respective groups. FFA, NEFA.

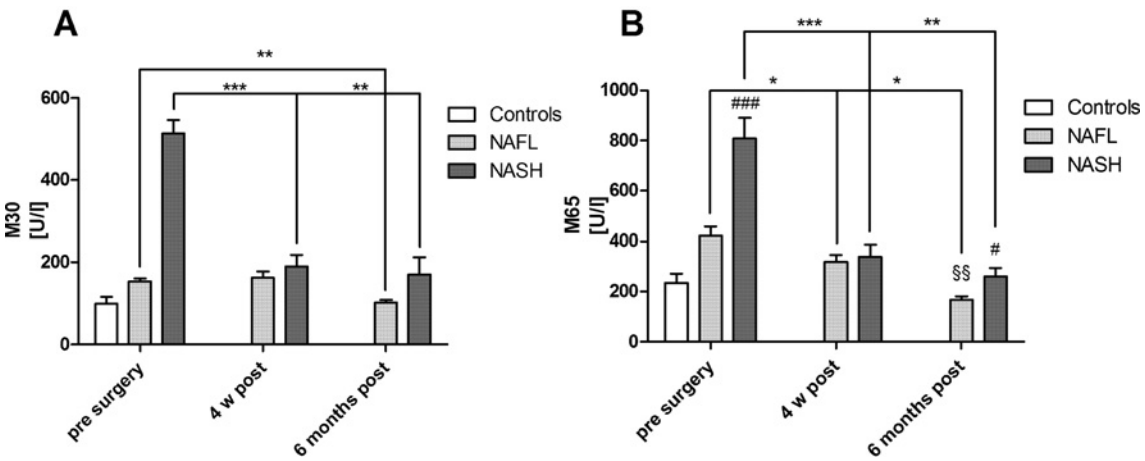

Figure 2 BAS reduces systemically detectable cell death

Apoptotic cell death determined by M30 revealed a decline in both groups, with a significant reduction in NAFL patients (**A**). M65 as marker for overall cell death was significantly higher in NASH patients compared with NAFL (**B**). Similar to M30, overall cell death significantly diminished at 4 weeks and 6 months post-operatively in NASH patients. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.0001$ compared with NASH pre-surgery; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.0001$ compared with NASF $^{\S\S}P < 0.01$ compared with NAFL 4 weeks post-operatively.

Course of fetuin-A in morbidly obese patients with NAFLD after BAS

Fetuin-A is a liver-derived glycoprotein that impairs insulin signalling and correlates with hepatic steatosis [21]. Although fetuin-A serum concentrations were not increased in NAFLD patients (or subgroups) compared with controls (controls 107.4 ± 10.6 μg/ml compared with NAFLD 117.7 ± 7.5 μg/ml; Figure 3B), at 4 weeks post-operatively a slight elevation of fetuin-A was observed. This was more prominent and reached significance in NASH, which is associated with higher liver injury. This might imply a 'third hit', inducing fetuin-A expression in pre-existing fatty liver injury. At 6 month post-surgery, fetuin-A levels were comparable with initial values and did not differ significantly to pre-operative values or values at 4 weeks post-operatively. In liver tissue, expression of fetuin-A (detected by qPCR, Figure 3C; and Western blot, Figure 3E) was significantly elevated in NAFL compared with controls. The NASH group exhibited an even stronger increased mRNA expression than NAFL patients. This was confirmed by immunohistochemistry for fetuin-A Figure 3F), demonstrating increasingly more fetuin-A-positive cells in NAFL and NASH. No positive cells were found in controls. To test, if serum fetuin-A may be an appropriate surrogate marker for hepatic fetuin-A expression, mRNA expression and serum concentrations were correlated (Figure 3D). A significant, positive correlation for liver mRNA and serum concentration of fetuin-A was found. As a possible connection between

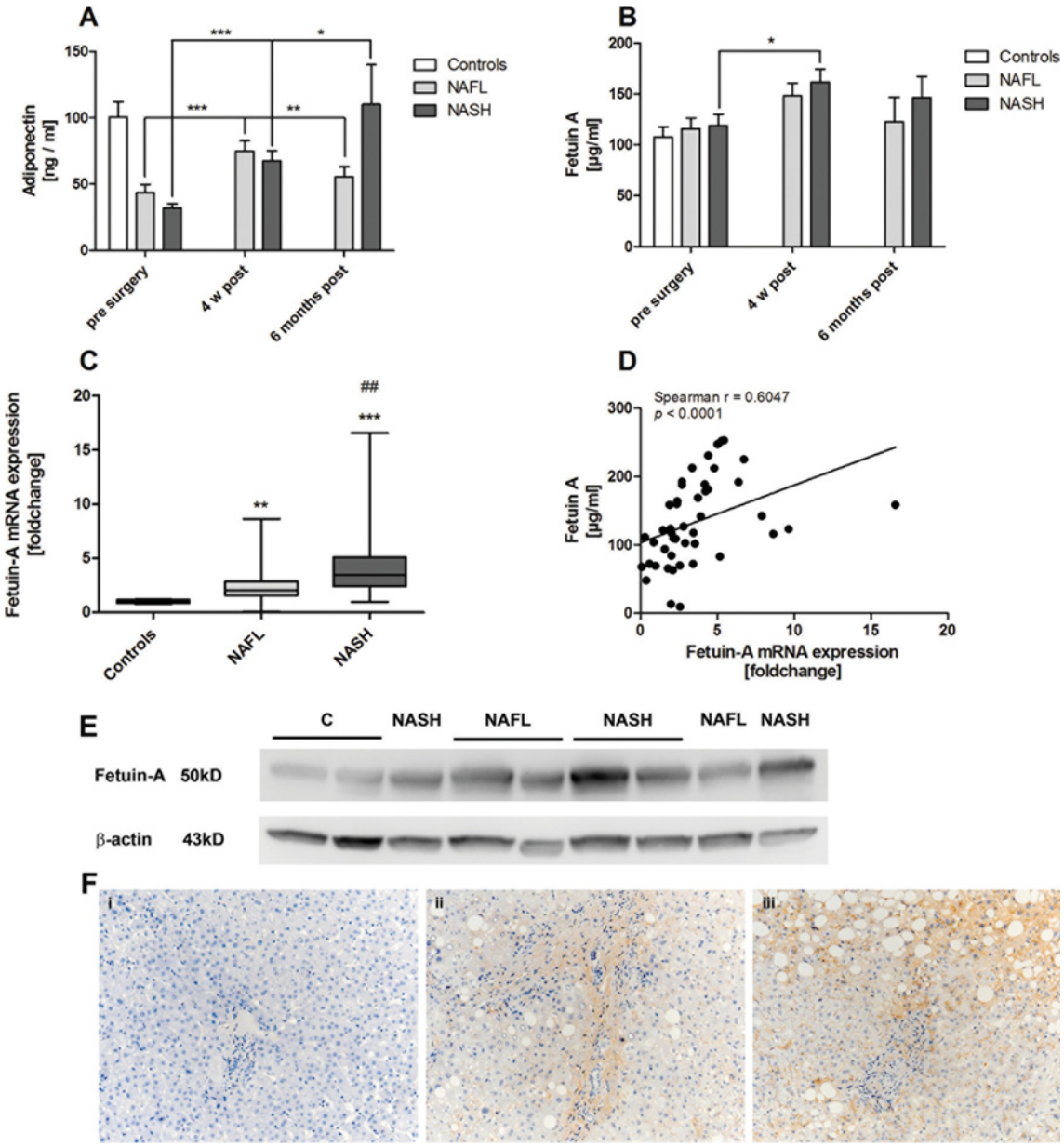

Figure 3 **BAS leads to enhanced adiponectin production and fetuin-A is increased in NAFLD**

Increased serum concentrations of adiponectin were found in patients with NAFL and NASH patients following surgery-induced weight loss (**A**). Although NAFL patients seemed to reach a plateau after 4 weeks post-operatively (4w post), NASH patients exhibited a further increase at 6 months post-operatively, although the difference between the subgroups was not significant ($P < 0.13$ compared with NASH). Serum fetuin-A slightly increased in NAFL as well as NASH patients at 4 weeks post-operatively, although significance could only be detected in NASH (**B**). In contrast, mRNA (**C**) and protein (**E**) expression of fetuin-A was significantly elevated in liver tissue of NAFL patients compared with controls ($^{**}P < 0.01$ compared with controls) and more prominently in NASH ($^{***}P < 0.0001$ compared with controls; $^{\#\#}P < 0.01$ compared with NAFL) before BAS. This was confirmed by immunohistochemistry for fetuin-A in liver tissue of controls (**F**, panel i), NAFL (**F**, panel ii) and NASH (**F**, panel iii). Representative results from each group are shown, demonstrating no positive staining in controls and increasing positive cell numbers from NAFL to NASH. In addition, fetuin-A serum concentrations correlated significantly positively with mRNA expression of fetuin-A in the liver (**D**).

fetuin-A as marker of liver injury and NAFLD in particular, histological signs for NAFLD were correlated with fetuin-A. A significant negative correlation was found for the fibrosis stage and serum fetuin-A concentrations (results not shown), while no further correlations were found.

Fetuin-A expression increased after treatment of primary human hepatocytes with NEFA

To test, whether NEFA induce fetuin-A expression in a cell culture model, primary human hepatocytes were treated with NEFA at a concentration of 1 mM *in vitro*. Subsequently, hepatocytes

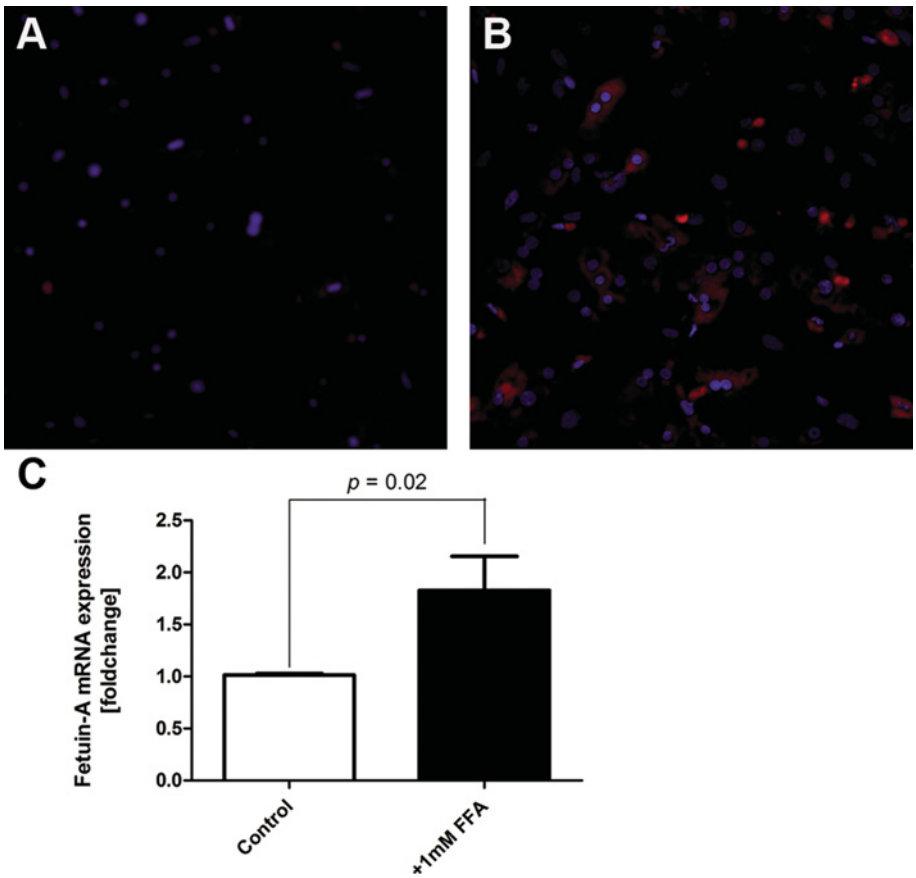

Figure 4 ***In vitro*** **treatment with NEFA enhances fetuin-A mRNA expression of hepatocytes**
Freshly isolated primary human hepatocytes cells were stained with Oil-Red-O to demonstrate fat uptake under normal (**A**) and NEFA-treated (**B**) conditions. Treatment of primary human hepatocytes with NEFA resulted in up-regulation of fetuin-A mRNA expression (**C**). FFA, NEFA.

were incubated with Oil-Red-O solution and mounted with DAPI to visualize fat uptake (Figures 4A and 4B). As shown in Figure 4(C), mRNA expression of fetuin-A was significantly increased in treated samples as compared with controls (1.0 ± 0.1-fold change compared with 1.85 ± 0.3-fold change, $P = 0.02$).

DISCUSSION

The clinical presentation of NAFLD is highly variable, which can be attributed to host, genetic and environmental factors [22]. Some patients develop only minimal hepatic damage that rarely progresses to a chronic hepatopathy [23]. As many of these patients maintain this status, medical treatment is often not required. However, some of the patients develop liver cirrhosis and HCC, even in the absence of cirrhosis [5] with the necessity of liver transplantation [24,25] – particularly when other risk factors, such as obesity, are present [26]. Within the past few years, BAS has become a novel and promising option in this cohort [27]. This trend towards surgery has grown as a result of reported benefits of BAS on various obesity-related co-morbidities such as NAFLD. Growing data on the underlying mechanisms of NAFLD improvement in obese patients after surgery may offer new opportunities to understand and treat this entity. This is of particular importance since obesity and NAFLD are both globally on the increase [28]. In the present study, we demonstrate a parallel decrease in BMI and cell death as well as an increase of adiponectin in NAFLD patients after BAS. In addition, we showed that fetuin-A expression in the liver before surgery was increased and correlated to serum fetuin-A. These findings will be discussed in detail below.

A study by Diab et al. [29] assessed the usefulness of M30 in a cohort of obese patients undergoing liver biopsy at the time of BAS and revealed a significant decrease in M30 levels in most of the patients 6 months after surgery. Likewise, in the present study, markers of cell death (M30 and M65) were significantly reduced in NAFL and NASH after surgery-induced weight loss. Indeed, reduced hepatocyte apoptosis after weight loss has been well established [19]. Since the liver contributes to a large extent to serum M30 in NAFLD, these findings suggest a reduction of fatty liver disease after BAS.

Adipokines are regarded as important mediators with pro- or anti-inflammatory effects, mainly produced and secreted by the adipose tissue [30]. Growing evidence suggests a key role for adipokines in the pathophysiology of NAFLD [7]. Although

most studies have been done on adipose tissue, some adipokines – and in particular their receptors – are also expressed in the liver. Reduced intrahepatic expression of adiponectin and its receptors has been demonstrated by Kaser et al. [31] in morbidly obese patients with NASH. Although adiponectin has mainly anti-inflammatory functions, leptin, resistin and visfatin are considered as pro-inflammatory mediators [32]. Moschen et al. [33] prospectively investigated the effects of weight loss on systemic levels and hepatic expression of these cytokines in severely obese patients following BAS. Pronounced weight loss was accompanied by a significant increase in serum adiponectin levels while both leptin and visfatin levels decreased significantly. In addition, hepatic adiponectin mRNA expression increased and weight loss resulted in a significant decrease in hepatic resistin mRNA expression. The same group examined alterations of interleukin-1 family cytokine members in obese patients following LAGB (laparoscopic adjustable gastric banding) [34]. The authors revealed that extensive weight loss shifted the adipose and hepatic expression profile of interleukin-1 family members towards a more anti-inflammatory pattern after LAGB. Results of the cohort described in the present study are in line with these previous findings as we also observed a significant increase in serum adiponectin levels in patients with NAFL and, more strikingly, in NASH after surgery. On the one hand, this supports a general positive effect of BAS on patient health. On the other hand, this suggests adiponectin as possible marker to monitor recovery from the metabolic syndrome after BAS. Previous findings of our group underscore this notion, as we could show a negative correlation of adiponectin with histological measures of NAFLD (NAS and ballooning) [35].

Fetuin-A is a marker of impaired insulin signalling, lipid metabolism and hepatic steatosis [36]. However, its exact course and effects after surgery-induced weight loss has not been fully elucidated yet, especially when differentiating between patients with NAFL and NASH [37]. In our study, plasma fetuin-A concentrations increased slightly after the initial weight loss (4 weeks post-operatively). However, 6 months after BAS no differences were observed compared with pre-operative status. Elevated mRNA and protein expression of fetuin-A and an overall higher serum concentration of fetuin-A in NAFLD patients compared with controls was still remarkable. According to previous publications fetuin-A might be associated with the metabolic syndrome [38] and may actually diminish adiponectin expression [10]. However, in our data, no correlations of serum fetuin-A levels and signs of the metabolic syndrome, histological signs of NAFLD or adiponectin concentrations were found. Previous studies suggest that BAS can facilitate lipid mobilization [39], which may be due to a reduction in adipocyte size [40]. In conjunction with the results described in the present paper, a sudden increase in lipids may lead to the increase in fetuin-A in the early post-operative period. This is supported by an increase in fetuin-A mRNA expression under NEFA treatment *in vitro*. Although fetuin-A may be a possible marker of NAFLD progression, regression of NAFLD, as assumed in the present study, may be too slow for a significant reduction after 6 months. Larger cohorts or longer follow-up periods could clarify whether fetuin-A is an appropriate marker to monitor NAFLD regression.

Owing to limitations in sample size of human-derived tissue and serum, most recent works relating fetuin-A to TLR4 (Toll-like receptor 4)-dependent insulin resistance [41] or ER (endoplasmic reticulum) stress [42] could not be included in our analyses. Although, complementary *in vitro* experiments clearly demonstrated up-regulation of fetuin-A mRNA expression in primary human hepatocytes treated with NEFA. This again suggests a possible usage of fetuin-A as NAFLD surrogate marker, probably in the long run of disease progression or regression.

In summary, BAS-induced weight loss has marked beneficial systemic effects in morbidly obese patients suffering from NAFLD. Weight loss simultaneously affects the constellation of metabolic, inflammatory and hepatic abnormalities involved in the pathogenesis of NAFLD. An early increase in adiponectin levels accompanied by a diminished cell death suggests contribution of metabolic factors and improved cell survival to a possible improvement of NAFLD after BAS. High fetuin-A expression before BAS and correlation of steatosis to fetuin-A serum levels should stimulate investigation on a possible use of fetuin-A as an NAFLD marker.

CLINICAL PERSPECTIVES

- BAS improves metabolic function and overall health, although underlying mechanisms remain unclear.
- In the cohort described in the present paper, high cell death was associated with increased hepatic fetuin A mRNA expression and elevated fetuin A serum concentrations. After BAS serum cell death markers diminished, whereas serum adiponectin levels increased over time. Serum fetuin A increased early after surgery but returned to pre-operative levels after 6 months.
- The course of serum fetuin A levels may allow monitoring reconstitution of fatty liver after BAS.

AUTHOR CONTRIBUTION

Alisan Kahraman participated in the study design, collected patient material, performed statistical analyses and drafted the paper. Jan-Peter Sowa participated in the study design, performed statistical analyses, drafted the paper and revised the paper for important intellectual content. Martin Schlattjan collected patient material and generated the data. Svenja Sydor generated the data and revised the paper for important intellectual content. Martin Pronadl collected patient material, participated in the study design and drafted the paper. Alexander Wree performed statistical analyses and revised the paper for important intellectual content. Anja Beilfuss generated the data. Alpaslan Kilicarslan collected patient material and drafted the paper. Akif Altinbaş collected patient material and the data. Lars Peter Bechmann collected patient material, performed statistical analyses and revised the paper for important intellectual content. Wing-Kin Syn participated in the study design and revised the paper for important intellectual content. Guido Gerken participated in study coordination and revised the manuscript for important intellectual content. Ali Canbay conceived of the study, and

participated in its design and co-ordination and revised the paper for important intellectual content. All authors read and approved the final paper.

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

Fetuin-A mRNA expression is elevated in NASH compared with NAFL patients

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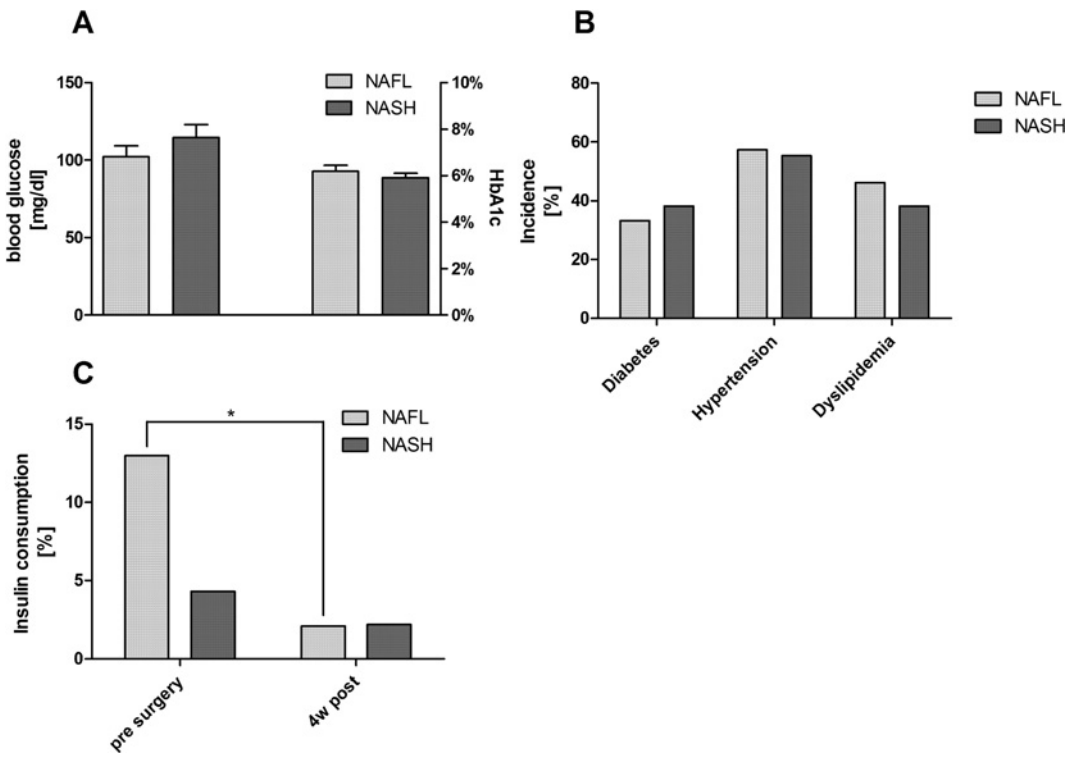

Figure S1 **Metabolic sequelae and glucose metabolism in NAFLD patients**
Blood glucose and HbA_{1c} (glycated haemoglobin) are shown for the NAFL and NASH subgroups of the analysed cohort (**A**), although no differences between the groups were observed. Characteristics of the metabolic syndrome were present to high proportions in NAFL as well as NASH patients (**B**). Usage of insulin was higher in the NAFL group compared with NASH patients before BAS (**C**). After surgical intervention (4 weeks) the use of insulin was reduced significantly in NAFL. The slight reduction of insulin usage in the NASH group did not reach significance.

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ORIGINAL ARTICLE

Vitamin D counteracts fibrogenic TGF-β signalling in human hepatic stellate cells both receptor-dependently and independently

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ABSTRACT

Objective Non-alcoholic fatty liver disease (NAFLD) is closely linked to obesity and constitutes part of the metabolic syndrome, which have been associated with low serum vitamin D (VD). Due to known crosstalk between VD and transforming growth factor (TGF)-β signalling, VD has been proposed as an antifibrotic treatment.

Design We evaluated the association between VD, the vitamin D receptor (VDR) and liver fibrosis in primary human hepatic stellate cells (phHSC) and 106 morbidly obese patients with NAFLD.

Results Treating phHSC with VD ameliorated TGF-β-induced fibrogenesis via both VDR-dependent and VDR-independent mechanisms. Reduction of fibrogenic response was abolished in cells homozygous for GG at the A1012G single nucleotide polymorphisms within the VDR gene. Compared with healthy livers, NAFLD livers expressed higher levels of VDR mRNA and VDR fragments. VDR mRNA was lower in patients homozygous for GG at A1012G and expression of pro-fibrogenic genes was higher in patients carrying the G allele.

Conclusions VD may be an antifibrotic treatment option early in the onset of fibrosis in specific genotypes for VDR. Known polymorphisms of the VDR may influence the response to VD treatment.

Significance of this study

What is already known on this subject?

- Vitamin D (VD) is reduced in obese subjects.
- Non-alcoholic fatty liver disease (NAFLD) and obesity are connected via the metabolic syndrome.
- VD signalling is antagonistic towards the transforming growth factor (TGF)-β pathway in various models.

What are the new findings?

- Serum VD is reduced in patients with NAFLD and correlates with a fibrogenic state in the liver. Liver fibrosis is associated with reduced full-length VD receptor (VDR) protein expression, but increased VDR protein fragments.
- VD exerts a VDR-independent effect on early-TGF-β-induced SMAD activation and protects VDR from trypsin digestion in primary human hepatic stellate cells.
- Reduction of TGF-β-induced profibrogenic gene expression by VD does not occur in cells homozygous for minor allele genotypes of the VDR.
- Profibrogenic mRNA expression is partially dependent on known polymorphisms of the VDR in patients with NAFLD.

How might it impact on clinical practice in the foreseeable future?

- The currently ineffective therapy of fibrotic processes by patients might benefit from a targeted approach to individuals without certain variants of the VDR gene. Targets within the TGF-β pathway might provide opportunities for patients with detrimental VDR single nucleotide polymorphisms.

INTRODUCTION

Liver fibrosis and cirrhosis are not only a hallmark of chronic liver disease, but also comprise severe risks for development of hepatocellular carcinoma and acute-on-chronic liver failure. The functional basis for fibrosis and cirrhosis during chronic liver injury is activation of non-parenchymal cells, such as hepatic stellate cells (HSC). These cells secrete extracellular matrix, which serves as a scaffold for cellular reconstitution.[1] Activated HSC produce collagen as well as tissue inhibitors of metalloproteinases that inhibit the degradation of extracellular matrix proteins.[2 3] They further secrete transforming growth factor (TGF)-β, which expands the profibrotic microenvironment by activating neighbouring HSC in paracrine and autocrine fashion.[4] Some studies demonstrated a beneficial effect for vitamin D (VD) as it inhibits TGF-β-induced fibrogenesis in vitro[5 6] and in vivo.[7] Accumulating evidence points to close interaction of VD and TGF-β signalling in animal models of fibrosis and in LX-2 cells.[8]

Increasing numbers of individuals worldwide are overweight or obese.[9] A cluster of conditions, commonly referred to as the metabolic syndrome, occurs in association with obesity. In the liver, the

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metabolic syndrome manifests as non-alcoholic fatty liver disease (NAFLD),[10] which is a major risk factor for type-2 diabetes mellitus, progressive alcoholic liver disease and drug hepatotoxicity.[11 12] Some transplantation centres exclude NAFLD livers as potential allografts,[13] although slightly steatotic organs may have an improved regenerative capacity.[14] Nevertheless, NAFLD and, in particular, non-alcoholic steatohepatitis (NASH) (NAFLD with cell death and inflammation) are major health concerns, as individuals with NASH are likely to develop fibrosis, cirrhosis and hepatocellular carcinoma, and the complex pathogenic mechanisms that drive disease progression remain to be elucidated. The increased level of free fatty acids (FFA) is one of the major drivers of lipotoxicity;[15 16] FFAs induce the formation of reactive oxygen species[17] which have been shown to promote NAFLD progression. Although the specific role of VD in NAFLD progression remains unclear, obese individuals (who are at risk for developing NAFLD) exhibit repressed levels of serum VD.[18] The low levels of VD in obese individuals may be an added risk factor for development of fibrosis and cirrhosis, as there is a loss of anti-TGF-β effects.

In the present study, we evaluated (1) if, and how low VD concentrations promote NAFLD progression; (2) if alterations in the vitamin D receptor (VDR) signalling modulate fibrogenic responses in human NAFLD and primary human HSC (phHSC); (3) if FFAs and VD interact in the fibrogenic response of phHSC; (4) if VD supplementation may inhibit the profibrogenic response by phHSC.

MATERIALS AND METHODS

Patients

Data of liver samples collected from 106 morbidly obese patients undergoing bariatric surgery with biopsy-proven NAFLD were analysed and compared with 10 healthy liver samples. All enrolled patients were physically and ultrasonographically examined, and a complete set of laboratory parameters as well as a liver biopsy were obtained.

The study protocol conformed to the ethical guidelines of the revised Declaration of Helsinki (2000, Edinburgh) and was approved by the Institutional Review Board (Ethics Committee) at the University Hospital of Essen. All patients provided written informed consent before enrolment. Patients' general characteristics are given in table 1.

Isolation of phHSC

For isolating phHSCs, explanted liver grafts or partially resected liver segments were perfused with Hank's Balanced Salt Solution (HBSS) and subsequently with HBSS containing 440–450 U/mL collagenase to digest the connective tissue. The obtained cell suspension was filtered through a 4 μm mesh and washed three times in HBSS. The supernatant was collected and subjected to density gradient centrifugation, prepared as 12.5% iodixanol solution (Optiprep, Axis-Shield, Wädenswil, Austria), overlayed with a 9% iodixanol solution and overlayed with GBSS (Sigma, Steinheim, Germany). The upper layer, between GBSS and 9% density, was carefully transferred into magnetic-activated cell sorting (MACS) buffer and washed twice. The cell suspension was incubated with CD133 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the CD133 HSC were separated by MACS.[19]

SNP PCR and sequencing

Genomic DNA was extracted from peripheral blood mononuclear cells (patients with NAFLD) or directly from frozen cell culture pellets (phHSC) with QIAamp DNA mini Kit (250) for genomic DNA (Qiagen, Hilden, Germany). PCR amplification and nuclease restriction was done according to protocols individually designed for each target single nucleotide polymorphism (SNP). A detailed overview of sequences, enzymes and conditions is given in extended experimental procedures (online material). Randomly chosen samples were sequenced to verify the correct restriction site. Sequencing was performed with Big Dye Terminator v1.1 Sequencing Kit on an ABI PRISM 3100-Avant Genetic Analyzer (ABI Applied Systems, life Technologies, Carlsbad, California, USA).

Table 1 Clinical and biochemical characteristics of patients with non-alcoholic steatohepatitis (NASH) and pre-bariatric surgery for obesity, individuals with NAFL and of healthy controls

	Healthy controls (n=10)	NAFL* (n=56)	NASH* (n=51)
Gender ratio	f:m=3:7	f:m=45:10	f:m=36:15
Age (years)	26±7.6	49.5±2.1	48.6±1.4
Weight (kg)	69.7±16.1	154.2±3.2†	155.9±4.7†
Height (cm)	171.4±11.5	170.6±1.2	171.1±1.2
BMI (kg/m^2)	22.5±1.3	53.2±1.2†	53.1±1.3†
AST (U/L)	24.5±1.7	25.5±2.0	32.4±2.3†‡
ALT (U/L)	28.7±2.8	29.5±2.6	42.9±3.8†‡
Total bilirubin (mg/dL)	0.5±0.3	0.58±0.06	0.52±0.03
Serum cholesterol (mg/dL)	141.3±17.3	200.2±5.0†	193.0±4.4†
HbA1c (%)	3.1±0.8	5.0±0.2	5.9±1.5
Fasting blood glucose		106.0±8.3	112.4±7.4

See also online supplementary table S1.
*Patients were grouped according to serum M30, with a threshold of 275 U/L (M30<275 U/L=NAFL; M30≥275 U/L=NASH) as described in.[23]
†Significant difference (p<0.05) compared with healthy controls.
‡Significant difference (p<0.05) compared with NAFL.
BMI, Body Mass Index; AST, alanine transaminase; AST, aspartate aminotransferase; f:m, female:male; NAFL, non-alcoholic fatty liver.

Statistics

All data of patients with NAFLD are expressed as means±SEM. Data of cell culture experiments represent at least n=4 independent measurements and are expressed as means±SEM unless specified otherwise. Statistical significance (p<0.05) was assessed by a two-tailed unpaired or paired Student t test, respectively. All statistical analyses were performed using GraphPad Prism (V.5.03, GraphPad Software, San Diego, California, USA).

For detailed experimental protocols, measurements and methods, please refer to the online supplementary methods.

RESULTS

Serum VD levels are decreased and hepatic VDR expression is elevated in obese patients with NAFLD

To assess the impact of VD serum levels on NAFLD progression, general characteristics, standard liver parameters and serum concentrations of FFAs, hyaluronic acid and VD were collected from 106 morbidly obese (NAFLD on liver biopsy) patients. In liver biopsies obtained from these patients, the expressions of collagen as well as the VDR were analysed.

Despite histological evidence of NAFLD, 84% of the patients exhibited near-to-normal serum transaminases (<50 U/mL for alanine transaminase and for aspartate aminotransferase). Table 1: please also refer to this table for general patient characteristics. According to histological scoring, 97.5%

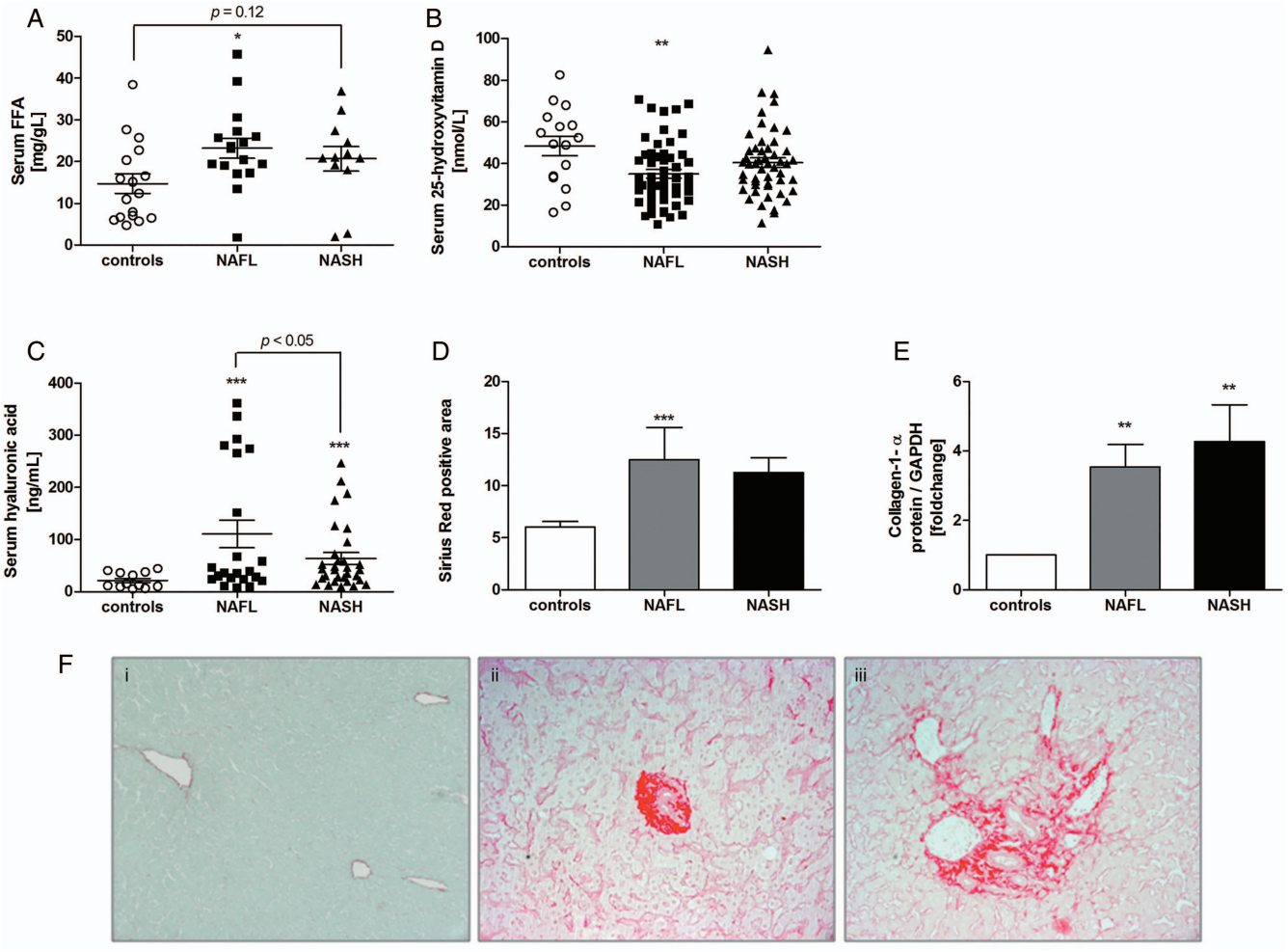

Figure 1 Serum parameters and fibrosis evaluation in patients with non-alcoholic fatty liver disease (NAFLD). The NAFLD cohort showed increased serum concentrations of free fatty acids (FFA) (A) and reduced vitamin D levels (B). Serum hyaluronic acid (C) as a surrogate marker for collagen production, Sirius red staining (D, Fi-Fiii) and collagen1-α protein expression (E) were found elevated. Representative stainings with Sirius Red are shown in panel F, depicting a control liver with slight collagen deposition around vessels (i), liver tissue from a patient with NAFL with increased collagen in the portal area (ii), and from a patient with non-alcoholic steatohepatitis (NASH) with abundant collagen deposition spreading into the periportal areas (iii). Patients with NAFLD were grouped into NAFL or NASH according to the M30 threshold of 275 U/L (<275 U/L=NAFL; ≥275 U/L=NASH), as described by Feldstein *et al.*[23]

exhibited early fibrosis while 2.5% had advanced fibrosis (see online supplementary table S1 for detailed breakdown). Compared with healthy individuals, patients with NAFLD had significantly higher serum FFA but lower serum VD levels, consistent with previously published reports[18 20 21] (figure 1A, B). No effect of the acquisition date (time of the year) on VD levels was found (data not shown). In addition to histological fibrosis assessment, increased serum levels of hyaluronic acid were found (figure 1C), which was accompanied by enhanced deposition of collagen fibres in the liver detected by Sirius red staining and western blot (figure 1D–F). In order to assess whether low serum VD concentrations correlate with hepatic VDR expression, mRNA and protein expression were analysed. While the hepatic VDR gene expression increased, the levels of full-length VDR protein were repressed compared with healthy livers (figure 2A, B). Intriguingly, VDR fragment in liver tissue of patients with NAFLD was significantly increased compared with controls (figure 2C).

Degradation of the VDR protein by the proteasome usually generates a 20 kDa fragment of the actual VDR.[22] To mimic physiological VDR digestion, protein isolates generated from phHSC were exposed to trypsin with or without VD. In all preparations, a decrease in the full-length VDR signal was observed when trypsin was added (see online supplementary figure S1). In the presence of VD, the full-length VDR signal was significantly stronger than without VD. Trypsin-catalysed VDR fragmentation was diminished in the presence of VD. The aggregate data confirm the presence of fibrogenesis in obese individuals with NAFLD. Low serum VD and changes in VDR protein expression suggest that availability of VD to liver cells may be severely impaired in NAFLD, possibly diminishing functioning VDR due to unchecked proteasomal digestion.

FFAs do not enhance fibrogenesis in phHSC

As described above, NAFLD slowly progresses to fibrosis and in the presented cohort, signs for ongoing HSC activation and fibrogenesis were found. Among other mechanisms, FFAs are considered putative 'hits' in the progression of NAFLD. As FFAs influence various metabolic pathways (ie, bile acid metabolism[24]), they might affect VDR expression, thereby leading to a reduction of bioavailable VD in liver tissue. To ascertain whether elevated FFA concentrations, in fact, altered the

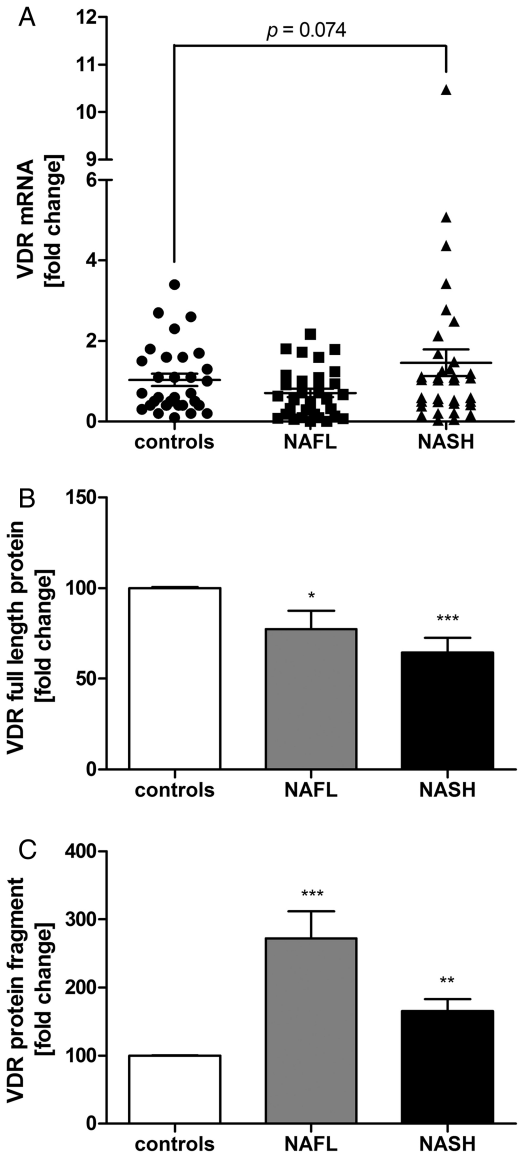

Figure 2 Vitamin D receptor expression in non-alcoholic fatty liver disease (NAFLD) liver. Within the liver tissue of patients with NAFLD, an increase of vitamin D receptor (VDR) mRNA was detected (A). Although the full-length VDR protein (B) was reduced, the fragment of proteasomal VDR degradation was elevated (C). Patients with NAFLD were grouped into NAFL or non-alcoholic steatohepatitis (NASH) according to the M30 threshold of 275 U/L (<275 U/L=NAFL; ≥275 U/L=NASH). Protein expressions of full length VDR or VDR fragment were normalised to glyceraldehyde 3-phosphate dehydrogenase as loading control. See online supplementary figure S1.

expression of VDR and/or enhanced fibrogenesis, phHSC cultures were pretreated with FFAs for 1 h, with or without VD supplementation for 24 h (total incubation time with FFA was thus 25 h). FFA treatment did not enhance fibrogenesis in phHSC (see online supplementary figure S2A–F). By contrast, FFA and VD supplementation appeared to repress profibrogenic genes. Consequently, at least in this in vitro system, fatty acids do not activate phHSC. As an additional known stimulant for HSC proliferation, platelet-derived growth factor (PDGF)-BB was employed. Though, in phHSC, no PDGF-BB-induced activation was observable via the analysed mRNA expressions (see online supplementary figure S2G, H).

VD supplementation counters TGF-β-induced fibrogenic response

TGF-β is a major HSC activator and a potent profibrogenic stimulus. To investigate whether VD can ameliorate TGF-β-induced fibrogenesis in primary human cells to a similar extent as described in various models,[5 7 8] phHSC were stimulated with TGF-β for 24 h and optionally treated with VD. Upon TGF-β treatment, mRNA expression of TGF-β, α-smooth muscle actin (SMA), PDGF, collagen1-α, and VDR were significantly upregulated by phHSC (figure 3A–C, see online supplementary figure S3A, B). Additional treatment with VD repressed PDGF gene expression to almost basal levels (figure 3C) and significantly attenuated the increases in TGF-β (figure 3A) and α-SMA (figure 3B). Protein expression of TGF-β, collagen1-α, and VDR, however, were hardly affected (see online supplementary figure S3C–E). Protein expression of SMAD2, a central regulator of TGF-β-induced processes, was similarly unaffected by TGF-β or VD treatment (figure 3E). As expected, phosphorylation of SMAD2 was upregulated considerably upon TGF-β stimulation and was slightly diminished by VD treatment (figure 3F). It has been suggested that VD effects could be mediated by PDIA3 in addition to VDR.[25] Upon stimulation with VD or TGF-β alone, no effect on PDIA3 mRNA was observable. Cotreatment with both VD and TGF-β led to a significant increase of PDIA3 expression (figure 3G). Changes in VDR expression on activated HSC and/or the timing of treatment in HSC cultures may modulate the extent of VD-mediated reduction in TGF-β-induced effects.

VDR knockdown abolishes VD-mediated anti-TGF-β effect

To evaluate if TGF-β signalling is affected by suppressed hepatic VDR expression, as observed in patients with NAFLD, RNA interference for VDR expression was performed in phHSC. Under optimal conditions, the knockdown efficiency was satisfactory (see online supplementary figure S4A, B) and did not lead to reduced expression of collagen1-α (see online supplementary figure S4C, D) or other tested parameters. Under VDR knockdown, effects of VD on TGF-β were blunted (figure 4A). By contrast, absence of VDR significantly enhanced TGF-β-induced expression of α-SMA (mRNA and protein, figure 4B, D) and pSMAD2 (figure 4F), irrespective of VD treatment. Of note, expression of total SMAD2 was not changed (figure 4E).

Taken together, our data suggest that (1) unimpaired VD–VDR signalling pathway modulates TGF-β signalling, while (2) VDR depletion results in the loss of VD activity (ie, antifibrotic actions). Since some VD–VDR effects might occur early during phHSC activation, we also checked whether VD could influence the initiation of HSC activation.

VDR-independent early effects of VD on SMAD signalling

PhHSC with or without VDR knockdown were treated with TGF-β or TGF-β and VD for 15 min, 30 min or 1 h, respectively. For the entire treatment period, SMAD2 protein expression was initiated and increased in cells irrespective of VDR knockdown (figure 5A, B). Independent of a VDR knockdown, VD treatment significantly reduced SMAD2 expression at 30 min after stimulation only. An even more prominent reduction by VD treatment was observed for phosphorylated SMAD2 at 15 min and 30 min (figure 5C, D). These findings demonstrate that VD is able to counteract TGF-β-induced SMAD2 expression and phosphorylation in a VDR-independent fashion.

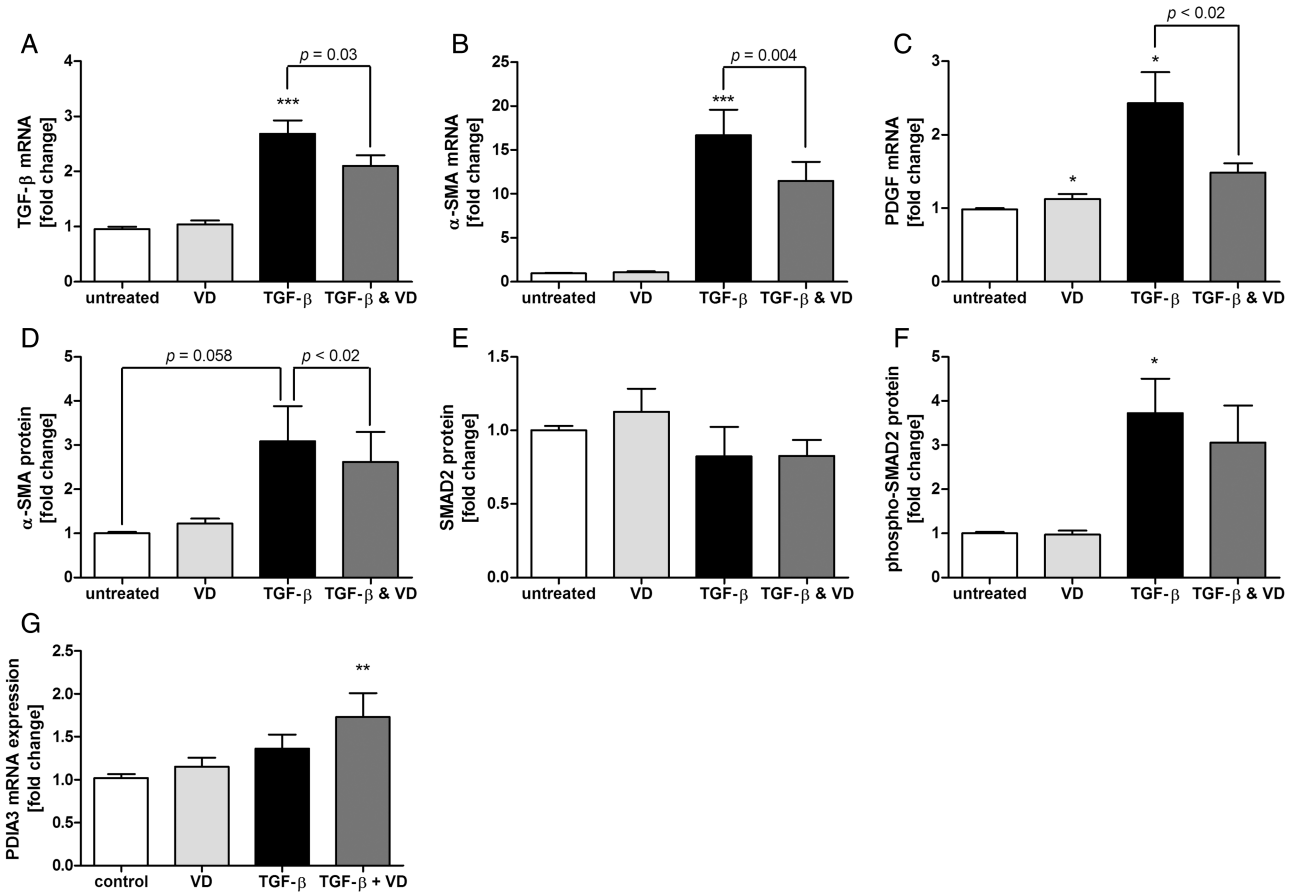

Figure 3 Fibrogenic and activation-related gene expression in primary human hepatic stellate cells (phHSC). phHSC from patients with non-viral liver diseases were isolated and treated with vitamin D (VD) and/or transforming growth factor (TGF)-β. TGF-β-induced expressions of the mRNAs of TGF-β (A), α- smooth muscle actin (SMA) (B) and platelet-derived growth factor (PDGF) (C) were diminished by VD. However, VD treatment only affected the TGF-β-induced protein expression of α-SMA (D). We found no effect of TGF-β or VD on the amount of SMAD2 protein, a central adaptor of TGF-β signalling (E). In contrast, SMAD2 phosphorylation mediated by TGF-β treatment was ameliorated by the addition of VD (F). Expression of the putative VD membrane receptor PDIA3 was increased only upon costimulation with VD and TGF-β (G). All protein expressions were normalised to glyceraldehyde 3-phosphate dehydrogenase as loading control. *, ***=p Versus untreated <0.05, <0.01 or <0.0001, respectively. See online supplementary figures S2 and S3.

Human stellate cell response to TGF-beta and vitamin D is partially dependent on allele distribution of the VDR

Since SNPs within the VDR gene are associated with liver stiffness,[26] a possible sign for liver fibrosis/cirrhosis, we investigated if the interaction of VDR and TGF-β in phHSC may also be associated with known SNPs of the VDR gene. To this end, in a subset of the experiments described above (see figure 3), known SNPs within the VDR gene were analysed (for details, please refer to online supplementary methods). Gene expressions under TGF-β and VD cotreatment (as shown in figure 3: condition TGF-β and VD) were categorised by individual genotype of phHSC for each SNP and evaluated for differential effects by 1-way analysis of variance with Tukey's Multiple Comparison Test. There were four known SNPs, where genotype influenced the reaction to VD treatment after TGF-β stimulation. For each of these four SNPs, there was one (homozygous) genotype, where mRNA expression of the respective target gene was not affected by VD treatment after TGF-β dosage (figure 6). Specifically, after stimulation with TGF-β, VD treatment did not lead to any reduction in TGF-β mRNA (corresponding to figure 3A), if cells harboured homozygous genotypes for A1012G (GG; figure 6A), BsmI G/A (AA; figure 6B) and TaqI T/C (TT; figure 6C). Similarly, TGF-β-induced expression of collagen1-α mRNA (corresponding to online supplementary figure S3A) was unaffected in cells from homozygous donors for Apl A/C (CC; figure 6D) when treated with VD.

Differential expression of profibrogenic genes depending on VDR polymorphisms in patients with NAFLD

As VD-mediated changes to TGF-β-induced gene expressions were significantly affected by VDR polymorphisms in phHSC, we aimed to connect possible genetic variants of VDR to the results found in patients with NAFLD. The same SNPs were analysed, as described above for phHSC. Distribution of genotypes for the analysed SNPs was not equal between phHSC and patients with NAFLD (see online supplementary figure S5).

No associations of the analysed SNPs with fibrosis (METAVIR Score) or with the NAFLD activity score were found. Though, for three SNPs (A1012G, BsmI, Tru9 I) significant differences of VDR mRNA or profibrogenic mRNA expressions were detected (figure 7). In detail, homozygous carriers of the GG allele of the A1012G SNP exhibited significantly lower VDR mRNA than AA homozygous or heterozygous patients (figure 7A). Conversely patients homozygous for the major allele (AA) exhibited significantly lower TGF-β (figure 7B) and α-SMA expression (figure 7C) compared with heterozygous/homozygous GG carriers. Additionally, patients homozygous for AA (at A1012G) exhibited a significant correlation of the mRNA

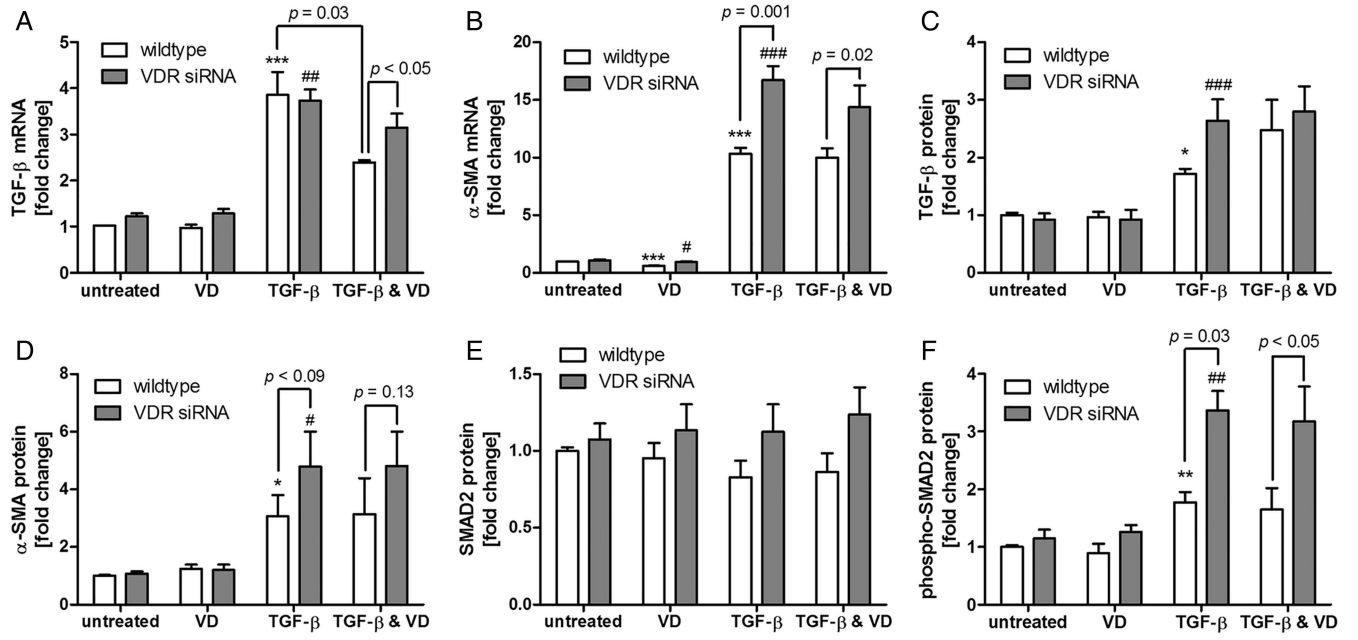

Figure 4 Vitamin D receptor counteracts transforming growth factor (TGF)-β-induced gene expression. By means of siRNA knockdown, primary human hepatic stellate cells (phHSC) were deprived of the vitamin D receptor (VDR). Stimulation of cells with TGF-β induced mRNA and protein expressions of TGF-β (A and C) and α- smooth muscle actin (SMA) (B and D). This effect was independent of VDR knockdown. TGF-β-induced TGF-β mRNA expression was reduced by vitamin D (VD) treatment only in the presence of VDR (A). VDR knockdown led to an increase of the TGF-β-induced expression of α-SMA (B and D). Furthermore, SMAD2 expression was slightly increased in cells with VDR knockdown compared with wild-type phHSC, independent of stimulation with TGF-β or VD (E). TGF-β-enhanced phosphorylation of SMAD2 in wild-type phHSC was stronger in cells with VDR knockdown (F). All protein expressions were normalised to glyceraldehyde 3-phosphate dehydrogenase as loading control. *, **, ***=p versus untreated wildtype <0.05, <0.01 or <0.0001, respectively. #, ##, ###=p Versus untreated VDR siRNA <0.05, <0.01 or <0.0001, respectively. See online supplementary figure S4.

expression of VDR and collagen1-α, which did not occur in the whole patient cohort or other genotypes (figure 7D). Similarly, patients homozygous for the major allele of BsmI (GG) had significantly lower TGF-β mRNA levels in comparison with heterozygous/homozygous AA carriers (figure 7E). For Tru9I, only patients either homozygous for the major allele (GG) or

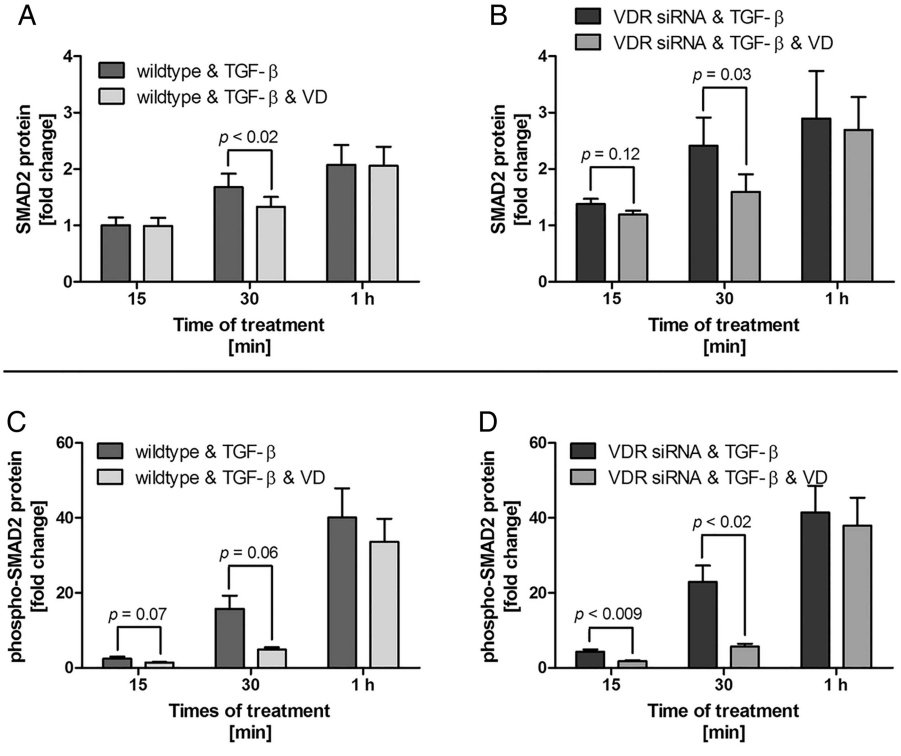

Figure 5 Rapid SMAD2 phosphorylation is receptor-independently reduced by vitamin D. Protein expression of the central transforming growth factor (TGF)-β pathway adaptor SMAD2 increased after TGF-β stimulation from 15 min to 1 h postadministration (A). Treatment with vitamin D (VD) slightly decreased TGF-β-induced SMAD2 expression. The absence of vitamin D receptor (VDR) did not affect this outcome (B). SMAD2 phosphorylation strongly increased due to TGF-β treatment from 15 min to 1 h postadministration (C). Again, and independently of the presence of VDR, VD was able to reduce the degree of SMAD2 phosphorylation (D). All protein expressions were normalised to glyceraldehyde 3-phosphate dehydrogenase as loading control.

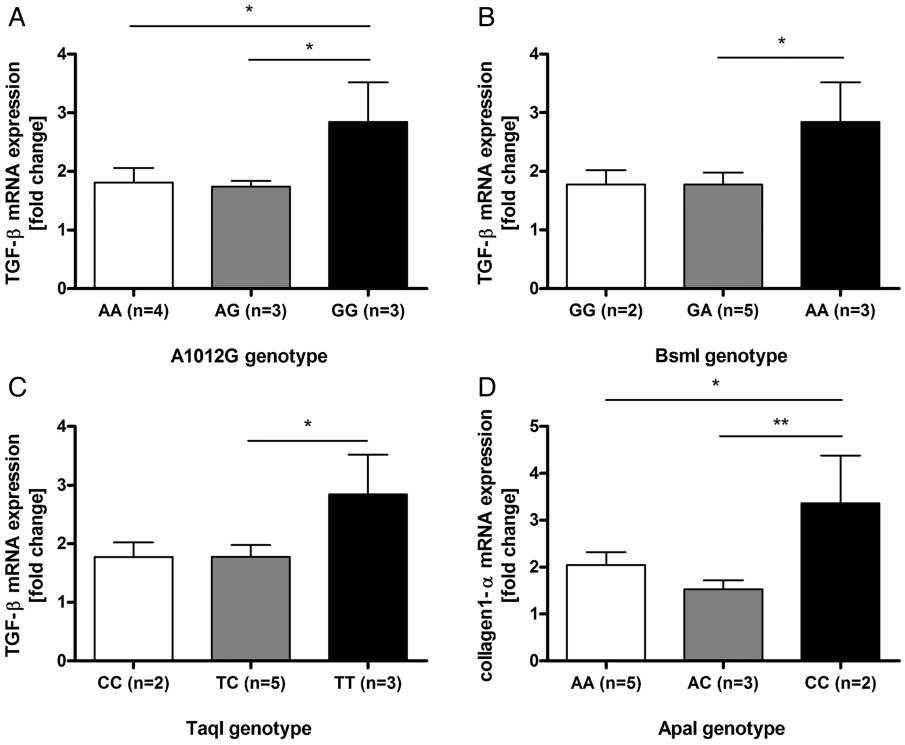

Figure 6 Single nucleotide polymorphisms (SNPs) in the vitamin D receptor (VDR) gene affecting fibrogenic mRNA expression. In a subset of the primary hepatic stellate cells (phHSC) stimulated with transforming growth factor (TGF)-β and treated with vitamin D (VD) (see figure 3 condition TGF-β and VD for complete results) known SNPs of the VDR gene were analysed. TGF-β-induced TGF-β mRNA expression (corresponding to figure 3A) was not reduced due to VD treatment in phHSC with the genotype GG at the A1012G SNP (A), AA at the BsmI SNP (B) and TT at the TaqI site (C). Cells homozygous for CC at the ApaI SNP exhibited no reduction in collagen1-α mRNA expression (D; corresponding to online supplementary figure S3A) when cotreated with TGF-β and VD. See online supplementary figure S5. Genotypes are depicted in white for homozygous major allele, grey for heterozygous and black for homozygous of the minor allele, respectively.

heterozygous (AG) were present in the cohort, probably due to the very low incidence of the minor allele. Homozygous GG carriers exhibited significantly higher mRNA expression of VDR (figure 7F) and α-SMA (figure 7G).

In summary, some known polymorphisms of the VDR gene affect mRNA expression of the VDR itself and of profibrogenic genes in HSC in vitro and in an NAFLD patient cohort. Response to VD treatment may be dependent on the genotype of certain VDR SNPs.

DISCUSSION

HSC are the main cell type responsible for extracellular matrix deposition, which involves activation of HSC and the TGF-β pathway. Earlier studies in intrauterine tumour cells[5] and HL-60 cells[6] focused on the crosstalk between TGF-β signalling as a hallmark of fibrogenesis and vitamin D metabolism. Abramovitch *et al*[7] demonstrated in a rat model of liver fibrosis and rat HSC, that VD can counter fibrosis. More recently, Ding *et al*[8] elegantly unravelled a close genetic interaction of VDR and TGF-β signalling via enhanced VDR-binding to VD response element in the presence of SMAD3 on the same binding site. Chen *et al*[27] added another piece of information, demonstrating interaction of VDR with the putative VD membrane receptor PDIA3. The present study corroborates these findings by showing that VD modulates TGF-β effects in cultured phHSC and that these effects occur during VDR deficiency and is partially dependent on VDR-SNPs.

Our aggregate data show that VD inhibits TGF-β-associated effects in phHSC. However, the weak effects observed might be explained by possible HSC preactivation and, thus, refractoriness to VD and/or VDR signalling. The individual aetiology of liver disease may further affect the response of isolated phHSC to VD treatment. For example, recent findings of Skoien *et al*[28] suggest multiple fibrogenic mechanisms. This may be also indicated by our preliminary studies, which showed that phHSC isolated from patients with viral hepatitis did not respond or responded only marginally to VD treatment (data not shown). Furthermore, it appears that an optimal therapeutic effect may only be achieved in the early stages of disease.

Interaction between VD and TGF-β may also depend on the time of stimulation and/or cellular uptake/binding of VD or TGF-β, respectively. While it has been known for some time that VD induces rapid responses (see review by Yang *et al*[29]), current studies have shown that these may be mediated by the membrane receptor PDIA3.[25] Moreover, the same group showed that PDIA3 and VDR interact on levels of gene expression and receptor binding of VD.[27] This is consistent with our results, as short-term suppression of SMAD2 phosphorylation was independent of VDR expression. PDIA3 mRNA expression itself was induced only upon costimulation by VD and TGF-β in our experiments, which may indicate a crosstalk between PDIA3 and TGF-β signalling. Future work will be needed to ascertain whether early changes in TGF-β-induced effects are mediated by VD-PDIA3 activation. We hypothesise that (very) early VD supplementation may have a beneficial antifibrotic effect. This notion is supported by Abramovitch *et al*,[7] who demonstrated a reduction of thioacetamide-induced liver fibrosis in rats only when VD was administered simultaneously with injury.

Earlier studies reported reduced serum VD levels in liver disease,[30–32] but these focused on individuals with chronic viral hepatitis while neglecting the role of VD in NAFLD. In obese patients with NAFLD, the reduced levels of serum VD may be related to its aberrant storage in adipose tissue.[24] VDR is itself degraded by proteasomal digestion, thereby amplifying a VD-deficient state within HSC.[22] In liver tissue of patients with NAFLD, we found increased VDR mRNA levels, but a reduced amount of protein, which was associated with elevated amounts of VDR fragments. Our data show that VD supplementation may reduce VDR degradation and attenuate TGF-β-induced HSC fibrogenesis. By contrast, VDR knockdown resulted in enhanced TGF-β-induced effects in phHSC, suggesting that VDR—independent of VD—may keep TGF-β signalling in check. This would imply diminished full-length VDR protein in patients with NAFLD to facilitate a profibrotic status of HSC.

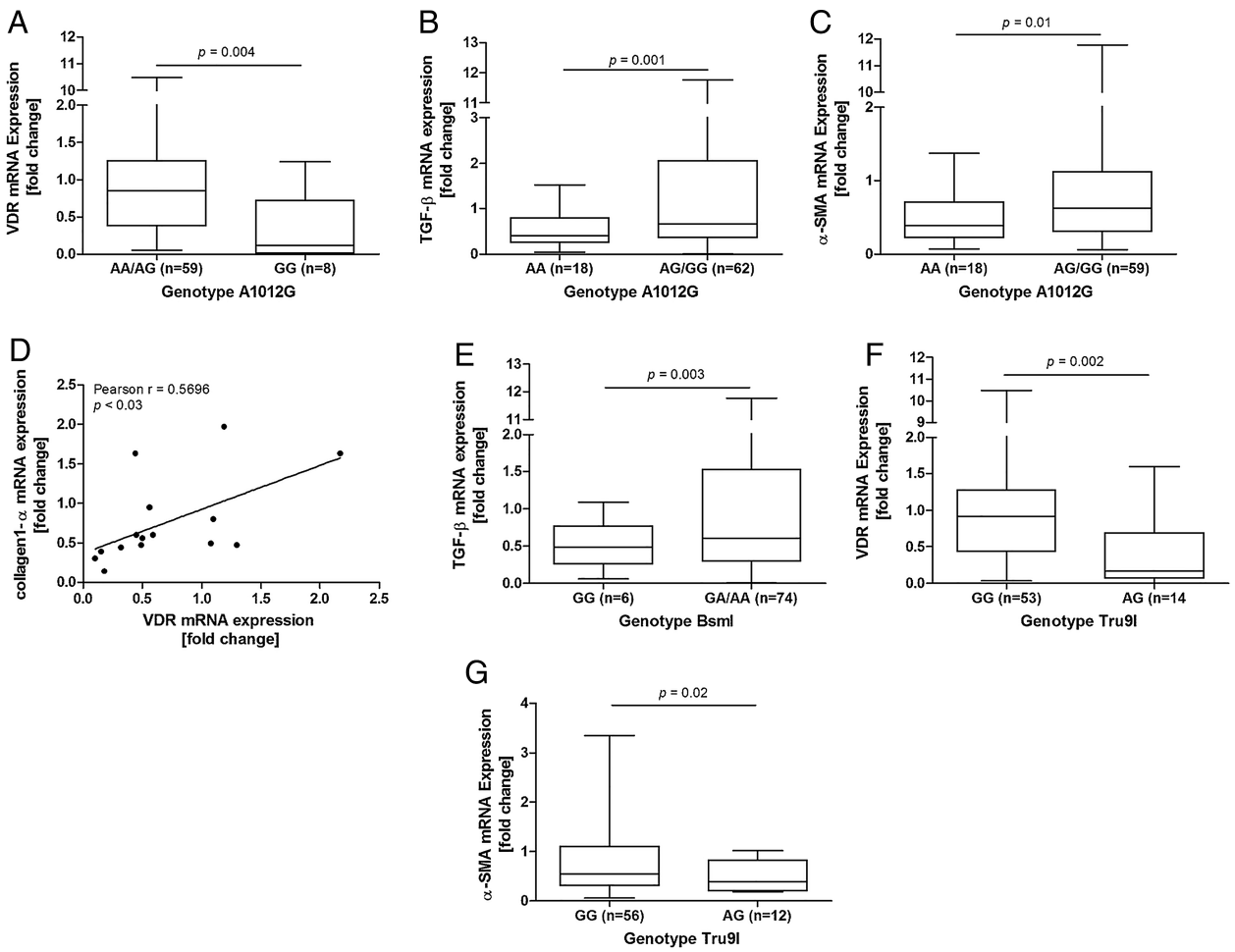

Figure 7 Effects of known polymorphism in the vitamin D receptor (VDR) gene on profibrotic gene expression in patients with non-alcoholic fatty liver disease (NAFLD). Expression levels of profibrotic genes in liver biopsies from patients with NAFLD were stratified according to genotypes of nine known single nucleotide polymorphisms (SNPs) within the VDR gene. Patients homozygous for the minor allele of the A1012G SNP (GG) had significantly lower VDR mRNA expression (A). By contrast, patients homozygous for the major allele of this polymorphism (AA) exhibited lower TGF-β (B) and α-smooth muscle actin (SMA) (C) mRNA levels. In the subgroup of these patients homozygous for A1012G AA, mRNA expressions of VDR and collagen1-α were significantly correlated. This effect did not occur in the whole patient cohort or other genotype combinations. NAFLD patients homozygous for the major allele of the BsmI polymorphism (GG) exhibited significantly lower TGF-β mRNA expression (E). Homozygous GG carriers for the Tru9I SNP had significantly higher mRNA expressions of VDR (F) and α-SMA (G).

The recent findings connecting genetic regulation of TGF-β via SMAD3 and VDR by enhanced binding activity of VDR on SMAD3-bound sites additionally demonstrate a close, though highly complex interaction of these signalling pathways.[8] SMAD3 bound to certain regulatory elements facilitated binding of the VDR and, thus, counter regulation of TGF-β activation in LX-2 cells and rat HSC. VD may be a valuable antifibrotic supplement, but probably in a rather limited time window during early fibrogenesis or in very high doses[33] to counteract permanently upregulated HSC activity. However, recent publications have described novel pharmaceutical VDR agonists,[34 35] which might overcome this problem by stronger binding to the VDR and enhanced activation of the VDR pathway. Moreover, combined treatment with VD and S-Farnesylthiosalicylic acid has been shown to reduce PDGF-induced HSC proliferation in vitro.[36] This synergistic effect may enhance the utility of VD treatment, especially in early stages of liver fibrosis. Further tests will have to demonstrate the efficacy of these components on possible cotreatment options in ongoing fibrosis and cirrhosis.

Differences in the protein stability under low VD conditions may indicate the risk of individual progression from simple steatosis (NAFL) to NASH. Additionally, certain VDR SNP(s) may pinpoint positive responders to VD treatment. Indeed, Grünhage *et al*[26] found that liver stiffness is associated with certain VDR SNPs. Among a French cohort with type 2 diabetes, VDR SNP in intron 8 and exon 9 were associated with obesity.[37] In the current NAFLD cohort, mRNA expression of VDR itself and of profibrogenic genes was significantly affected by presence of homozygous alleles for A1012G (major/minor), BsmI (major) and Tru9I (major). The A1012G polymorphism is located in the promoter region (at a putative GATA-3 binding site), possibly affecting VDR mRNA expression. Strikingly, this SNP also affected the influence of VD on TGF-β-treated phHSC. VD could not ameliorate TGF-β-induced TGF-β expression in phHSC homozygous for GG at the A1012G site. Another SNP affecting fibrogenesis in patients with NAFLD and phHSC response to VD treatment was BsmI. BsmI is considered a silent SNP without change of the amino acid sequence, though possibly affects mRNA stability of the VDR. However, we found reduced TGF-β expression in patients with NAFLD with the major allele for BsmI (GG) and no effect of VD on TGF-β-induced TGF-β expression in phHSC homozygous for the minor allele (AA). The Tru9I polymorphism is located in intron 9 and has currently no clear effect, but may influence

translation. Patients with NAFLD with homozygous major allele for Tru9I (GG) exhibited higher VDR as well as α-SMA mRNA expression, again suggesting a complex interaction of these pathways. The reduction of TGF-β-induced fibrogenesis by VD in phHSC was affected by two additional SNPs. The incidence of some SNPs differed to a significant extent between the phHSC and the NAFLD cohort. This might explain why different SNPs affect the outcome in NAFLD and phHSC. Still, A1012G had similar impact on the VDR-TGF-β interaction in both phHSC and patients with NAFLD and may thus be at a crucial site within the VDR gene. Taken together it becomes clear, that the genetic basis affects expression and function of the VDR in patients with NAFLD. This has to be taken into account when discussing or investigating VD as possible antifibrotic therapy. There may indeed be patients, who could benefit from such a treatment, while others could lack a genetic basis for effectively countering fibrosis via the VD-VDR axis.

Our findings in freshly isolated phHSC and in patients with NAFLD corroborate earlier reports suggesting a potential therapeutic effect of vitamin D on the progression of this disease entity. Future studies will be needed to elucidate how VD interacts with putative receptors, VDR and PDIA3, and their effects on TGF-β signalling, and how genetic VDR variants influence fibrogenic outcomes. The combination of genetic and mechanistic insights could provide us with a rational approach to treating NAFLD.

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Contributors Study concept and design: AB, JPS, RKG, AC. Acquisition of data: AB, SS, MB, MS, IW. Analysis and interpretation of data: AB, JPS, LPB, CJ, RKG, AC. Drafting of the manuscript: JPS, RKG, WKS, AC. Critical revision of the manuscript for important intellectual content: JPS, WKS, RKG, AC. Statistical analysis: AB, JPS, LPB. Obtained funding: GG, AC. Administrative, technical, or material support: SS, MB, LPB, MS, IW, ZM, CJ, RKG. Study supervision: GG, RKG, AC.

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Competing interests None.

Ethics approval Ethics Committee at the University Hospital Essen.

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Original Article

MOLECULAR METABOLISM

Acid sphingomyelinase deficiency in Western diet-fed mice protects against adipocyte hypertrophy and diet-induced liver steatosis

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ABSTRACT

Objective: Alterations in sphingolipid and ceramide metabolism have been associated with various diseases, including nonalcoholic fatty liver disease (NAFLD). Acid sphingomyelinase (ASM) converts the membrane lipid sphingomyelin to ceramide, thereby affecting membrane composition and domain formation. We investigated the ways in which the Asm knockout (Smpd1$^{-/-}$) genotype affects diet-induced NAFLD.
Methods: Smpd1$^{-/-}$ mice and wild type controls were fed either a standard or Western diet (WD) for 6 weeks. Liver and adipose tissue morphology and mRNA expression were assessed. Quantitative proteome analysis of liver tissue was performed. Expression of selected genes was quantified in adipose and liver tissue of obese NAFLD patients.
Results: Although Smpd1$^{-/-}$ mice exhibited basal steatosis with normal chow, no aggravation of NAFLD-type injury was observed with a Western diet. This protective effect was associated with the absence of adipocyte hypertrophy and the increased expression of genes associated with brown adipocyte differentiation. In white adipose tissue from obese patients with NAFLD, no expression of these genes was detectable. To further elucidate which pathways in liver tissue may be affected by Smpd1$^{-/-}$, we performed an unbiased proteome analysis. Protein expression in WD-fed Smpd1$^{-/-}$ mice indicated a reduction in Rictor (mTORC2) activity; this reduction was confirmed by diminished Akt phosphorylation and altered mRNA expression of Rictor target genes.
Conclusion: These findings indicate that the protective effect of Asm deficiency on diet-induced steatosis is conferred by alterations in adipocyte morphology and lipid metabolism and by reductions in Rictor activation.

Keywords Ceramide; NAFLD; Rictor; Western diet

1. INTRODUCTION

Western societies are experiencing an increase in the prevalence of obesity, which leads to a parallel increase in the prevalence of related diseases, such as the metabolic syndrome, type II diabetes, and nonalcoholic fatty liver disease (NAFLD) [1]. NAFLD is primarily associated with excessive accumulation of fat in the liver and ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which is accompanied by cell death, tissue inflammation, and a high risk of the development of fibrosis, with further progression to cirrhosis or hepatocellular carcinoma [2]. Obesity is also associated with the development of systemic inflammation and insulin resistance as part of the metabolic syndrome, which promotes the progression of NAFLD. In particular, cytokines derived from adipose tissue, also known as adipokines, play an important role in this systemic disease [3].
Hypertrophy of adipose tissue alters adipokine secretion and enhances the release of free fatty acids (FAAs) into the circulation [4,5]. This effect seems to be caused specifically by visceral adipocyte

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hypertrophy, which is associated with serum inflammation markers and altered adipokine secretion, indicating an interaction between adipose tissue and liver tissue in the metabolic syndrome [6–8]. Thus, these changes in the concentrations of circulating lipid components and in (hepato)cellular lipid metabolism seem to play an important role in the formation of NAFLD.

Ceramides are sphingolipids that are important components of cell membranes. The ceramide content of a membrane affects fluidity and the signal transduction pathways regulating cell differentiation or apoptosis. The effects of ceramide are mediated by the formation of ceramide-rich microdomains that facilitate the trapping and clustering of receptors and, thereby, the induction of apoptosis [9,10]. Altered ceramide levels in cell membranes have been associated with many human diseases, such as neurodegenerative and skin disorders, pulmonary and cardiovascular diseases, and hormonal disorders and liver diseases [10–14].

Ceramide can be derived from membrane-bound sphingomyelin that is cleaved by an enzyme called acid sphingomyelinase (ASM), coded by the *SMPD1* gene. The conversion of sphingomyelin to ceramide within cell membranes is essential for various signaling pathways [15,16]. ASM deficiency has also been discussed as a possible mechanism in the development of obesity, the metabolic syndrome, diabetes, and various liver diseases, such as steatosis or fibrosis [14,17–19]. It has also been reported that sphingolipids, especially ceramide, play a pivotal role in obesity and the metabolic syndrome [20,21]. Boini and colleagues found that excessive accumulation of sphingolipids, ceramide, and the metabolites of ceramide contribute to the development of obesity and associated kidney damage in mice fed a high-fat diet (HFD). Treatment with amitriptyline, a functional ASM antagonist, diminishes both the steatosis associated with HFD and the accumulation of fat in this murine model.

A protective effect against diet-induced liver steatosis has also been observed in Asm knockout (Smpd1$^{-/-}$) mice and in Asm and low-density lipoprotein receptor (Ldlr) double knockout mice [22,23]. Although the results of these studies indicate that ASM deficiency may protect from diet-induced liver steatosis by reducing autophagy and endoplasmic reticulum stress, many other processes may also be involved. Therefore, we aimed for a broader view of processes potentially affected by ASM knockout, processes that may even protect from steatosis. In addition, we specifically investigated the contribution of adipose tissue to the development of NAFLD. We found that reductions in the activation of rapamycin-insensitive companion of mTOR (Rictor, or mTORC2) in the liver and alterations in adipocyte physiology may contribute to the protective effect of Smpd1$^{-/-}$ against diet-induced steatosis.

2. MATERIAL AND METHODS

2.1. Animals and sample collection

Four- to six-week-old C57Bl/6 and Smpd1$^{-/-}$ mice [11] were fed a standard diet (SD; n = 6 animals per group) or a Western diet rich in carbohydrates and fat (WD; TD.88137, details are given in Supplementary Table 1; ssniff Spezialdiäten, Soest, Germany) *ad libitum* for six weeks (n = 6 animals per group). Food intake was not measured. After six weeks, mice were sacrificed, blood was drawn from the *vena cava* and centrifuged, and serum was stored at −80 °C. Total protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were measured with a Spotchem II system (Akray, Kyoto, Japan). Liver tissue and white adipose tissue were collected for isolation of RNA and protein and for histopathological processing. All mice were bred and housed in the Central Animal Facility (ZTL) of the University Hospital Essen, University of Duisburg-Essen (Germany), according to the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). All procedures were approved by the State Agency for the Protection of Nature, the Environment, and Consumers, North Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen; LANUV NRW).

2.2. Histopathology and sample handling

Liver and adipose tissues were stored in a 4.5% formalin solution, embedded in paraffin, and sectioned. Staining was performed as previously described [24]. The size of adipocytes was determined in paraffin-embedded sections stained with hematoxylin and eosin (H&E), as described previously [6].

Liver and adipose tissues for the isolation of RNA and protein were immediately frozen in liquid nitrogen. Total RNA was isolated by TRIzol extraction (Invitrogen, Darmstadt, Germany) and purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with the QuantiTect RT kit (Qiagen) with 1 μg of total RNA.

2.3. Patients

The study protocol conformed to the revised Declaration of Helsinki (Edinburgh, 2000) and was approved by the local Institutional Review Board (Ethik-Kommission am Universitätsklinikum Essen; file number 09-4252). Before enrollment, all patients provided written informed consent for participation in the study.

Data from liver and matched adipose tissue samples were collected from morbidly obese patients with biopsy-proven NAFLD who were undergoing bariatric surgery. The samples were analyzed and compared with four non-steatotic liver samples. All enrolled patients underwent physical and ultrasound examinations, a complete set of laboratory studies, and liver biopsy. Supplementary Tables 2 and 3 present detailed demographic and clinical information.

Subjects reporting excessive alcohol consumption (>20 g/day for men or >10 g/day for women) and those with other known causes of secondary fatty liver disease (e.g., viral hepatitis, metabolic liver disease, toxic liver disease) were excluded from the study. An experienced pathologist (HAB) used the NAFLD Activity Score (NAS) to determine the degree of NAFLD in wedge liver biopsy samples obtained during a surgical procedure and stained with H&E [25]. The mean adipocyte diameter in representative slides was calculated from multiple (>50) individual measurements of adipocyte diameter (ImageJ, National Institutes of Health, Bethesda, MD, USA), as previously described [6].

2.4. Quantitative real-time polymerase chain reaction

Gene expression levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR) with succinate dehydrogenase complex subunit A (*Sdha*) as a reference gene; oligonucleotide sequences are presented in Supplementary Table 4. Relative gene expression was calculated from the threshold cycles in relation to the reference gene and to untreated controls. Reactions were performed on a CFX96 Touch qPCR System (Biorad Laboratories, Munich, Germany), as previously described [26].

2.5. Quantitative proteome analysis

Mass spectrometry–based proteome analysis was performed according to a previously described protocol [27,28]. Briefly, liver tissue samples were lysed in 30 mM Tris HCl, 2 M thiourea, 7 M urea, and 4% CHAPS detergent (pH 8.5) and subjected to in-gel digestion with trypsin. Trypsinized peptides were then analyzed online with an Ultimate 3000 RSLCnano system coupled to an Orbitrap Elite mass

spectrometer (both from Thermo Fisher Scientific, Bremen, Germany). Liquid chromatography—tandem mass spectrometry (LC-MS/MS) data were analyzed with Proteome Discoverer software (version 1.4; Thermo Fisher Scientific) for protein identification and Progenesis LC-MS (version 4.1; Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) for protein quantification based on two unique peptides per protein. Statistical significance was determined with ANOVA and Tukey's honestly significant difference test for post hoc analysis with RStudio software (version 0.99; RStudio Inc., Boston, MA, USA). Proteins with fold changes higher than 1.5 or lower than −1.5 and with p values lower than 0.05 were considered to be differentially expressed between two experimental groups and were used for further analysis. In total, six biological replicates were analyzed per experimental group.

2.6. Pathway analysis

Data from quantitative proteomics experiments were analyzed with Ingenuity® Pathway Analysis (IPA; QIAGEN, Redwood City, CA, USA; www.qiagen.com/ingenuity). The proteins differentially expressed between the experimental groups were analyzed for enriched canonical pathways and associated upstream regulators. Upstream regulators and canonical pathways with z-scores higher than 2 were considered to be activated, and those with z-scores lower than −2 were considered to be inhibited.

2.7. Western blotting

Protein lysates generated during proteomics experiments were used for Western blotting. Protein concentrations were assessed with Pierce BCA kit (Thermo Fisher, Braunschweig, Germany), and 30 μg of total protein was separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed according to standard procedures with the following primary antibodies: Nrf2/Nef2l2 (Cell Signaling Technology, Danvers, MA, USA); pERK (Cell Signaling Technology); or ERK (Santa Cruz Biotechnology, La Jolla, CA, USA). For a loading control, we used β-Actin (13E5) (Cell Signaling Technology).

2.8. Biochemical assays and enzyme-linked immunosorbent assays

Biochemical assays for FFAs in serum were performed with the Fatty Acid Quantification Kit (Biovision/BioCat GmbH, Heidelberg, Germany). Serum leptin and adiponectin levels were determined with Quantikine mouse enzyme-linked immunosorbent assay (ELISA) kits for leptin and adiponectin (R&D Systems Inc., Minneapolis, MN, USA).

2.9. Ceramide content in liver tissue

Liver samples were subjected to cryoconservation in liquid nitrogen and were lysed in TN3 buffer. The samples were extracted in $CHCl_3$:CH_3OH:1N HCl (100:100:1; v/v/v), and the organic phase was collected and dried. Diacylglycerol (DAG) was degraded in 0.1 N methanolic potassium hydroxide (KOH) at 37 °C for 60 min; the samples were re-extracted, and the organic phase was dried and resuspended in 20 μL of a detergent solution containing 7.5% (w/v) n-octyl glucopyranoside and 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid (DETAPAC). The samples were sonicated for 10 min in a bath sonicator to facilitate micelle formation. Phosphorylation of ceramide was initiated by the addition of 70 μL of a buffer consisting of 0.1 M imidazole/HCl (pH 6.6), 0.1 M NaCl, 25 mM $MgCl_2$, 2 mM ethylene glycol tetraacetic acid (EGTA), 2.8 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), 10 μCi [32P]γATP, and DAG kinase, according to the vendor's instructions (GE Healthcare Europe, Freiburg, Germany). The samples were incubated for 60 min at room temperature; the incubation was stopped by extraction in 1 mL $CHCl_3$:CH_3OH:1N HCl (100:100:1; v/v/v), 170 μL buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES [pH 7.2]), and 30 μL of a 100-mM ethylenediaminetetraacetic acid (EDTA) solution. The organic phase was collected, dried, and dissolved in 20 μL $CHCl_3$:CH_3OH (1:1; v/v). Lipids were separated on Silica G60 thin-layer chromatography (TLC) plates with $CHCl_3$:CH_3OH:CH_3COOH (65:15:5; v/v/v); each plate was dried and analyzed by autoradiography. Ceramide spots were identified by co-migration with a C16-ceramide standard, scraped from the plate, and quantified by liquid scintillation counting. Ceramide amounts were determined from a standard curve using C16-ceramide.

2.10. Statistical analysis

Statistical significance was determined by two-way ANOVA with the Bonferroni correction for multiple comparisons with Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). For patient data, unpaired two-tailed *t*-tests were performed. Statistical significance set at the level of $p \leq 0.05$. If not otherwise stated, all data are presented as means ± SEM. Correlations between parametric data were measured with the Pearson product-moment correlation coefficient; correlations between nonparametric data were measured with Spearman's rank correlation coefficient.

3. RESULTS

3.1. A Western diet does not aggravate steatosis in Smpd1$^{-/-}$ mice

Consuming excess calories in the form of fat and carbohydrates leads to higher serum levels of FFA because the storage capacity of adipocytes is exceeded [7], resulting in fat accumulation in liver tissue and, subsequently, NAFLD. To test the role of Asm in diet-induced steatosis of liver tissue, we fed WT mice and Smpd1$^{-/-}$ mice either a SD or WD *ad libitum* for as long as six weeks so that we could focus on the early stages of NAFLD. Histologic assessment of liver tissue sections confirmed robust steatosis in WD-fed WT mice (Figure 1). SD-fed Smpd1$^{-/-}$ mice exhibited a mild steatotic phenotype (Figure 1B), probably because of the accumulation of sphingomyelin. However, this phenotype was not aggravated by WD feeding (Figure 1). Steatosis was not as severe in Smpd1$^{-/-}$ mice as in WD-fed WT mice. In addition, the liver-to-body weight ratio (LBWR) was significantly higher in Smpd1$^{-/-}$ mice than in corresponding WT mice ($p = 0.0003$; Figure 1E). In particular, the LBWR was higher in WD-fed Smpd1$^{-/-}$ mice than in WD-fed WT mice ($p < 0.001$).

3.2. Serum indicators of liver damage are unaltered by short-term steatosis

Liver damage was determined by liver serum indicators. No significant differences in routine liver damage variables were found between the groups (Suppl. Fig. 1). Albumin production was significantly lower in Smpd1$^{-/-}$ mice than in WT mice (Suppl. Fig. 1E; $p < 0.04$), and WD feeding significantly reduced serum albumin levels independent of genotype ($p < 0.02$). Short-term WD feeding did not affect serum markers of liver damage in WT or Smpd1$^{-/-}$ mice.

3.3. Western diet feeding increases profibrotic gene expression but not fibrosis in liver tissue of Smpd1$^{-/-}$ mice

Steatosis may progress to steatohepatitis and finally to fibrosis and cirrhosis, changes that are indicated by the expression of typical

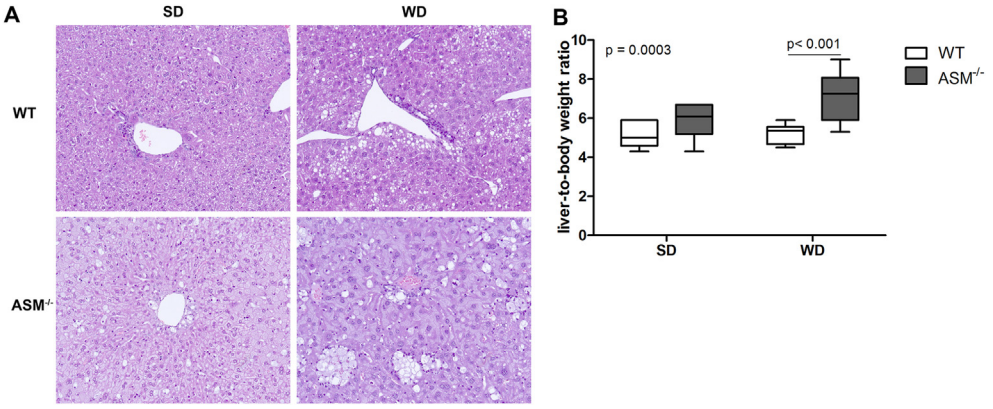

Figure 1: Acid sphingomyelinase deficiency ameliorates diet-induced hepatocellular steatosis. (A) After 6 weeks of eating either a standard diet (SD) or a Western diet (WD), liver tissue from acid sphingomyelinase deficient ($Smpd1^{-/-}$) mice and C57BL/6 mice (wild-type, WT) was sectioned and stained with hematoxylin and eosin (H&E). SD-fed $Smpd1^{-/-}$ mice exhibited slight steatosis, which seems to be mostly based on formation of foam cells (macrophages with lipid accumulation). Although WD-fed WT animals exhibited robust steatosis of hepatocytes, WD did not aggravate hepatic steatosis in $Smpd1^{-/-}$ mice. Foam cell formation seemed to be slightly increased. (B) $Smpd^{-/-}$ animals exhibited a slightly higher liver-to-body weight ratio, which increased with WD consumption. This effect did not occur in WT animals.

markers such as transforming growth factor beta 1 (Tgfβ1), collagen type I-alpha 1 (Col1a1), and α-smooth muscle actin (Acta2). Our results show that mRNA expression of both the profibrotic growth factor Tgfβ1 (Suppl. Fig. 2A) and Col1a1 (Suppl. Fig. 2B) was significantly higher in liver tissue from WD-fed $Smpd1^{-/-}$ than in WT mice. No alterations were observed in the expression of Acta2, a marker of hepatic stellate cell activation, or in collagen deposition, as assessed by Sirius Red staining (Suppl. Fig. 2C, D).

3.4. Altered adipocyte physiology may contribute to diminished steatosis in $Smpd1^{-/-}$ mice fed a Western diet

To identify the molecular mechanisms that protect $Smpd1^{-/-}$ mice from steatosis, we investigated visceral white adipose tissue. Adipose tissues store energy in the form of fat in adipocytes. In addition, white adipose tissue has been shown to be an important endocrine organ, producing and secreting specific adipokines that influence liver inflammation and the progression of NAFLD [6,29]. Thus, we investigated the adipose tissue and factors released from adipocytes in the present model. $Smpd1^{-/-}$ animals either lacked or had a reduced amount of adipose tissue [30]. All of the data presented below on the expression of mRNA in adipocytes and adipose tissue refer to WT animals and those $Smpd1^{-/-}$ animals that had at least a minimal amount of adipose tissue (SD, $n = 4$; WD, $n = 5$).

Although WD consumption led to a significant increase in the size of adipocytes in WT mice, it did not lead to adipocyte hypertrophy in $Smpd1^{-/-}$ mice (Figure 2A,B). The levels of FFAs in sera from WD-fed WT mice were higher than those in SD-fed WT mice (Figure 2C), although this effect was not statistically significant. $Smpd1^{-/-}$ mice exhibited higher serum levels of FFAs than did WT mice ($p < 0.05$), but WD feeding did not further increase serum FFA levels in $Smpd1^{-/-}$ mice. Serum adiponectin concentrations were similar in SD-fed WT and $Smpd1^{-/-}$ mice (Figure 2D). WD feeding led to a slight, statistically significant reduction irrespective of genotype ($p = 0.008$). Levels of leptin, an adipokine that signals satiety, were increased in WD-fed WT mice, but this increase was not observed in WD-fed $Smpd1^{-/-}$ mice ($p < 0.01$; Figure 2E).

These findings show that WD-fed $Smpd1^{-/-}$ mice do not exhibit adipocyte hypertrophy or hyperleptinemia.

3.5. Levels of mRNA expression of genes associated with browning of adipocytes and cell cycle control are elevated in $Smpd1^{-/-}$ mice

On the basis of the observation of altered adipocyte physiology and adipokine release, we hypothesized that the expression of genes associated with lipid metabolism is altered in adipose tissue of $Smpd1^{-/-}$ mice. Thus, we investigated the expression of metabolism-associated genes and fatty acid transporters. In adipose tissue from $Smpd1^{-/-}$ mice, the mRNA expression of caveolin 1 (Cav1), a central fatty acid transporter of adipocytes, was lower (n.s.) than in WT animals, irrespective of diet (Figure 3A). WD consumption increased the expression of solute carrier family 27 member 1 (Slc27a1) mRNA ($p = 0.004$; Figure 3B), independent of genotype.

The expression of genes catalyzing molecules within β-oxidation, e.g., carnitine palmitoyltransferase 1a (*Cpt1a*; Figure 3C; $p = 0.03$) and peroxisome proliferator—activated receptor gamma coactivator 1 alpha (*Ppargc1a*; Figure 3D; $p < 0.04$), was significantly higher in $Smpd1^{-/-}$ mice than in WT mice, independent of dietary group. Of note, elevations in the expression of both genes have been associated with differentiation of brown adipocytes [31—34]. The mRNA expression of cyclin-dependent kinase inhibitor 1A (Cdkn1a), which is involved in cell cycle arrest, senescence, and adipogenesis [34,35], was significantly higher in $Smpd1^{-/-}$ animals ($p < 0.04$; Figure 3E). WD consumption enhanced the expression of apolipoprotein E (apoE), a molecule important for the catabolism of triglycerides and lipoproteins [36], in adipose tissue from WT and $Smpd1^{-/-}$ mice ($p < 0.05$, Figure 3F). Although the effect was larger in adipose tissue from $Smpd1^{-/-}$ mice than in adipose tissue from WT mice, this difference was not statistically significant.

These findings show that the mRNA expression profile of adipose tissue from $Smpd1^{-/-}$ mice differs significantly from that of adipose tissue from WT mice, in particular regarding genes of adipocyte differentiation.

3.6. Expression of genes that may protect from NAFLD is lacking in human adipose tissue

To relate the described findings in $Smpd1^{-/-}$ mice to humans, we analyzed the ceramide content of liver tissue from obese subjects and control subjects (Supplementary Table 2). Additionally, we analyzed

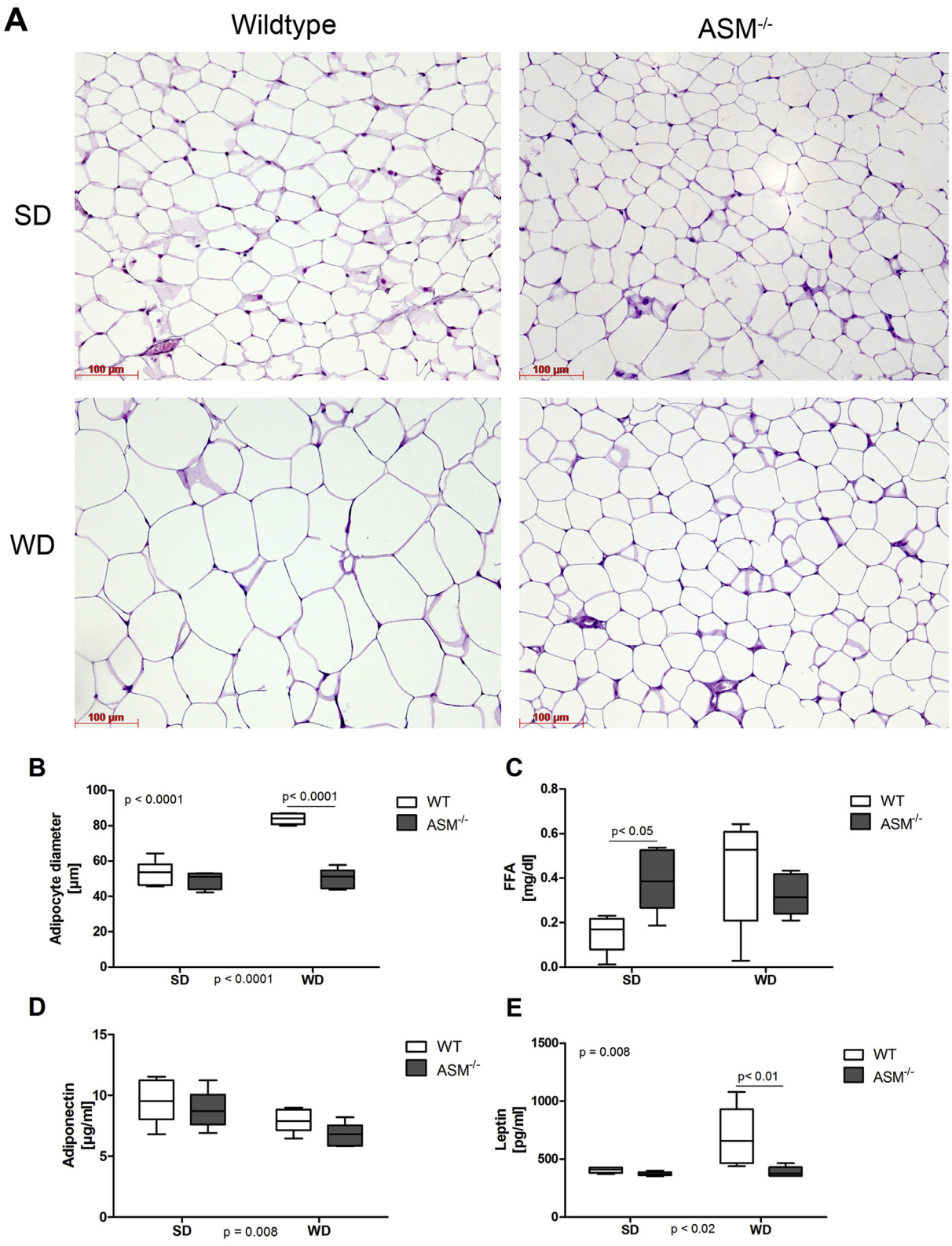

Figure 2: Acid sphingomyelinase deficiency protects from diet-induced adipocyte hypertrophy. (A) Adipose tissue from acid sphingomyelinase–deficient ($Smpd^{-/-}$) mice and C57BL/6 mice (wild-type, WT) fed either a standard diet (SD) or a Western diet (WD) for 6 weeks was sectioned and stained with hematoxylin and eosin (H&E). WD consumption led to hypertrophy of adipocytes in WT mice but not in $Smpd^{-/-}$ mice. (B) Mean adipocyte diameter was significantly larger in WD-fed WT mice than in WD-fed $Smpd^{-/-}$ mice. (C) Although SD-fed $Smpd^{-/-}$ mice exhibited higher serum concentrations of free fatty acids (FFAs), no statistically significant differences were observed between the WD-fed groups because of higher FFA concentrations in WT mice. (D) Serum adiponectin concentrations were lower in WD mice than in SD mice, irrespective of genotype. (E) WD-fed WT mice exhibited substantial hyperleptinemia; this condition did not occur in $Smpd^{-/-}$ mice.

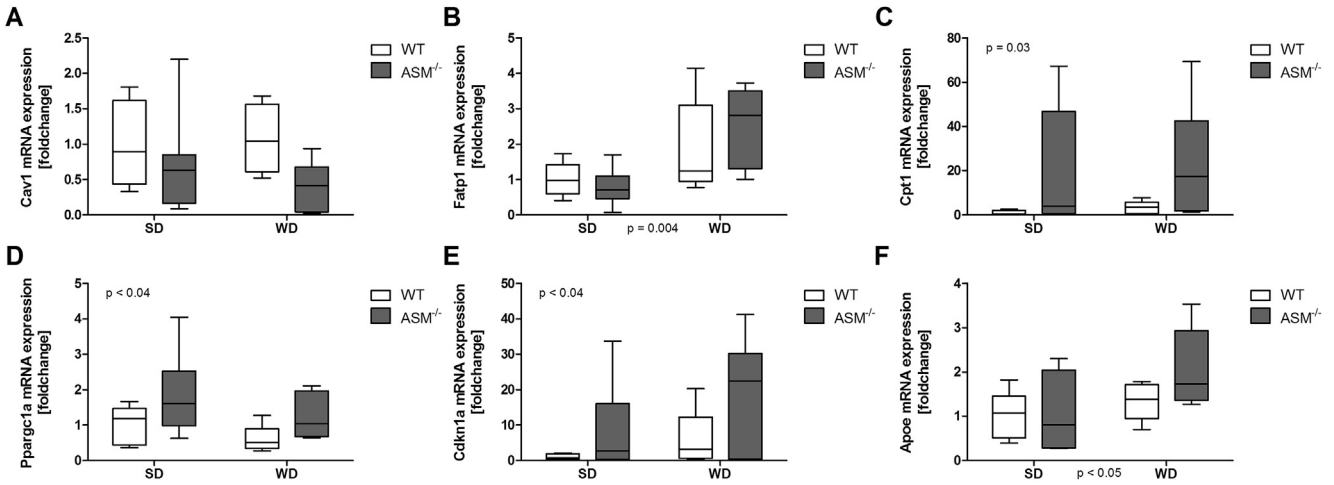

Figure 3: Acid sphingomyelinase deficiency affects adipocyte expression of genes related to beta-oxidation and cell differentiation in adipose tissue. RNA was isolated from adipose tissue from acid sphingomyelinase–deficient mice ($Smpd^{-/-}$) mice and C57BL/6 (wild-type, WT) mice fed either a standard diet (SD) or a Western diet (WD) for 6 weeks. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess mRNA expression. (A) Caveolin1 (Cav1) expression did not differ between the groups. (B) WD consumption increased the expression of the fatty acid transporter solute carrier family 27 member 1 (Slc27a1, fatty acid transport protein 1), irrespective of genotype. The expression of genes associated with β-oxidation (C, carnitine palmitoyltransferase 1a, *Cpt1a*; D, peroxisome proliferation-activated receptor, gamma, coactivator 1 alpha, *Ppargc1a*) and adipocyte differentiation (E, cyclin-dependent kinase inhibitor 1A, *Cdkn1a*) was significantly higher in $Smpd^{-/-}$ mice than in WT mice, independent of dietary group ($p = 0.03$; $p < 0.04$). (F) WD consumption enhanced apolipoprotein E (apoE) expression in adipose tissue from WT and $Smpd^{-/-}$ mice.

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gene expression in adipose and liver tissues from obese patients (Supplementary Table 3). The ceramide content in liver tissue was significantly lower in obese subjects than in healthy control subjects (Suppl. Fig. 3A). Obese subjects were grouped by maximum diameter of adipocytes (<125 μm or ≥125 μm). As we have previously shown, the NAS was significantly lower in patients with adipocyte diameters smaller than 125 μm compared to patients with adipocyte diameters of 125 μm or larger (Suppl. Fig. 3B). The mRNA expression of *PPARGC1A*, *CDKN1A*, and *APOE*, the expression of which has been found to be increased in adipose tissues of Smpd$^{-/-}$ mice, was not detectable in human adipose tissue, although these genes were expressed in liver tissue (not shown). *CPT1A* mRNA expression in adipose tissue did not differ by size of adipocytes (Suppl. Fig. 3C). Because we found no changes in the expression of genes of adipocyte differentiation in human adipose tissue, we assessed the expression of abhydrolase domain containing 5 (*ABHD5* or *CGI-58*), a lipid droplet—associated protein that may be involved in long-chain fatty acid oxidation. Expression of *ABHD5* was significantly higher in the patients with larger adipocytes (≥125 μm; Suppl. Fig. 3D). However, this difference was not found in liver tissue (not shown). This finding suggests that human adipocyte biology in obesity does not resemble the situation in Smpd1$^{-/-}$ mice. However, more severe liver injuries are observed in patients with larger adipocytes.

3.7. Quantitative proteome analysis of liver tissue identifies Rictor as a possible downstream mediator of the antisteatotic effects of the Smpd$^{-/-}$ genotype

To explore the differences between the genotypes in the effects of SD or WD on a wide unbiased range of regulatory processes, we determined protein regulation by label-free quantitative proteome analysis with mass spectrometry. Relevant alterations in canonical pathways between WD-fed WT and WD-fed Smpd1$^{-/-}$ mice are shown in Figure 4A. We performed IPA to identify upstream regulators in sets of altered proteins. The strongest indications of affected regulators (Figure 4B, Supplementary Table 5) were detected for Rictor (down-regulated in WD-fed Smpd1$^{-/-}$ mice; Figure 4C), nuclear factor, erythroid derived 2, like 2 (Nfe2l2; up-regulated in Smpd1$^{-/-}$ mice), leptin (up-regulated in Smpd1$^{-/-}$ mice), peroxisome proliferator—activated receptor alpha and gamma (Ppara and Pparg; both down-regulated in Smpd1$^{-/-}$ mice). To confirm these findings, we assessed the protein expression of Nfe2l2, which is involved in the activation of genes regulating the inflammatory response, in liver tissue with Western blot analyses (Figure 4D). In liver tissue from WD-fed Smpd1$^{-/-}$ mice, Nfe2l2 expression was lower than in liver tissue from WD-fed WT mice. Rictor regulates the activation of Akt by phosphorylating Akt at Serin473. Western blot analysis for phosphorylation of Akt-Ser473 showed that Akt phosphorylation was lower in WD-fed Smpd1$^{-/-}$ mice than in WD-fed WT mice (Figure 4E). Both quantitative proteome analysis and Akt phosphorylation suggest lower Rictor activity in WD-fed Smpd1$^{-/-}$ mice.

3.8. Confirmation of altered Rictor signaling by qRT-PCR assessment of relevant target genes

To further support the findings of quantitative proteome analysis, we analyzed the mRNA expression levels of various target genes for altered regulatory pathways or factors. Selected target genes for Rictor were proteasome subunit alpha type 1 (*Psma1*), ATPase H^+ transporting V0 subunit D1 (*Atp6v0d1*), nicotinamide adenine dinucleotide (NADH) dehydrogenase 1 alpha subcomplex 6 (*Ndufa6*), and fatty acid binding protein 5 (*Fabp5*). Because Rictor inhibits the expression of these targets, we expected to find increased expression of these genes with reduced Rictor activation in Smpd1$^{-/-}$ mice, as observed for quantitative proteome analysis (Figure 4C). The aldo-keto reductase family 1, member A1 (*Akr1a1*) and glucan (1,4-alpha-), branching enzyme 1 (*Gbe1*) genes served as exemplary target genes for Nfe2l2 with regard to mRNA expression. For Ppara, we chose the targets acyl-CoenzymeA oxidase 1, palmitoyl (*Acox1*), fatty acid synthase (*Fasn*), *Fabp4*, *and Fabp5*. *Acox1*, *Fasn*, and *Fabp4* served as targets for Pparg. We observed no statistically significant alterations in the mRNA expression of *Pparg*, *Acox1*, *Fasn*, *Psma1*, *Akr1a1*, or *Gbe1* (Suppl. Fig. 4). In contrast, the expression of *Fabp4* and *Fabp5* was significantly higher in the liver of WD-fed Smpd1$^{-/-}$ mice than in the liver of WD-fed WT mice (Figure 5C,D; $p < 0.05$ for *Fabp4*; $p < 0.01$ for *Fabp5*). The expression of *Ndufa6* did not differ significantly between Smpd1$^{-/-}$ mice and WT mice irrespective of diet (Figure 5A). *Atp6v0d1* expression was slightly lower in SD-fed Smpd1$^{-/-}$ mice than in SD-fed WT mice (Figure 5B). WD consumption further reduced *Atp6v0d1* expression in Smpd1$^{-/-}$ mice but increased *Atp6v0d1* expression in WT animals. Genes associated with fatty acid metabolism or transport were differentially expressed in WD-fed WT mice and WD-fed Smpd1$^{-/-}$ mice and may be associated with the protective effect of diet-induced steatosis. Furthermore, the regulation of downstream targets and effectors seems to be consistent with diminished Rictor activation in WD-fed Smpd1$^{-/-}$ mice.

Our observations of gene expression were tested in part in human NAFLD (in liver tissue of obese subjects; Suppl. Table 3). The mRNA expression of *FABP4* and *FABP5* was significantly higher in patients with adipocytes larger than 125 μm in diameter (Figure 5E,F). Moreover, the expression of *FABP4* and *FABP5* exhibited statistically significant positive correlations with NAS as a measure of liver injury (*FABP4*, Spearman $r = 0.4146$, $p = 0.003$; *FABP5*, Spearman $r = 0.4976$, $p = 0.0004$).

4. DISCUSSION

ASM deficiency results in the lysosomal storage of sphingolipids, which disrupts a variety of signal transduction pathways [37] and causes various cardiovascular, neurological, infectious, metabolic, and hepatic diseases [10,23]. ASM inhibition has been described as a potential therapeutic agent that may reduce the progression of liver diseases [23] and may reduce liver toxicity due to defective transduction of death receptor and apoptosis signals [9,22,38]. Although some effects of ASM knockout have been demonstrated in the context of hepatic steatosis, fibrosis, diabetes, and fat disorder [20,22,23,39], the interaction of the liver with adipose tissue and its derived cytokines, also known as adipokines, has not been described. With this study, we aimed to determine whether the interaction between liver tissue and adipose tissue in Asm-deficient mice protects them from diet-induced steatosis apart from other mechanisms, such as endoplasmic reticulum (ER) stress and autophagy. We found that adipocyte hypertrophy, which is associated with reduced leptin release and altered adipocyte expression profiles, does not occur in WD-fed Smpd1$^{-/-}$ mice. In addition, we found that Rictor may be a regulator within the liver and may confer protection from diet-induced liver steatosis in a murine model.

As has been previously shown [30], we found that Smpd1$^{-/-}$ mice are resistant to WD-induced steatosis after a feeding duration of 6 weeks and that, instead of accumulating lipids by hepatocytes (steatosis), Asm-deficient mice accumulate lipids within foam cells in various tissues. We also observed elevated mRNA expression of early pro-fibrogenic genes, although we found no signs of actual collagen deposition or stellate cell activation. These seemingly contradictory

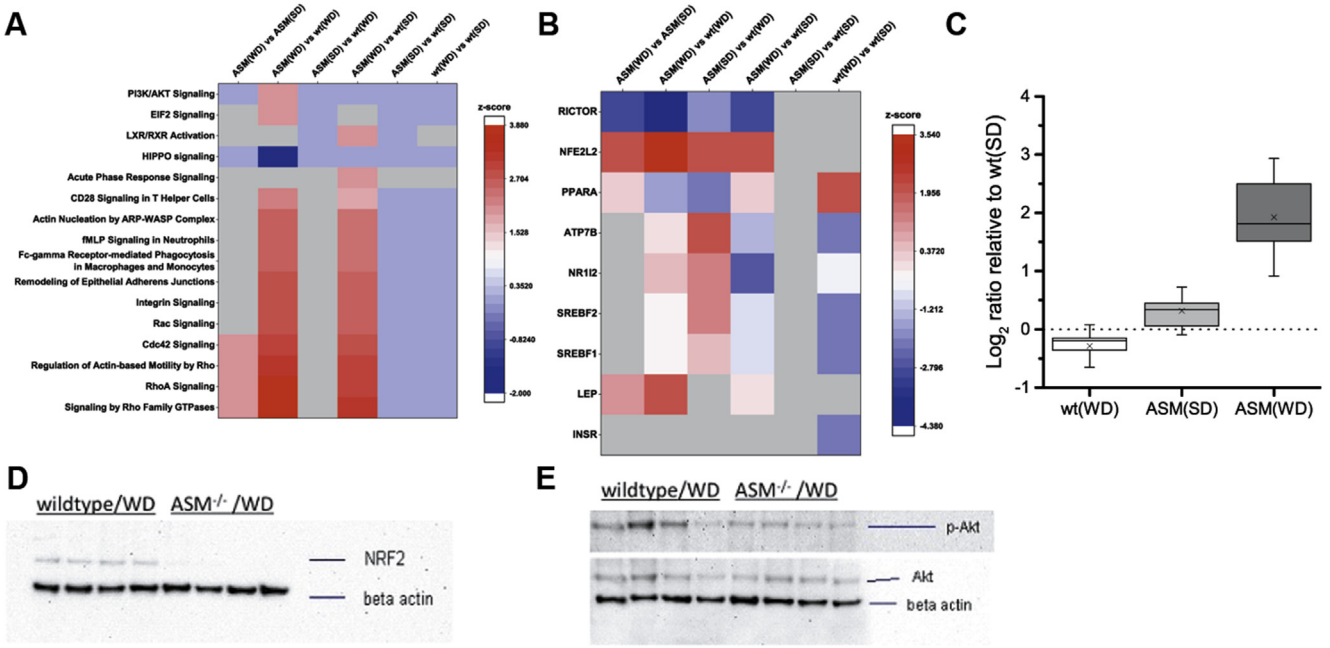

Figure 4: Reduced Rictor activity in liver tissue is associated with protection from diet-induced liver steatosis by acid sphingomyelinase knockout. Quantitative proteome analysis was performed to identify alterations between acid sphingomyelinase–deficient mice ($Smpd^{-/-}$) and C57BL/6 (wild-type, WT) mice consuming a standard diet (SD) or a Western diet (WD). (A) Among canonical pathways whose activation was stronger in WD-fed $Smpd^{-/-}$ mice than in WD-fed WT mice, Rho signaling pathways were prominent. (B) Ingenuity® Pathway Analysis, performed to detect upstream regulators from sets of altered proteins, found that the activity of rapamycin-insensitive companion of mTOR (Rictor) was lower and that of nuclear factor, erythroid derived 2, like 2 (Nfe2l2/Nrf2) was higher in WD-fed $Smpd^{-/-}$ mice than in WD-fed WT mice (see also Supplementary Table 5). (C) Combined Log_2 ratio of genes found altered in quantitative proteome analysis and identified as downstream targets of Rictor related to SD-fed WT mice. A significant upregulation of Rictor target genes was observed for WD-fed $Smpd^{-/-}$ mice, indicating reduced Rictor activity (targets are negatively regulated by Rictor). (D) Protein expression of Nfe2l2/NRF2 in liver tissue, as determined by Western blot, was lower in WD-fed $Smpd^{-/-}$ mice than in WD-fed WT animals. (E) Because Rictor regulates the activation of Akt by phosphorylation at Serin473, we performed Western blot analysis for Akt-Ser473 phosphorylation. We found that Akt phosphorylation was lower in WD-fed $Smpd^{-/-}$ mice than in WD-fed WT mice, a finding confirming the findings of quantitative proteome analysis.

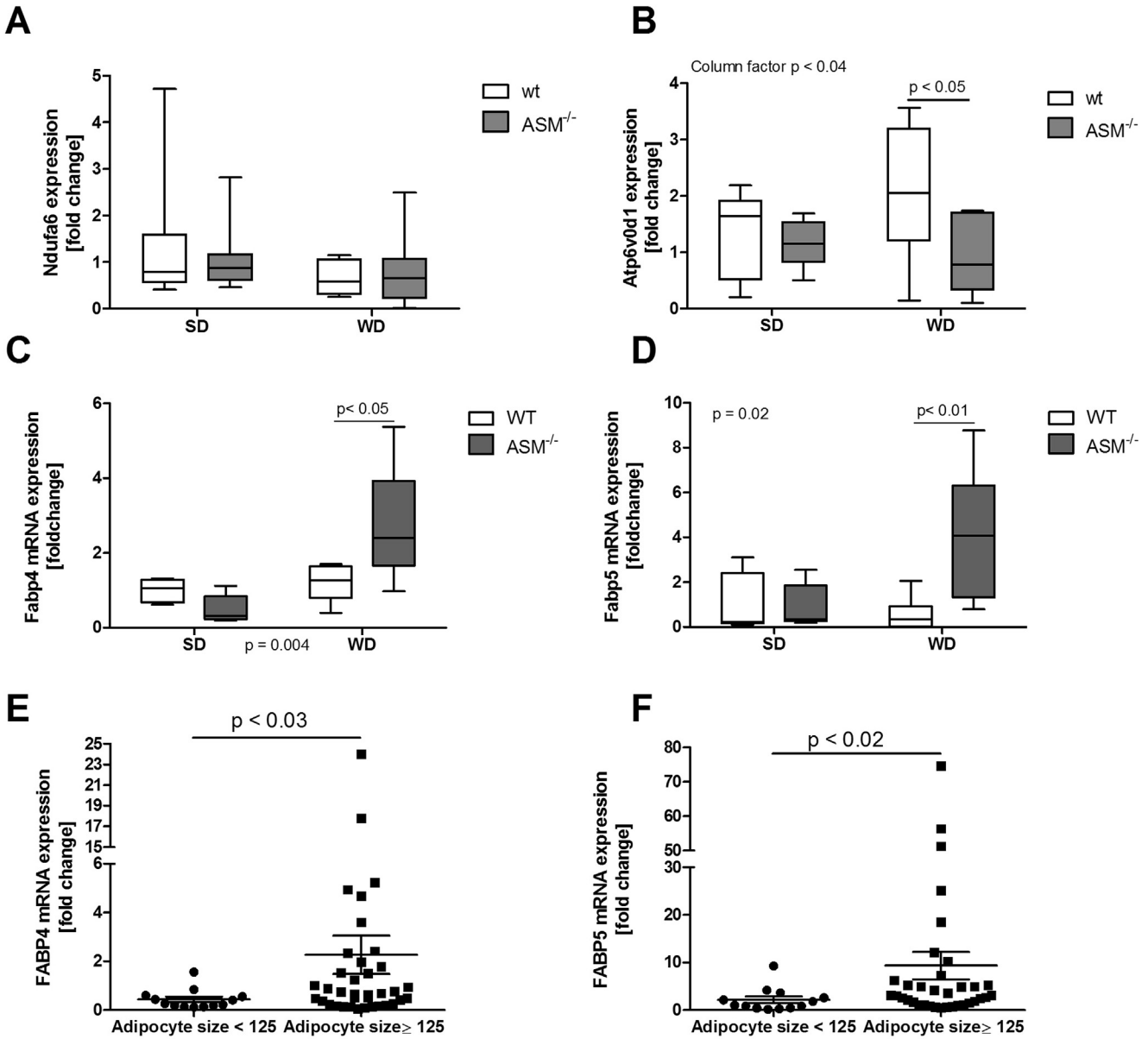

Figure 5: Alterations in the expression of genes related to fatty acid transport and oxidation in liver tissue are associated with protection against diet-induced liver steatosis by acid sphingomyelinase knockout. We used quantitative real-time polymerase chain reaction (qRT-PCR) to quantify RNA in liver tissue from acid sphingomyelinase–deficient ($Smpd^{-/-}$) mice and C57BL/6 (wild-type, WT) mice fed a standard diet (SD) or a Western diet (WD). (A) *Ndufa6* mRNA expression was not significantly different between $Smpd^{-/-}$ mice and WT mice irrespective of diet. (B) Expression of ATPase H^+ transporting V0 subunit D1 (*Atp6v0d1*), a target of Rictor, whose activation was found to be altered, according to the results of quantitative proteome analysis, was slightly lower in SD-fed $Smpd^{-/-}$ mice than in SD-fed WT mice. WD consumption diminished *Atp6v0d1* expression in $Smpd^{-/-}$ mice but increased its expression in WT mice; this difference was statistically significant. (C, D) The expression of *Fabp4* (target gene of Ppara and Pparg) and *Fabp5* (target gene of Rictor, Ppara, and Pparg) mRNA target genes was significantly higher in the liver of WD-fed $Smpd^{-/-}$ mice than in the liver of WD-fed WT mice. (E, F) In liver tissue samples from obese subjects (see Suppl. Table 3), mRNA expression of *FABP4* and *FABP5* was significantly higher in patients with maximal adipocyte size (≥125 μm).

results may indicate an early response to dietary stress that may not lead to established fibrosis. These observations are in line with previous studies demonstrating that the Smpd1$^{-/-}$ genotype exerts a protective effect against fibrosis and HSC activation with longer durations of HFD consumption [23,39,40]. Because the genes activated early in fibrosis were clearly up regulated, the feeding period we chose may have been too short to allow the formation of fibrosis. Additional studies are necessary for addressing our contradictory findings related to fibrogenesis in Smpd1$^{-/-}$ mice. Taken together, however, our findings show that Smpd1$^{-/-}$ mice are protected from diet-induced liver steatosis.

Current lines of research have brought attention to the interaction between adipose tissue and the liver as a driving force for NAFLD and the progression to NASH. The current study showed that WD-fed Smpd1$^{-/-}$ mice do not exhibit adipocyte hypertrophy in visceral adipose tissue. This observation is supported by the finding of reductions in body weight among patients with Niemann-Pick disease, a condition that results from deficient ASM activity. A study using an Asm$^{-/-}$/ldrl$^{-/-}$ double knockout mouse model found that these animals exhibit lower body weight and do not accumulate fat in white adipose tissue after eating a WD for ten weeks [22]. Because ldlr$^{-/-}$ animals exhibit hypertrophy in visceral adipocytes when eating a HFD, our findings indicate that their resistance to adipocyte hypertrophy is probably due to Asm deficiency alone. In addition, WD-induced increases in the release of leptin were found in WT animals but not in Smpd1$^{-/-}$ mice. This finding was associated with an mRNA expression profile that suggested altered adipocyte differentiation or proliferation, hinting at a "browning" of adipocytes. Indeed, increased energy expenditure or fatty acid oxidation could explain the resistance of these mice to both adipocyte hypertrophy and liver steatosis. In contrast, the mRNA expression of fatty acid transporters and inflammatory genes in adipose tissue did not differ significantly between WD-fed Smpd1$^{-/-}$ mice and WD-fed WT mice. These findings show that one mechanism that may protect Smpd1$^{-/-}$ mice from liver steatosis is an altered visceral adipocyte profile leading to diminished leptin release and changes in fatty acid metabolism.

To translate these findings from Smpd1$^{-/-}$ mice to humans, we analyzed ceramide content in the liver and mRNA expression in adipose tissue from obese subjects. Liver ceramide content was lower in obese subjects than in control subjects of normal weight, a finding that corresponds to diminished ASM activity. Adipose tissue expression of *PPARGC1A*, *CDKN1A*, and *APOE* mRNA was not detectable, irrespective of the extent of adipocyte hypertrophy. When patients were grouped by adipocyte size, we observed no difference in the mRNA expression of *CPT1*. The lack of expression of *PPARGC1A* and *APOE* indicates diminished breakdown or mitochondrial oxidation of lipids. Unaltered *CPT1* expression hints at unchanged β-oxidation in adipocytes despite increased availability of long-chain fatty acids. Unfortunately, it was not possible to obtain exact measures of adipose tissue metabolic rates or fatty acid composition. CGI-58 is associated with lipid droplets and activates lipolysis by adipocyte triglyceride lipase (ATGL) in adipocytes. Because mRNA expression of *CGI-58* was significantly higher in adipose tissue from patients with larger adipocytes, lipolysis may be affected not by adipocyte size but rather by oxidation of fatty acids. This finding merits deeper analysis of human adipocyte biology in various states of nutrient (over-)supply.

Because it is still not clear what causes the protection from WD-induced steatosis in Smpd1$^{-/-}$ mice, we performed quantitative proteome analysis for unbiased identification of alterations in proteins and pathways. IPA showed that Rictor, among other regulators, was downregulated because of many significantly regulated target proteins. Reductions in Akt phosphorylation at Serin473 indirectly confirmed this assumption. The mRNA expression of target genes was also partly elevated in Smpd1$^{-/-}$ mice, supporting the finding of lower Rictor activation in WD-fed Smpd1$^{-/-}$ mice than in WD-fed WT mice. Quantitative proteome analysis showed that many proteins associated with fatty acid transport, fatty acid oxidation, and glucose metabolism were affected, converging on the few known pathways in IPA.

These findings show that the reductions in the activity of Rictor may contribute to the protective effect of the Smpd1$^{-/-}$ genotype against steatosis in WD-fed mice. Currently, the only known connection between ceramide metabolism and mTORC2 has been demonstrated in yeast [41]. This finding suggests that Rictor and mammalian target of rapamycin complex 2 (mTORC2) are promising targets in ceramide- or ASM-associated diseases.

Rictor is an integral part of mTORC2. Both mTORC1 and mTORC2 are central regulators of multiple cellular functions. In particular, mTORC2 has been associated with metabolic functions such as autophagy, glycolysis, and lipogenesis, but also with actin cytoskeleton formation and insulin/Akt signaling. A liver-specific knockout of Rictor induces hepatic insulin resistance and leads to diminished phosphorylation of Akt and Pkc and, subsequently, to reductions in glucose flux and maturation of Srebp-1c [42]. A diminished association of Rictor with mTORC and diminished mTORC2 activity has also been linked to hepatic glucose intolerance [43]. Similar findings have been observed with adipose tissue—specific Rictor knockout, which leads to impaired tolerance of glucose and insulin, hyperinsulinemia, and increased body size [44,45]. A more recent study on adipose tissue—specific RICTOR knockout mice demonstrated that hepatic glucose and lipid metabolism were altered [46]. Moreover, HFD feeding in adipose tissue—specific RICTOR knockout mice did not lead to increased body weight or aggravate insulin resistance. It was concluded that adipose tissue ablation of RICTOR mimics the effects of HFD and that RICTOR dependent *de novo* lipogenesis in adipose tissue could be an early target during development of insulin resistance. This is in line with our finding that the effects of WD on adipose tissue were reduced in an Smpd1$^{-/-}$ model, leading to reduced activity of Rictor in the liver. Another possible explanation may be that reduction of RICTOR in the lineage of brown adipocytes protects against WD-induced obesity and seems to increase energy expenditure at thermoneutrality [47], although Rictor knockout also inhibited the differentiation of brown adipocytes [47]. Thyroid hormone signaling also seems to co-activate FOXO1 target genes *via* deacetylation of RICTOR [48]. Diminished RICTOR activity leads to reduced AKT phosphorylation, FOXO1 phosphorylation, and subsequently increased nuclear localization and DNA binding of FOXO1. These recent findings suggest mTORC2/RICTOR as an important regulating element in different metabolic signaling pathways. RICTOR might also be a promising target to prevent tumor formation in metabolic conditions [49]. Knockout of FASN in a murine model of hepatocarcinogenesis and in human cell lines abolished AKT-dependent carcinogenesis. This was associated with diminished mTORC2 activity and Rictor knockout could reproduce the effect of FASN knockout. Taken together current findings of other groups and our own data suggest RICTOR as a possibly valuable target to counter metabolic alterations in adipose tissue and liver.

In summary, we have shown that the protective effect of ASM knockout could be associated with altered adipocyte morphology and metabolism. Although it is known that the effects of RICTOR knockout in specific tissues may lead to insulin resistance and lipolysis, reductions in RICTOR activation in adipose tissue could be beneficial with regard to energy expenditure and the reduction of adipocyte hypertrophy. Unfortunately, it was not within the scope of the presented work to

assess RICTOR activity in murine or human adipose tissue. It remains to be determined whether diminished but not completely abrogated hepatic mTORC2 activity could protect against WD-induced steatosis.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Study concept and design: SS, JPS, EG, AC; Acquisition of data: SS, JPS, DAM, MS, SJ, LW, ACar, LPB; Analysis and interpretation of data: SS, JPS, DAM; Drafting of the manuscript: SS; Critical revision of the manuscript for important intellectual content: JPS, EG, AC; Statistical analysis: JPS, DAM; obtained funding: GG, AC; Technical or material support: HAB, BS, EG; Study supervision: AC.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.03.002.

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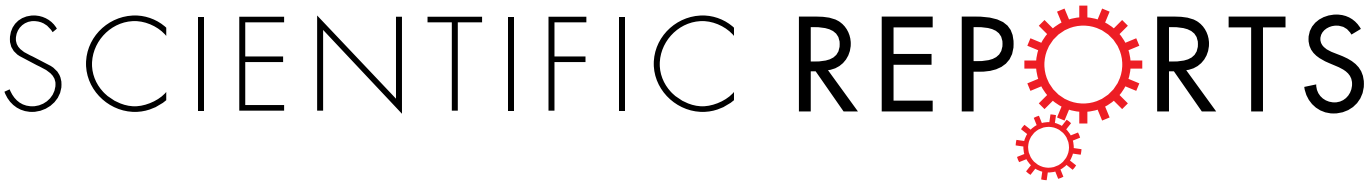

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Normal liver enzymes are correlated with severity of metabolic syndrome in a large population based cohort

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Key features of the metabolic syndrome are insulin resistance and diabetes. The liver as central metabolic organ is not only affected by the metabolic syndrome as non-alcoholic fatty liver disease (NAFLD), but may contribute to insulin resistance and metabolic alterations. We aimed to identify potential associations between liver injury markers and diabetes in the population-based Heinz Nixdorf RECALL Study. Demographic and laboratory data were analyzed in participants (n = 4814, age 45 to 75y). ALT and AST values were significantly higher in males than in females. Mean BMI was 27.9 kg/m² and type-2-diabetes (known and unkown) was present in 656 participants (13.7%). Adiponectin and vitamin D both correlated inversely with BMI. ALT, AST, and GGT correlated with BMI, CRP and HbA1c and inversely correlated with adiponectin levels. Logistic regression models using HbA1c and adiponectin or HbA1c and BMI were able to predict diabetes with high accuracy. Transaminase levels within normal ranges were closely associated with the BMI and diabetes risk. Transaminase levels and adiponectin were inversely associated. Re-assessment of current normal range limits should be considered, to provide a more exact indicator for chronic metabolic liver injury, in particular to reflect the situation in diabetic or obese individuals.

The liver as central organ for glucose and lipid metabolism is strongly affected by the metabolic syndrome. Thus, non-alcoholic fatty liver disease (NAFLD) represents the hepatologic consequence of Western lifestyles. NAFLD is the most common chronic liver disease in industrialized nations with a prevalence of up to 30% and probably the most common cause of elevated liver enzymes[1,2]. According to the National Health and Nutrition Examination Survey, the prevalence of major causes of chronic liver diseases remained stable from the years 1988 to 2008 except for NAFLD. Incidence of NAFLD increased steadily during this time, contributing to the burden of chronic liver disease in the United States[3]. NAFLD is associated with obesity as well as diabetes and could be not only a result of insulin resistance (IR) and metabolic syndrome but rather a major contributor to systemic IR[4,5]. NAFLD ranges from simple hepatic steatosis (NAFL) to non-alcoholic steatohepatitis (NASH) with hepatocyte ballooning and inflammatory components. Simple hepatic steatosis generally has a good prognosis, however NASH can lead to cirrhosis and hepatocellular carcinoma (HCC) in up to 15% of patients[6]. Metabolic

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	Male (n = 2395)	Female (n = 2419)
age (y)	59.7 ± 7.8	59.6 ± 7.8
height (cm)	174.8 ± 6.8	162.1 ± 6.2***
weight (kg)	86.2 ± 13.2	72.6 ± 13.8***
BMI (kg/m^2)	28.2 ± 4.0	27.7 ± 5.2***
waist circumference (cm)	100.3 ± 10.8	88.5 ± 12.9***
Diabetes n (%)	418 (17.5)	238 (9.8)***
smoking status		
current	614 (25.6%)	514 (21.3%)
former	1105 (46.1%)	557 (23.0%)
never	669 (27.9%)	1345 (55.6%)

Table 1. Demographic data of the analyzed study cohort (Heinz Nixdorf Recall). ***$p < 0.0001$ *vs.* males.

injury to the liver will probably constitute a major burden for health care systems worldwide in the near future.

By now the metabolic syndrome is a common term and high cholesterol, high blood pressure, or obesity raise the attention of internists regarding the risk for cardiovascular or metabolic complications. Though, despite the very obvious link of NAFLD and metabolic syndrome, the awareness of metabolic liver injury and its connection to cardiovascular risk remains low. Several studies have shown, that normal transaminase levels do neither exclude NAFLD (or NASH) nor progression to advanced fibrosis[7,8]. However, aminotransferases are regarded as the main alarm signal for liver diseases or injury before enrolling further diagnostic. Previous studies already discuss the idea to lower normal values and to take metabolic factors into account, especially body mass index (BMI) and sex, which have a significant effect on ALT values[9–11]. Since the origin of obesity may be based in early childhood, hepatologists already claim to revise the values in pediatrics[11]. Still the classic liver serum parameters (ALT, AST, GGT), elevated in most chronic and acute liver diseases, may not be ideal markers for liver injury in NAFLD.

Liver function is crucial for glucose- and fatty acid metabolism and vice versa[12]. Enzymes and signaling pathways involved in hepatic glucose homeostasis contribute to insulin sensitivity. Reciprocally peripheral IR and lipolysis contribute to hepatic steatosis[13,14]. Other factors known to contribute to systemic IR and to development of NAFLD are adipokines such as adiponectin. Low adiponectin is associated with obesity, IR, and severity of NAFLD[15–17]. Vitamin D (VD) is also discussed to play an important role in the pathophysiology of IR and VD serum concentrations above 25 ng/ml were associated with a lower risk of type 2 diabetes[18].

In the present study we aimed to investigate in a large population based cohort, whether serum transaminase levels within normal ranges are associated with metabolic risk and prevalence of diabetes. Associations of adiponectin, systemic inflammation, and VD levels with transaminases and diabetes were analyzed. Previously we were able to generate effective classification models by machine learning techniques[19–21]. Thus, we also aimed to build a model for diabetes prediction from non-invasive parameters.

Results

Demographic data. The initial cohort included 4814 participants (2419 female), aged 45 to 75 years (female 59.6 ± 7.8y, male 59.7 ± 7.8y). Detailed demographics are given in Table 1. Men exhibited significantly higher weight (86.2 ± 13.2 kg; $p < 0.0001$) and waist circumference (100.3 ± 10.8 cm; $p < 0.0001$) than women. The mean BMI of the cohort was 27.9 kg/m^2, suggesting large parts of this population to be overweight or obese. Both women and men were considered overweight by BMI, with men reaching higher values (m: 28.2 ± 4.0 kg/m^2 *vs.* w: 27.7 ± 5.2 kg/m^2; $p < 0.0001$). Type 2 diabetes was present in 656 individuals of the HNR cohort (13.7%; Fig. 1). Thereof 397 (8.2%) had previously known type 2 diabetes and 259 (5.4%) had unknown diabetes. Type 2 diabetes was more common in men with 418 (17.5%) male subjects affected compared to 238 female subjects (9.8%). The highest proportion of diabetes was found in subjects with BMI above 40 (approx. 45%).

Transaminase levels in the HNR Study Cohort were within normal limits, but gradually increase with BMI. Mean ALT (16 ± 8.8 U/l) and mean AST (13 ± 4.6 U/l) remained both well below the common threshold for normal values (<50 U/l for males; <35 U/l for females). ALT was significantly

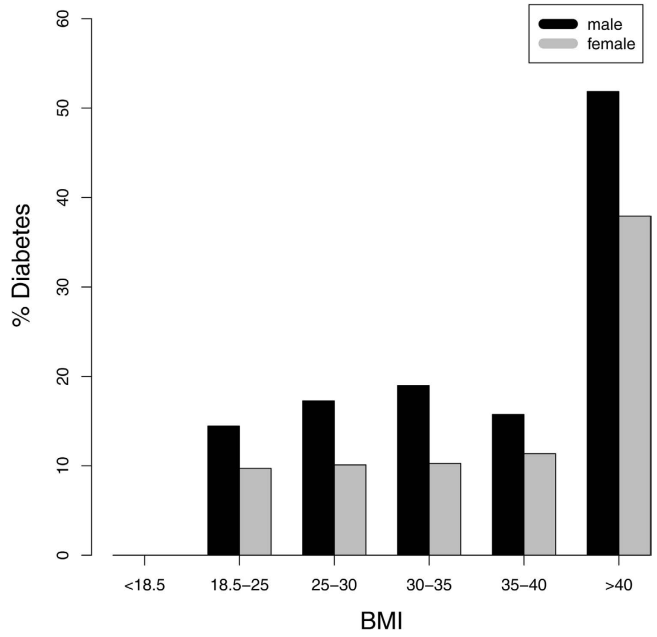

Figure 1. Prevalence of diabetes in the Heinz Nixdorf Recall study by BMI groups. Diabetes was present in below 20% of males and below 10% in females of all BMI groups up to 40 kg/m^2. In the highest BMI group (obesity grade III; >40 kg/m^2) more than 50% male subjects and almost 40% female subjects had overt diabetes.

higher in male subjects than in females (19 ± 9.7 U/l vs. 14 ± 7.1 U/l; $p < 0.0001$), which was also observable for AST (14 ± 4.8 U/l vs. 12 ± 4.2 U/l; $p < 0.0001$; Fig. 2).

Subjects were grouped by BMI according to common ranges for underweight, normal weight, overweight, and obesity grades I-III. Serum concentrations of ALT and GGT tended to be higher in higher BMI ranges, though this trend did not reach significance (Fig. 2). Moreover, even in the highest BMI group (>40 kg/m^2) the mean concentrations were still within normal ranges. A comparison of demographic and metabolic data of individuals with ALT in normal ranges and those with elevated ALT is given in Supplementary Table 1. AST levels were similar in all BMI groups.

Adiponectin and vitamin D levels are inversely correlated with BMI. For all BMI groups VD was around the threshold for a deficit. Only in the highest BMI category (above 40 kg/m^2) the mean VD values indicated a true deficit (<20 ng/ml; Fig. 3A). Slightly higher VD levels were found in males compared to females. Serum adiponectin, an adipocytokine with known hepatoprotective and insulin sensitizing properties[22–24], was low (<15 μg/ml) in all BMI groups. The lowest concentrations were again observed in the highest BMI group (Fig. 3B). Female subjects exhibited higher adiponectin levels than males. While no significant differences between BMI groups were observed for adiponectin and VD, both parameters correlated inversely with BMI (Table 2).

Transaminase levels correlate with BMI and HbA1c, while adiponectin was inversely correlated with transaminase levels. ALT, AST, and GGT correlated positively with BMI (Table 2). To link transaminase levels with a surrogate parameter of IR, HbA1c was quantified in this cohort. ALT, AST, and GGT significantly correlated with HbA1c. In contrast, adiponectin was inversely correlated with AST, ALT, and GGT. Interestingly, GGT was significantly associated with CRP, a marker of systemic inflammation and associated with obesity, linking hepatocellular injury to systemic inflammation.

HbA1c, adiponectin, and BMI are efficient predictors of diabetes in the HNR study cohort. Utilizing machine learning techniques a computational model was built to identify the most important parameters for prediction of diabetes from the available serum parameters. The model identified HbA1c, adiponectin, and BMI as highly important for the prediction of diabetes (Table 3). These were followed by GGT, vitamin D, and transaminases. Logistic regression models were built, using HbA1c, adiponectin, and/or BMI to predict diabetes from the presented cohort. The model using all three parameters (HbA1c, adiponectin and BMI) as well as the reduced models reached AUC values of 0.85. A model using only HbA1c reached an AUC of 0.83. The ROC curves of the models HbA1c and BMI, HbA1c and adiponectin and HbA1c only are shown in Fig. 4. Comparing the AUC values by the method of De Long *et al.*[25], it turned out that both, the model of HbA1c + BMI and HbA1c + adiponectin have significant higher AUC values compared to the model that uses only HbA1c ($p < 0.001$). The differences

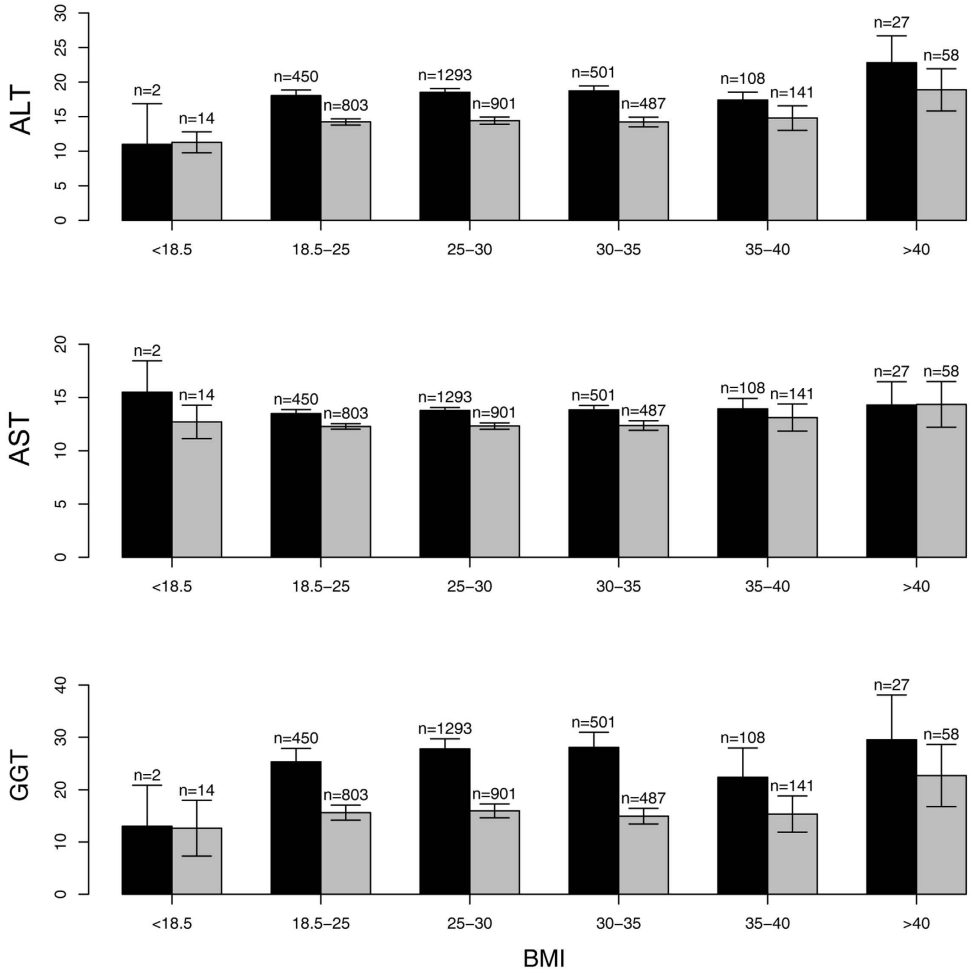

Figure 2. Distribution of classic serum liver parameters in the Heinz Nixdorf Recall study by BMI groups. Alanine aminotransferase (ALT/GPT) and gamma Glutamyltransferase (GGT) exhibited a trend of increased concentrations with higher BMI. Aspartate aminotransferase (AST/GOT) showed no differences between the BMI groups.

between the AUC values of the models HbA1c + BMI and HbA1c + adiponectin were not significant ($p = 0.7044$). By comparing the models at FPRs of 0.1, 0.05, and 0.001 using McNemar's tests, both, the model using HbA1c + adiponectin as well as the model using HbA1c + BMI showed significant differences at FPR = 0.05 ($p < 0.001$ and $p = 0.002$, respectively) compared to the model using only HbA1c. However, only the model using HbA1c and adiponectin showed significant differences for very low FPRs (FPR = 0.01, $p = 0.005$). A model using HbA1c, adiponectin and BMI did not significantly improve the overall performance compared to the models that use only two parameters. Adding the parameters GGT and vitamin D did not significantly improve the models further (not shown).

Discussion

Metabolic syndrome is alongside the obesity epidemic of industrialized countries increasingly common. It is hardly surprising that in parallel NAFLD has become the most predominant chronic liver disease in Europe[26] with up to 75% of NAFLD patients suffering from diabetes[27]. Mild elevations of aminotransferases are a common finding in NAFLD[28], though in over 25% of patients with advanced NAFLD and NASH, transaminase levels remain within normal levels[7]. In the presented cohort a very low proportion of transaminase levels above normal limits were detected (<2% for ALT). Though, with increasing BMI a parallel increase in ALT and GGT levels was observed. Mean BMI of the cohort suggests a substantial proportion of the population is overweight or obese. Moreover, overt type 2 diabetes was found in roughly 14%, while current German cross-sectional studies indicate a prevalence of approximately 7.2% in the general population[29,30], though in these cases only known diabetes was taken into account. This data suggests that a relevant proportion of the analyzed collective may have undiagnosed metabolic syndrome. These individuals are at a greater risk to develop NASH and consecutively cirrhosis or HCC[31]. The main indicator to enroll further diagnostics for liver injury in clinician's daily routine are liver transaminases. Thus, current normal values might miss a significant amount of individuals already

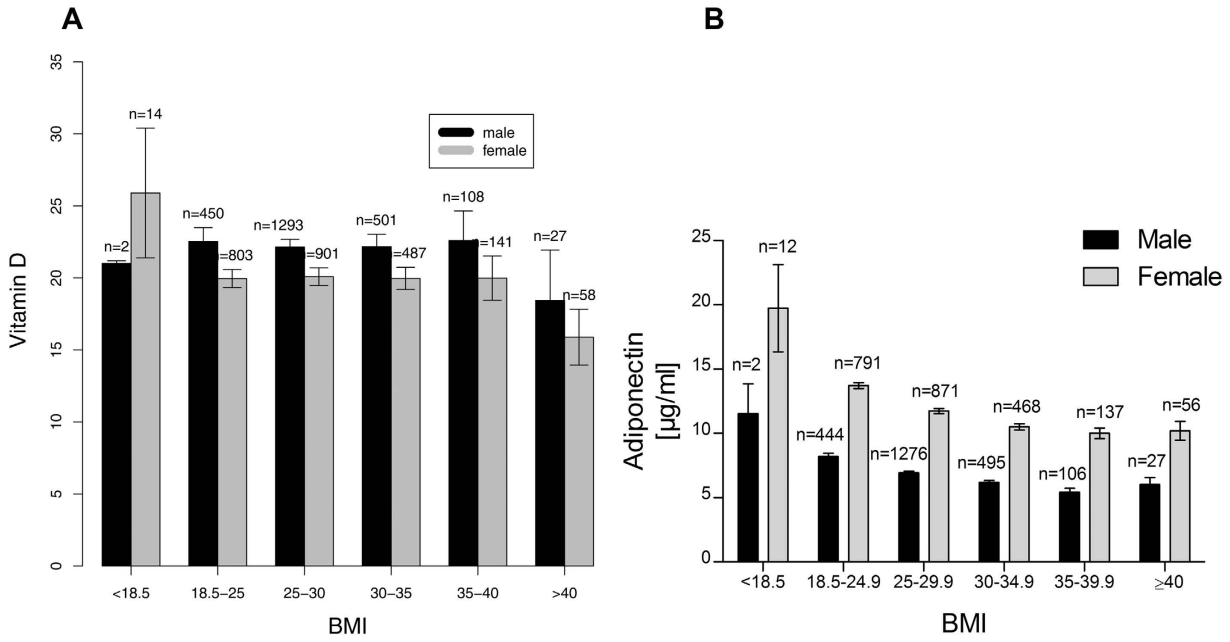

Figure 3. Distribution of metabolic serum markers in the Heinz Nixdorf Recall study by BMI groups. (**A**) Vitamin D serum levels were close to the threshold to insufficiency (20 ng/ml) in all BMI groups $<40\,kg/m^2$. In the highest BMI group ($>40\,kg/m^2$) the lowest vitamin D values were observed, with a mean concentration indicating insufficiency. (**B**) Adiponectin was distributed in a similar way as vitamin D with lowest serum concentrations found in the highest BMI group.

Parameters		r	p
BMI	Adiponectin	−0.2195	<0.0001
BMI	Vitamin D	−0.1243	<0.0001
BMI	ALT/GPT	0.2399	<0.0001
BMI	AST/GOT	0.127	<0.0001
BMI	GGT	0.1221	<0.0001
HbA1c	ALT/GPT	0.1237	<0.0001
HbA1c	AST/GOT	0.0448	<0.01
HbA1c	GGT	0.0907	<0.0001
HbA1c	Adiponectin	−0.1456	<0.0001
HbA1c	Vitamin D	−0.0678	<0.0001
GGT	CRP	0.0798	<0.0001
Diabetes	ALT/GPT	0.1394 (rpb)	<0.0001
Diabetes	AST/GOT	0.0973	<0.0001
Diabetes	GGT	0.1362	<0.0001

Table 2. Correlations of classic liver serum markers and metabolic parameters in the Heinz Nixdorf Recall study population.

developing chronic metabolic liver disease and presenting with transaminase values in the upper normal levels[32,33].

Despite mostly marginal elevations in patients with overweight and obesity, serum liver enzymes have previously been associated to metabolic syndrome or its components. In the Cyprus Metabolism study, Liu *et al.* demonstrated a clear association between ALT and GGT with metabolic risk factors, including IR[34]. Similar associations between transaminase levels and diabetes have been shown, however, data is still scarce[35]. ALT, AST, and GGT in the presented cohort were each correlated with HbA1c levels and BMI. Additionally, GGT was associated with CRP, suggesting a connection to systemic inflammation beyond metabolic associations. This may support previous suggestion of GGT as biomarker for atherosclerosis[36].

Parameter	Mean decrease of Gini impurity*	SD[4]
HbA1c	189.69	5.01
Adiponectin	67.72	3.43
BMI[1]	63.49	3.11
GGT[2]	55.47	2.70
Vitamin D	50.98	1.63
CRP[3]	46.43	1.65
ALT	41.43	1.42
AST	34.07	1.40

Table 3. Importance of parameters for diabetes prediction from the Heinz Nixdorf Recall cohort. *The Gini impurity gives an estimate, which parameters are most important for the random forest to predict the condition of interest (in this case: diabetes). A higher decrease of the Gini impurity represents a higher importance for this parameter. 1: Body mass index; 2: gamma-Glutamyltranerase; 3:C-reactive protein; 4: standard deviation.

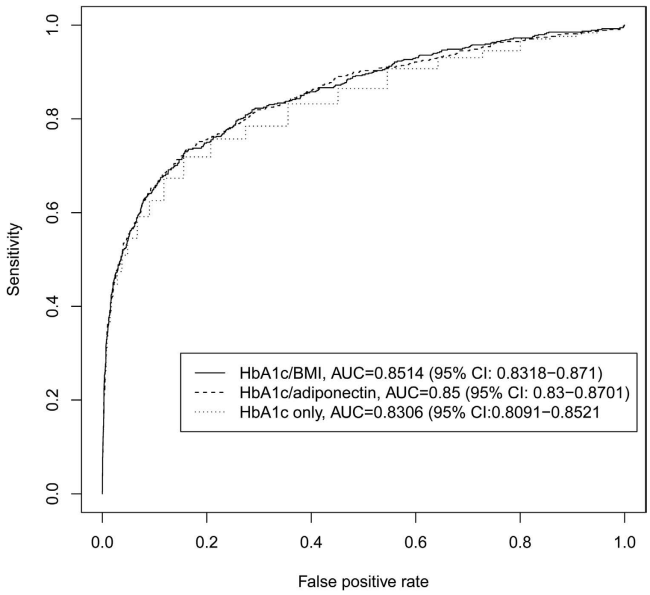

Figure 4. ROC curves of the logistic regression models generated from non-invasive markers. The models generated from serum markers and BMI (see table 3 for list) were able to predict diabetes with a high accuracy.

Although classic serum markers for liver damage were not elevated above normal ranges, higher values may still indicate metabolic alterations or even injury by IR and diabetes.

Apart from the classic serum parameters for liver injury, markers related to the metabolic syndrome might be more valuable to assess damage to the liver in this particular setting. Among possible candidates is adiponectin, an adipose tissue derived adipokine with insulin sensitizing properties, found reduced in obesity. Several study groups elucidated the importance of adiponectin for diabetes[37,38]. Adiponectin was shown to increase the insulin-induced tyrosine phosphorylation of the insulin receptor in skeletal muscle as well as to increase whole-body sensitivity to insulin[39]. Hui *et al.* found that hypoadiponectinemia predicts carotid intima media thickness progression, independent of known predictive factors such as age, smoking, hyperlipidemia, and hypertension[40]. Carotid intima thickness is associated with diabetes and NAFLD[41,42]. In the present study serum adiponectin concentrations were inversely correlated to BMI and classic parameters of liver damage. Moreover, adiponectin was one of three most important factors for random forests to predict diabetes from serum derived markers. Interestingly performance of logistic regression models to predict diabetes with simple parameters was almost similar for HbA1c + adiponectin and HbA1c + BMI. Addition of both parameters to the model (HbA1c, adiponectin, and BMI) did not further improve the performance. This result suggests, that adiponectin may represent an objective marker for adipocyte function, with similar relevance as BMI. Moreover, the differences between the

models HbA1c and HbA1c + adiponectin were also significant at very low FPRs, while this was not true for HbA1c + BMI. Thus the HbA1c + adiponectin model should be preferred in settings where a very high specificity is needed. Generally BMI can be determined easier, faster and cheaper than adiponectin. Though, there are cases (i.e. in highly trained athletes, very small or very large individuals), where BMI is not a reliable estimate for body composition (or adipocyte function)[43,44], although it is a valid estimate in most situations. This might explain the slightly better performance of adiponectin, which needs confirmation in larger studies. Collection of adiponectin data in large cohorts may also enhance our understanding of this marker for adipocyte function and mechanisms leading from obesity to insulin resistance and metabolic syndrome.

Another factor associated with metabolic syndrome is vitamin D, which was found reduced in obesity and has been linked to type 1 and 2 diabetes[45–47]. Histological and clinical stages of NAFLD have also been associated with VD levels in several studies[48]. However, these observations could not be confirmed as causal relationship in some studies[49,50]. Among other factors this might be due to the highly complex interaction of the VD and the TGF-β pathway[51]. In particular polymorphisms in the VD receptor gene might impact the influence of VD on liver disease progression[52]. Additionally VD supplementation has been shown to affect adipocytokine levels[53]. This effect might contribute indirectly to the association of low VD with adverse metabolic profiles. In the HNR cohort we were able to show an association between VD levels and serum ALT and AST. VD was also among possible relevant predictors of diabetes. Taken together serum markers related to metabolic syndrome, as adiponectin or VD, can predict prevalence of diabetes in a population based cohort. Moreover, these parameters might also represent candidates for non-invasive markers of NAFLD or NASH. Due to the correlation to classic liver damage markers, even within normal ranges, and the connection to the metabolic syndrome further studies are warranted to elucidate the potential of adipokines and VD to assess metabolic liver injury.

In summary a strong association between transaminase levels, BMI, adiponectin, VD, and diabetes was found in the HNR study. This association was present despite transaminase concentrations within normal range. Adiponectin and HbA1c can predict diabetes with high accuracy in this population based cohort. Re-assessment of current normal range limits should be considered for classic liver transaminases to provide an improved focus concerning chronic metabolic liver injury. Adipokines or other markers related to the metabolic syndrome should be evaluated as possible NAFLD-specific liver injury markers, especially in individuals at risk for metabolic syndrome.

Patients and Methods

Study population. The Heinz Nixdorf Recall (HNR) Study is an ongoing population-based prospective cardiovascular cohort study of the Ruhr area in Germany. Random samples of the general population were drawn from residents' registration offices including both genders aged 45–75 years. People were invited by mail (one invitational letter plus a maximum of two reminder letters) and phone calls to participate. Most people decided to participate after one invitational letter (52.6%)[54]. Blood tests were performed in fasting state, risk factors for coronary artery disease were analyzed by standardized questionnaires. A detailed description of the study design and population has been published previously[55]. Informed consent was obtained from the included participants. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institution's human research committee (Ethikkommission am Universitätsklinikum Essen).

Diabetes mellitus was defined as a prior physician diagnosis of diabetes or taking an anti-diabetic drug. Unknown diabetes was considered when (1) fasting glucose was $\geq$7.0 mmol/L (60% of study participants with fasting status) or random blood glucose $\geq$11.1 mmol/L (remaining subjects with less than 8 hours non-fasting status) and (2) subjects had not reported a diagnosed diabetes or antidiabetic medication. For the purpose of the present study known diabetes and unknown diabetes were grouped together as "diabetes".

Statistical data analysis and mathematic models. For correlation analyses Pearson product-moment correlation r, point-biserial correlation coefficient r_{pb}, and the phi coefficient ϕ were employed, depending on the type of parameters used (quantitative or dichotomous).

Random forests (RFs) we used to identify the most important parameters for the prediction of diabetes. We used the RF implementation in the R package randomForest (http://www.r-project.org/). Earlier studies have shown that RFs are excellent non-linear classifiers, which are highly stable and robust in comparison to other classifiers[19,20]. Additionally, RFs provide an importance analysis, which can be used to identify the most important positions for the classification process. The theoretical complexity of the random forest is $\Theta(MK\tilde{N}\log^2\tilde{N})$, which is based on the complexity of building single trees ($\Theta(KN\log^2N)$), with K: the number of variables at each node, N: the number of samples. Due to the fact that the random forests use bootstrap replicates, N is reduced to $\tilde{N} = 0.632N$. However, a random forest uses M randomized trees (bagging). In the current study, we used the random forests only for variable importance measurement, thus they have only been calculated once and are not used for prediction. For the importance analysis in our dataset the random forest needed 4 seconds on an Intel-Core i7-4700MQ CPU @ 2.40 Ghz. To reduce the bias due to the class imbalance in the dataset, we repeated sub-sampling for 100 times[56]. The most important parameters were then used to build logistic regression models to identify patients with diabetes (as defined above) within the analyzed cohort. For evaluation

of the models, we calculated the Receiver Operating Characteristics (ROC) and the corresponding Area Under the Curve (AUC) from a leave-one-out cross-validation. For comparisons between the different models we used the method of De Long *et al.* on the AUC values as well as McNemar's tests at certain false-positive rates (FPR), namely 0.01, 0.05, and 0.1.

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Author Contributions

J.K., L.P.B., J.P.S., S.M., K.H.J., R.E. and A.C. designed the study. J.K., J.B., P.M. and H.K. collected data. L.P.B., D.H., J.P.S., S.M., U.S. and K.H.J. performed data analyses. D.H., S.M., U.S., K.H.J., R.E. and G.G. provided material or technical support. J.K., D.H., J.B., P.M. and H.K. drafted the manuscript. L.P.B., J.P.S., K.H.J., R.E. and A.C. revised the manuscript for important intellectual content. S.M., U.S., K.H.J., R.E., G.G. and A.C. supervised the study. All authors approved the final version of the manuscript.

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Patients with ultrasound diagnosis of hepatic steatosis are at high metabolic risk

Patienten mit durch Ultraschall diagnostizierter hepatischer Steatose haben ein hohes metabolisches Risiko

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Schlüsselwörter
- hepatische Steatose
- metabolisches Syndrom
- kardiovaskuläre Erkrankungen
- Leber

Key words
- hepatic steatosis
- metabolic syndrome
- cardiovascular disease
- liver

Zusammenfassung

Hintergrund und Ziele: Fettakkumulation (Steatose) in der Leberzelle stellt die Grundlage der nicht-alkoholischen Fettlebererkrankung (NAFLE) dar. Die einfache Steatose bei Hepatozyten wird als milde Form der NAFLE betrachtet, kann jedoch zur progressiv-entzündlichen Form, der Steatohepatitis (NASH), übergehen. Die NASH kann über Fibrose weiter zur Zirrhose oder zu einem hepatozellulären Karzinom (HCC) führen. Dennoch stellt bereits die einfache Steatose ein Risiko für Herzkreislauferkrankungen (KHK) dar.

Patienten und Methodik: In der aktuellen Studie wurden 106 Patienten aus einer gastroenterologischen und hepatologischen Ambulanz untersucht, sowohl mit bekannter NAFLE (n = 60) oder anderen typischen Diagnosen (n = 46). Mittels Sonografie wurde bei 77 Patienten eine Steatosis hepatis diagnostiziert. Leberenzyme, Lipidprofil, Surrogatmarker des Zelltodes und Adiponektin wurden bestimmt. Transiente Elastografie (Fibroscan®) und Bioimpedanzanalyse (BIA) wurden durchgeführt.

Ergebnisse: Das mittlere Alter der Patienten lag bei 46 Jahren (23 – 62; nicht-NAFLE) bzw. bei 53 Jahren (18 – 71; NAFLE). AST und ALT zeigten keine signifikanten Unterschiede zwischen den Gruppen. Adiponektin und HDL waren signifikant niedriger bei NAFLE ($p < 0,05$) und BIA-Profile zeigten sowohl höhere Fett- als auch fettfreie Masse. Auch nicht-NAFLE Patienten mit Steatose wiesen ein deutlich verändertes metabolisches Profil auf. Insgesamt war Steatose häufiger mit Faktoren des metabolischen Syndroms (MS) und mit KHK assoziiert. Die Prävalenzen der KHK und Faktoren des MS weisen darauf hin, dass Leber-Steatose ein frühes Ereignis bei der Entwicklung dieser Erkrankungen darstellt.

Zusammenfassung: Patienten mit Leber-Steatose haben ein deutlich höheres kardiovaskuläres und metabolisches Risiko, ohne Veränderung der Transaminasen im Vergleich zu Patienten ohne Steatose.

Abstract

Background and aims: Hepatic steatosis is the basis of non-alcoholic fatty liver disease (NALFD). Mere fat accumulation within hepatocytes is considered the mild form of NAFLD, but can progress in some patients to advanced steatohepatitis (NASH), which may lead to fibrosis, cirrhosis or hepatocellular carcinoma. However, even hepatic steatosis alone may be a risk factor for cardiovascular disease (CVD).

Patients and methods: In the present real life study 106 patients from the outpatient clinic of the Department for Gastroenterology and Hepatology with either NAFLD (n = 60) or other typical diagnoses (n = 46) were included. Ultrasound examination identified 77 patients with hepatic steatosis. Liver enzymes, lipid profile, surrogate cell death markers, and adiponectin were determined. Transient elastography (Fibroscan®) and bioelectrical impedance analysis (BIA) were performed.

Results: Mean patient age was 46 years (23 – 62) for non-NAFLD and 53 years (18 – 71) for the NAFLD group. ALT and AST did not differ significantly between the two groups. Adiponectin and HDL were significantly lower in NAFLD ($p < 0.05$) and BIA profiles showed higher fat and fat free mass. Non-NAFLD patients with steatosis also exhibited an adverse metabolic profile. Overall steatosis was associated with factors of metabolic syndrome (MS) and CVD. Prevalence of CVD and factors of MS hint to steatosis as an early event for these conditions.

Conclusion: Patients with steatosis are at higher cardiovascular and metabolic risk without differences in transaminases levels compared to those without steatosis. Steatosis diagnosed by ultrasound needs to rise attention for further metabolic alterations including CVD.

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sonografisch diagnostizierte Steatose sollte die Aufmerksamkeit für weitere metabolische Veränderungem, inklusive KHK, erhöhen.

Introduction

Hepatic steatosis, as an accumulation of lipids within hepatocytes represents the basis of non-alcoholic fatty liver disease (NAFLD). Simple steatosis may lead to non-alcoholic steatohepatitis (NASH), the progressive form of NAFLD with hepatocyte ballooning and inflammatory alterations [1]. Simple steatosis is generally considered as having a good prognosis, however often discussed as the "first hit" in disease progression [2, 3].

Prevalence of NAFLD shows a steady increase and is currently the most common chronic liver disease with a prevalence of up to 30 % in Western countries [4, 5]. As the central organ for glucose and lipid metabolism, the liver is strongly affected by metabolic imbalances such as obesity or hyperlipidemia [6, 7]. While NAFLD has long been supposed a bystander-effect of the metabolic syndrome, NAFLD itself might cause or promote metabolic alterations, such as insulin resistance (IR) [8]. Despite this yet unsolved chicken-egg conundrum, clinicians should be on the alert either way: any type of metabolic change warrants investigation on liver status and liver steatosis warrants deeper analysis of the metabolic status. Moreover, the metabolic syndrome (MS) but also liver steatosis alone are risk factors for cardiovascular diseases (CVD), which represent the most common cause of mortality in MS or NAFLD related context [9, 10]. In clinical practice it is still challenging to identify patients at high risk for either metabolic alterations or CVD, based on routine diagnostics. Since steatosis of the liver is still seen as benign by many clinicians, abnormalities in liver related factors are overlooked or go without consequences for the patients.

In the present study we investigated the situation in a large tertiary outpatient clinic. Since non-invasive markers for NAFLD are still not available for clinical routine, diagnosis for NAFLD was based on ultrasound and prevalence of metabolic alterations indicative of MS as basis for liver disease. Not only NAFLD patients but any patient with steatosis on ultrasound exhibited an adverse metabolic profile, suggesting a higher risk for progression to diabetes or CVD.

Patients and methods

Study population and sera sample preparation

In the present study, 106 patients (mean age 50.88 ± 11.8 years; 36 females) from the outpatient clinic of the Department for Gastroenterology and Hepatology were enrolled (consecutive patients with hospital attendance from 05.2013 to 05.2014). Patients suffered from NAFLD (n = 60) or various other diagnosis (n = 46) routinely treated at the department (see **Table 1** and **Supplementary Table 1** for a detailed breakdown). NAFLD diagnosis was based on ultrasound as described in the following. Liver biopsy was not indicated in any patient and thus not performed. The non-NAFLD diagnoses comprised chronic virus hepatitis B (n = 5) or C (n = 9), autoimmune hepatitis (n = 10), and alcoholic hepatitis (n = 4). Other diagnoses related to the gastrointestinal system, such as dyspepsia or diffuse abdominal pain amounted to 18 patients (given in detail in **Supplementary Table 1**). Progressive liver disease was present as hepatocellular carcinoma (n = 6) and liver cirrhosis (n = 16) (distribution of etiologies see **Supplementary Table 2**). Two patients had received a liver transplantation during their disease course (one with HBV, one with alcoholic hepatitis). Serum samples were collected according to the standard procedure for outpatients and routine laboratory parameters were obtained; additionally, enzyme-linked immunosorbent essays (ELISA) were performed to detect adiponectin (Human Adiponectin/Acrp30 Quantikine ELISA Kit; R&D, Minneapolis, MN) and the cell death markers M30 and M65 (M30 Apoptosense ELISA or Epideath ELISA; both Tecomedical group, Switzerland). Clinical courses as well as demographic data were analyzed retrospectively. The study conformed to the amended declaration of Helsinki (Fortaleza 2013). The institutional review board waived need for written informed consent due to the retrospective nature of the study. All protocols and procedures adhered to the requirements of the institutional review board (Ethikkommission am Universitätsklinikum Essen) and the declaration of Helsinki.

Diagnostic procedures and patient data

All examinations were performed by hepatologists or trained technicians (BIA). Abdominal ultrasound was performed with a 5 MHz transducer as standard procedure for every outpatient. Fatty liver was diagnosed if abnormally intense, high-level echoes were detected from the hepatic parenchyma in comparison to the renal parenchyma of the right intercostal sonogram [11]. Diagnose was either absence or presence of steatosis; no grading scale was used.

Transient elastography (Fibroscan®, Echosens device) measures shear wave velocity of a wave passed into the liver from a transducer. The measurement is converted into liver stiffness, expressed in kilopascals [12]. Fibroscan® was performed as standard procedure.

Bioelectrical impedance analysis was used to estimate body mass components. A three-component model including fat mass (FM), extracellular mass (ECM) and body cell mass (BCM) was used [13].

Information about presence of CVD, diabetes and hypertension were assessed retrospectively from patient records. Known presence of coronary artery disease was considered as established CVD.

Analysis of data

All data shown are mean ± SEM, if not stated otherwise. Student´s t test was used to calculate differences between parameters, if Gaussian distribution was given. The Mann-Whitney U test was

Table 1 Etiologies of patients recruited from the outpatient clinic.

etiology	number of patients
NAFLD	60
hepatitis B virus infection	5
hepatitis C virus infection	9
autoimmune hepatitis	10
alcoholic hepatitis	4
other (gastrointestinal) diagnoses[1]	18
total	106

[1] A detailed list of other etiologies can be found in **Supplementary Table 1**.

performed for variables not distributed normally. Statistical significance was considered, if p was ≤0.05. Analyses were performed with Prism, version 5 (GraphPad Software Inc., CA, USA).

Results

NAFLD patients show mostly normal liver function tests and moderately elevated cell death

Table 2 summarizes the characteristics of 60 patients with previously known NAFLD (43 m /17f) in comparison to individuals without NAFLD (19 f/37 m). The median age was 53 years (18 – 71 years), significantly above the age of non-NAFLD individuals (48 years; 23 – 69). Standard liver function tests aspartate aminotransferase (AST) and alanine aminotransferase (ALT) did not differ between the two groups (**Table 2**). Of note, in both groups AST and ALT remained below the normal threshold in the majority of patients (for male patients 50 U/l; for female patients 35 U/l). NAFLD patients exhibited elevated AST in 25 % and elevated ALT in 43 % of cases. Non-NAFLD had elevated AST in 30 % and ALT in 32 %. Apoptotic cell death, assessed by caspase cleaved CK18 quantification in patient serum (M30), has been described as a non-invasive marker for severity of NAFLD or to separate steatosis from NASH [14, 15] but also to assess severity of liver disease in other settings [16, 17]. Surprisingly the surrogate markers for overall cell death (M65) and apoptosis (M30) were nominally higher in NAFLD than in non-NAFLD patients, but did not differ between NAFLD and non-NAFLD patients (**Supplementary Fig. 1A, B**). Though, when comparing all patients with liver-related diseases to those of gastrointestinal or other etiologies, M30 was significantly higher in liver diseases (**Supplementary Fig. 1C**). Similarly M65 was higher in liver related etiologies, though significance was not reached (**Supplementary Fig. 1D**).

NAFLD patients exhibit adverse metabolic profile and body composition

Since classic liver injury markers and cell death were not different between NAFLD and non-NAFLD patients, serum markers related to metabolic changes and body composition analysis (BIA) were analysed. Adiponectin is an adipocytokine known for insulin sensitizing properties and is found reduced in obese individuals [18, 19]. Adiponectin was significantly lower in NAFLD patients compared to other etiologies (**Fig. 1A**). Similarly serum HDL was reduced in NAFLD in comparison to other diseases (**Fig. 1B**). HDL is an important prognostic factor for CVD risk. Serum triglyceride concentrations were significantly higher in NAFLD than in patients with other disease etiologies (**Fig. 1C**). During routine diagnostics body composition analysis has become an important tool to assess metabolic status. As expected BMI was significantly higher in the NAFLD group compared to other patients (**Table 1**). Liver stiffness detected by Fibroscan® was lower in NAFLD than other diseases, but significance was not reached (**Fig. 2A**). Assessment of body composition by BIA led to significantly higher fat mass (**Fig. 2B**), fat free mass (FFM; **Fig. 2C**), body cell mass (BCM; **Fig. 2D**), and a significantly lower extracellular mass (ECM)-to-BCM ratio (**Fig. 2F**) in NAFLD compared to all other patients. While ECM was nominally higher in NAFLD, the difference to other etiologies did not reach significance (**Fig. 2E**).

NAFLD patients show higher proportions of progressive metabolic alterations

Since NAFLD has been recognized as liver manifestation of the metabolic syndrome, prevalence of the medical conditions associated to the MS was calculated (**Table 1**). Among NAFLD patients 28 % had diabetes and 20 % diagnosed CVD. Non-NAFLD etiologies reached 17.4 % diabetic individuals and 8.7 % patients with CVD (both n.s.). Diagnosis of hypertension was available in only 51.9 % of the patient cohort. Within those patients with data on hypertension 59.4 % of the NAFLD patients and 29.2 % of other etiologies had hypertension (p = 0.03).

Steatosis in ultrasound confers metabolic risk independent of etiology

Steatosis was found in 16 patients without NAFLD during ultrasound diagnostics of the liver. In one patient no liver ultrasound was performed. Distribution of general and clinical parameters

Table 2 General and clinical parameters of the presented patient cohort.

variable	all patients (n = 106)	NAFLD patients (n = 60)	patients with other etiologies (n = 46)	p value NAFLD vs. non-NAFLD
sex distribution (m/f)	70 / 36	43 / 17	27 / 19	0.17
age (y)	50.9 ± 1.1	52.8 ± 1.5	48.3 ± 1.7	0.049
BMI (kg/m²)	27.3 ± 4.4	28.7 ± 4.0	25.5 ± 4.2	0.0002
ALT (U/L)	64.3 ± 10.1	65.3 ± 13.6	62.9 ± 15.2	0.91
AST (U/L)	45.1 ± 3.8	44.9 ± 5.1	45.1 ± 5.8	0.98
GGT (U/L)	99.5 ± 10.6	116.2 ± 15.3	77.7 ± 13.8	0.07
fasting blood glucose concentration (mg/dL)	107.4 ± 4.1	105.9 ± 5.1	109.4 ± 6.6	0.67
LDL (mg/dL)	111.3 ± 3.7	112.8 ± 5.0	109.4 ± 5.4	0.65
total cholesterol (mg/dL)	189.1 ± 4.7	187.5 ± 5.8	191.3 ± 7.8	0.69
systolic blood pressure (mm Hg)	119.6 ± 2.6 (n = 48)	123.8 ± 3.4 (n = 25)	115.2 ± 3.9 (n = 23)	0.11
diastolic blood pressure (mm Hg)	72.1 ± 1.8 (n = 48)	74.9 ± 2.5 (n = 25)	69.1 ± 2.6 (n = 23)	0.12
diabetes	25 (23.6 %)	17 (28.3 %)	8 (17.4 %)	0.25
CVD	16 (15.1 %)	12 (20.0 %)	4 (8.7 %)	0.17
hypertension	26 (46.4 %) known for 55	19 (59.4 %) known for 32	7 (29.2 %) known for 24	0.03

All data are presented as mean ± standard error of mean. BMI: Body mass index; CVD: cardiovascular disease; NAFLD: non-alcoholic fatty liver disease.

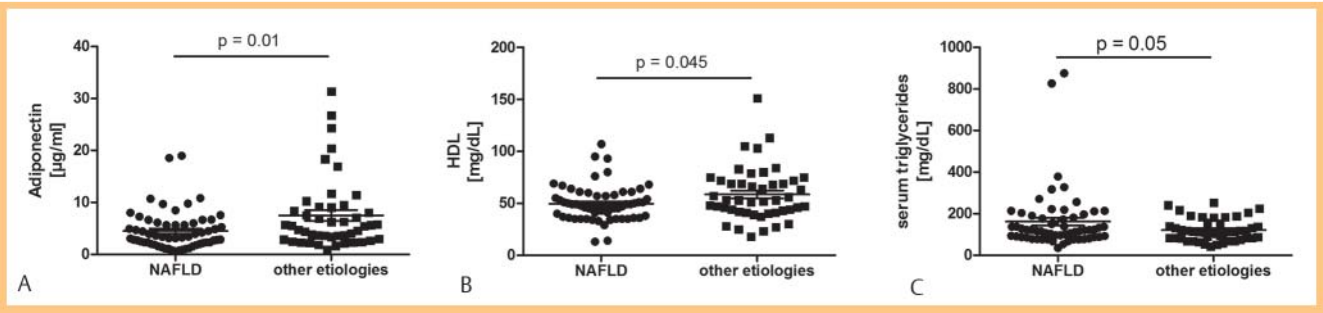

Fig. 1 Serum adiponectin and lipid profiles differ significantly between NAFLD and other etiologies. Concentrations of serum adiponectin **A** and HDL **B** were significantly lower in NAFLD compared to non-NAFLD patients, indicating a higher cardiovascular risk. Conversely, NAFLD patients exhibited higher serum triglycerides, again suggesting an adverse metabolic profile. Shown are scatter dot plots with mean and standard error of mean.

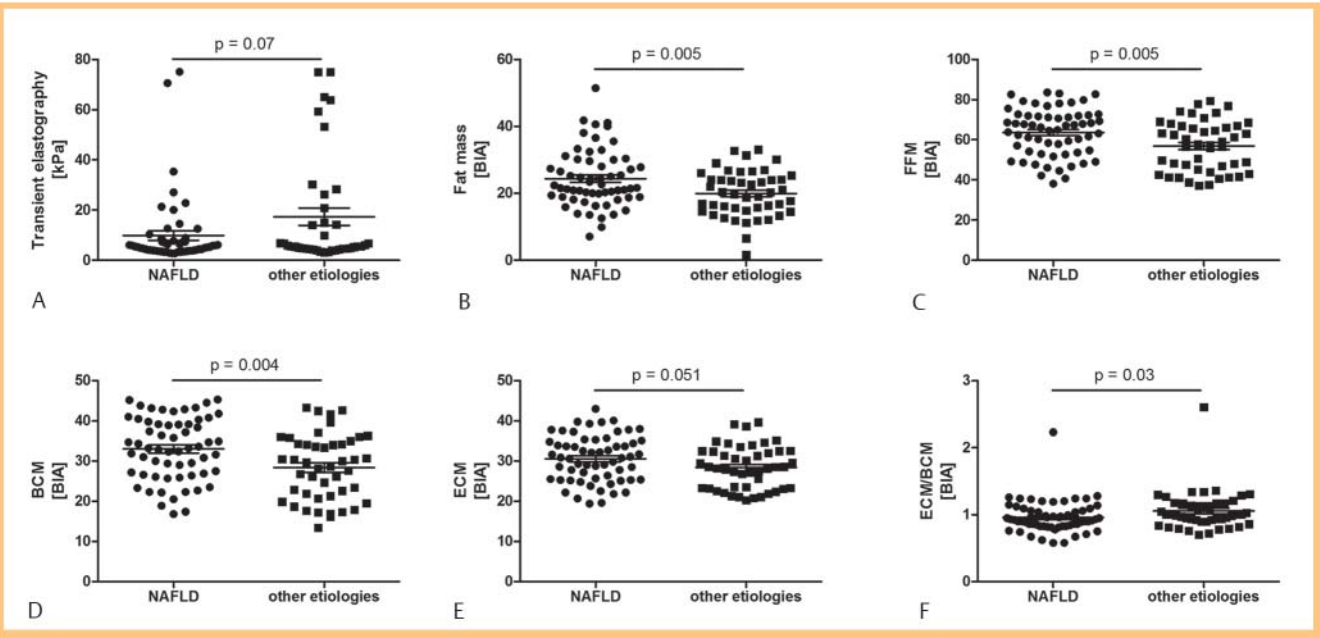

Fig. 2 NAFLD is associated with altered compartment masses in bioelectrical impedance analysis. Liver stiffness was assessed by transient elastography **A** and yielded lower stiffness in NAFLD than other diseases, though no significance was reached. Body composition was determined by bioelectrical impedance analysis (BIA) in patients with NAFLD and other etiologies. Surprisingly, not only fat mass **B** but also fat free mass (FFM; **C**) and the subcompartments of FFM, and body cell mass (BCM; **D**), were significantly higher in NAFLD patients. The ECM-to-BCM ratio **F** was significantly lower in NAFLD. Extracellular mass (ECM; **E**) was not different between the groups. Shown are scatter dot plots with mean and standard error of mean.

for the overall 76 patients with steatosis and 29 patients without steatosis are given in **Table 3**. Patients with steatosis were significantly older and had a higher BMI. Although, ALT and γGT were nominally higher in steatotic patients compared to those without steatosis, significance was not reached. Again cell death markers (M30, M65) did not differ between the groups (data not shown).

Similar to NAFLD, metabolic markers were significantly altered in steatosis. HbA1c (but not fasting glucose) was significantly higher in steatotic patients in comparison to non-steatotic individuals (**Fig. 3A**). Serum adiponectin (**Fig. 3B**) and HDL (**Fig. 3C**) were significantly lower in patients with steatosis compared to those without steatosis of the liver. Conversely, triglyceride concentrations were significantly higher in steatotic individuals than in non-steatotic patients (**Fig. 3D**).

Body composition differed also to a significant extent in steatosis from non-steatotic patients. Liver stiffness was significantly lower in steatosis compared to patients without steatosis (**Fig. 4A**). Compartments determined by BIA were all significantly higher in steatotic patients than in non-steatotic patients (**Fig. 4B–E**). No difference was found for the ECM-to-BCM ratio (**Fig. 4D**).

Of note, when patients without the diagnosis of NAFLD were grouped by prevalence of steatosis, again an altered metabolic profile was observed in steatotic individuals. Adiponectin (**Fig. 5A**; n.s.), HDL (**Fig. 5B**), and total cholesterol (**Fig. 5C**) were lower in steatotic patients without NAFLD than in patients without steatosis. Lower liver stiffness was observed in steatotic patients without NAFLD compared to non-steatotic patients (**Fig. 5D**). Measurement of body composition showed significantly higher FFM (**Fig. 5E**) and ECM (**Fig. 5F**) in steatotic patients without NAFLD compared to patients without steatosis. Metabolic changes are also present in non-NAFLD patients with liver steatosis in ultrasound, although these are not as extensive as in NAFLD patients.

variable	patients with steatosis (n = 76)	patients without steatosis (n = 29)	p value
sex distribution (m/f)	54 / 22	15 / 14	0.06
age (y)	52.9 ± 1.3	45.6 ± 2.0	0.004
BMI (kg/m²)	28.4 ± 4.0	24.4 ± 4.1	< 0.0001
ALT (U/L)	67.4 ± 12.9	55.9 ± 13.6	0.54
AST (U/L)	45.5 ± 4.7	43.9 ± 6.5	0.85
GGT (U/L)	109.4 ± 12.6	73.2 ± 19.3	0.13
fasting blood glucose concentration (mg/dL)	109.0 ± 5.2	103.8 ± 5.7	0.51
LDL (mg/dL)	110.2 ± 4.2	114.3 ± 7.6	0.62
total cholesterol (mg/dL)	183.9 ± 4.9	203.1 ± 11.0	0.12
systolic blood pressure (mm Hg)	121.9 ± 3.0 (n = 32)	113.5 ± 5.1 (n = 15)	0.15
diastolic blood pressure (mm Hg)	74.0 ± 2.1 (n = 32)	69.1 ± 3.6 (n = 15)	0.22
diabetes	20 (26.3 %)	5 (17.2 %)	0.44
CVD	14 (18.4 %)	2 (6.9 %)	0.22
hypertension	22 (55.0 %) known for 40	3 (20.0 %) known for 15	0.03

All data are presented as mean ± standard error of mean. BMI: Body mass index; CVD: cardiovascular disease.

Table 3 Distribution of general and clinical parameters depending on liver steatosis in ultrasound diagnosis.

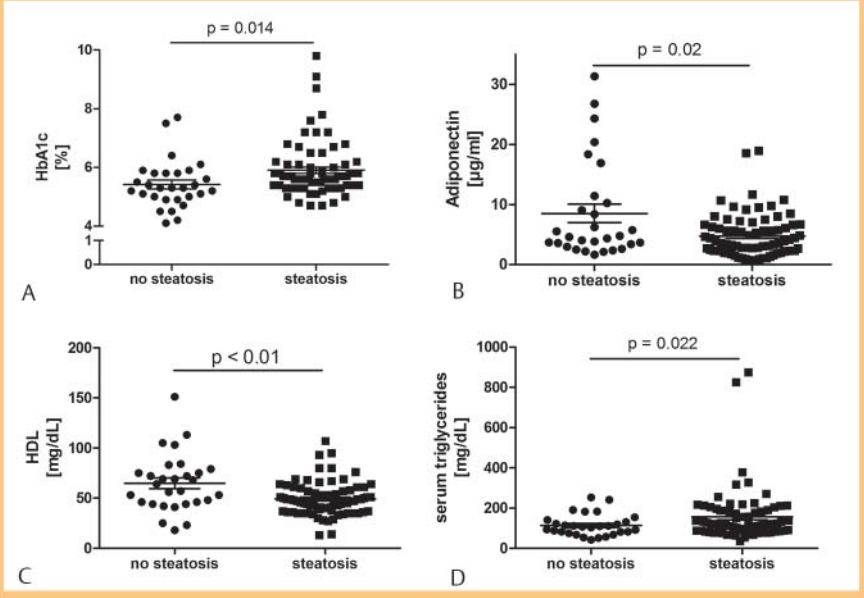

Fig. 3 Steatosis is associated with an adverse metabolic profile independent of etiology. Patients were grouped for steatosis diagnosed by ultrasonography (independent of underlying etiology). Serum concentrations of HbA1c **A** and serum triglycerides **D** were significantly higher in steatotic patients compared to those without steatosis. Conversely, the cardioprotective factors adiponectin **B** and high density lipoprotein (HDL; **C**) exhibited significantly lower concentrations in sera from patients with steatosis. Shown are scatter dot plots with mean and standard error of mean.

Higher prevalence of metabolic syndrome conditions in patients with ultrasound diagnosed steatosis

Medical conditions comprising the MS were evaluated for patient subgroups with or without steatosis. Of steatotic patients 26.3 % were diabetic and 18.4 % had CVD. Patients without steatosis had a prevalence of 17.2 % for diabetes and 6.9 % for CVD (both not significant). For 55 patients with ultrasound of the liver also data on hypertension were available. Of this subgroup, 55.0 % steatotic individuals had hypertension and 20.0 % of patients without steatosis exhibited hypertension (p = 0.03). MS was defined according to the IDF 2006 [20]. Since waist circumference was not routinely collected, obesity was determined by BMI > 30 kg/m². Among steatotic patients all individual conditions comprising MS were present in higher proportions than in non-steatotic individuals (**Supplementary Table 3**), though no significant differences were found. MS was not found in any patient without liver steatosis (0 %) but occurred in 14 steatotic individuals (18 %; p < 0.01).

Steatosis as possible baseline risk for cardiovascular disease onset

Since liver steatosis was per se closely associated to metabolic alterations and risk factors, individuals with steatosis were grouped by established CVD. Remarkably, only two factors were found significantly different between steatotic patients with and without CVD. The ECM-to-BCM ratio was significantly higher in patients with CVD and steatosis (n = 14; 1.09) than in patients with steatosis alone (n = 62; 0.97; p = 0.047). Moreover, patients with steatosis and established CVD were significantly older than those without CVD (50.7 ± 1.4 vs. 63.0 ± 2.0; p = 0.0002).

Discussion

In the present study we describe a real life outpatient cohort, with significant metabolic risk for the majority of patients. On

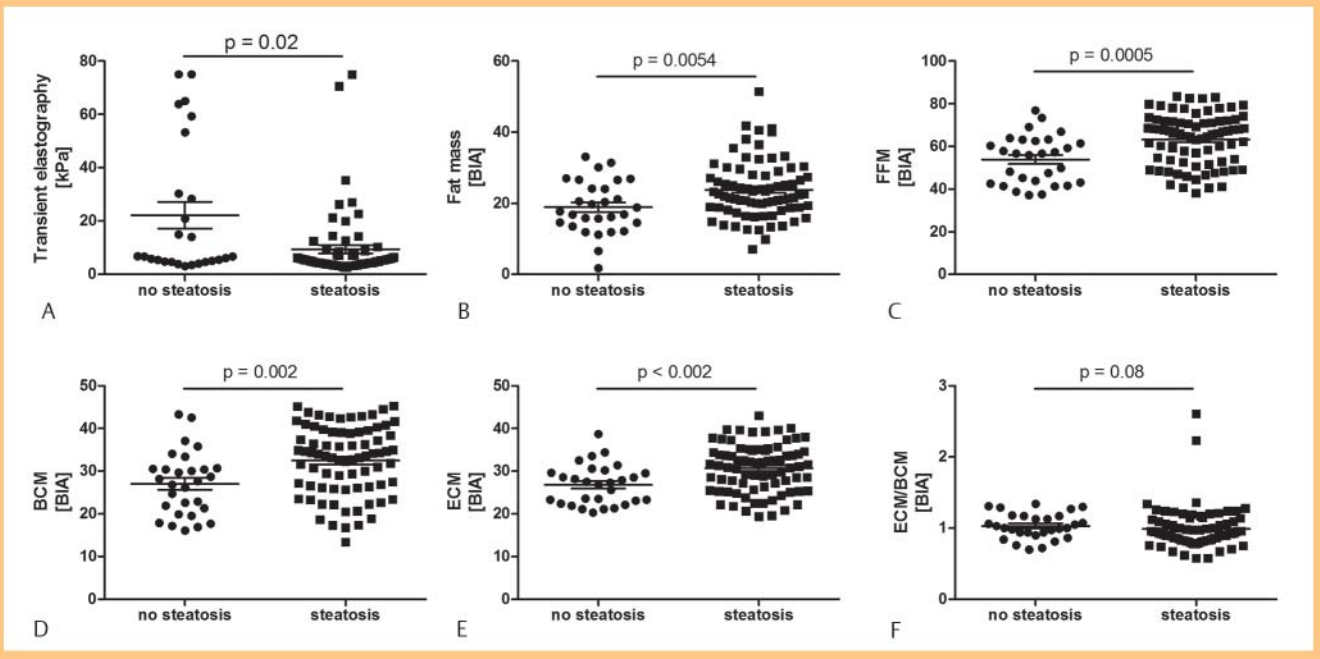

Fig. 4 Steatosis occurs in parallel to changes in body composition. Liver stiffness (by transient elastography) and body composition were determined for patients with or without steatosis detected by ultrasonography. Liver stiffness **A** was significantly lower in steatotic patients. Body composition assessed by bioelectrical impedance analysis (BIA) was significantly changed towards greater fat mass **B** and fat free mass (FFM; **C**) in steatotic individuals. Also, the subcompartments of FFM, body cell mass (BCM, **D**) and extracellular mass (ECM; **E**), were significantly higher in steatotic patients compared to patients without steatosis. The ECM-to-BCM ratio **F** did not differ between the groups. Shown are scatter dot plots with mean and standard error of mean.

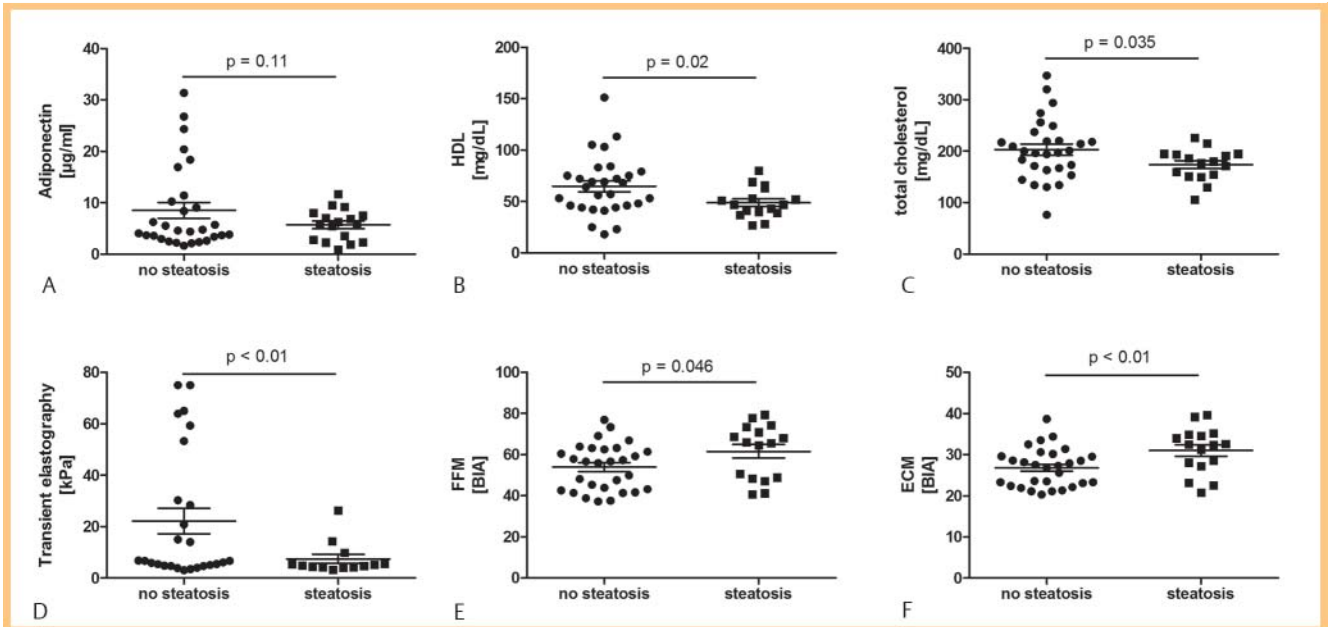

Fig. 5 Steatosis is associated with an adverse metabolic status in patients without NAFLD. The subgroup of patients without NAFLD was grouped by sonographically diagnosed steatosis. While serum adiponectin **A** was nominally lower in patients with steatosis, significance was not reached. High density lipoprotein (HDL; **B**) and total cholesterol **C** serum concentrations were significantly lower in patients with steatosis. Transient elastography **D** identified significantly lower liver stiffness in steatotic patients. Measurement of body composition by bioelectrical impedance analysis (BIA) showed significantly higher fat free mass (FFM; **E**) and extracellular mass (ECM; **F**) in patients with steatosis. Thus, finding of liver steatosis during ultrasound diagnosis was accompanied by alterations in metabolic profile in non-NAFLD patients. Shown are scatter dot plots with mean and standard error of mean.

the one hand patients with NAFLD, based upon ultrasound diagnosis, exhibited higher metabolic and therefore cardiovascular risk. On the other hand, diagnosis of liver steatosis by ultrasound suggests an adverse metabolic profile, independent of etiology. Bioelectrical impedance analysis (BIA) as a commonly used method to estimate body composition, revealed for patients with NAFLD or steatosis significantly higher fat mass and fat free mass, respectively. In summary the presented findings imply an increase of steatosis not only as a separate entity in NAFLD but also in other disease etiologies. Furthermore, relative prevalences of conditions related to MS and of CVD in the presented cohort as well as age distribution of patients with CVD and steatosis suggests liver steatosis as early event in development of metabolic syndrome and associated diseases. These findings will be discussed in greater detail below.

The metabolic profile of patients with NAFLD or with steatosis on ultrasound demonstrated a reduction of adiponectin and HDL, which are generally considered protective against insulin resistance and CVD [18, 21]. Conversely, hypertriglyceridemia was present indicating an overall shift towards metabolical impairment. Medical conditions comprising MS and MS itself were present in higher proportions of steatotic (and NAFLD) patients than in non-steatotic patients. In particular established CVD had a higher prevalence among steatotic individuals. Moreover, patients with CVD were significantly older than those with steatosis alone. This indicates that steatosis precedes other metabolic changes, as alterations in serum lipid components, hypertension, and CVD. Based on these findings we cannot provide a causal link between liver steatosis and CVD. Though, a recent large study was able to show with longitudinal data, that hepatic steatosis contributes to early atherosclerosis and its progression [22]. In our cohort also patients without the diagnosis of NAFLD but presence of steatosis in ultrasound exhibited metabolic alterations. In light of our findings we believe that ultrasound diagnosis of liver steatosis should be recognized as an important and far from benign warning sign for ongoing metabolic changes. While these changes may not lead to end stage chronic liver disease or CVD in all affected individuals, a large proportion will progress to MS and further. Liver steatosis thus would mark the urgent starting point for preventive measures to counter MS.

A limitation of the presented study is that steatosis was diagnosed only by ultrasound, since for most patients no biopsy was indicated. This actually reflects the current situation for most clinicians, which have to rely on non-invasive measures to assess liver status. To detect hepatic steatosis by ultrasound roughly 20 – 30 % fatty infiltration is required to have sufficiently increased echogenicity for a definite diagnosis [23]. Liver biopsy remains the diagnostic gold standard for steatosis. In liver tissue it is possible to quantify hepatic lipid droplets of any extent and to differentiate between macro- and microvesicular steatosis. A minimum of 5 % steatotic infiltration is widely accepted for histological diagnosis of hepatic steatosis [24, 25], which is much lower than the approximate 30 % required for ultrasound detection. Thus, ultrasound diagnosis of fatty liver implies a sufficiently long exposure to metabolic alterations to reach this substantial fat accumulation. This notion again underscores that steatosis identified in ultrasound must rise attention to follow up on metabolic alterations and other sequelae.

Another central finding are mild or largely absent elevations of classic liver serum markers, co-occurring with manifest metabolic alterations confirming previous studies from us and other groups [26, 27]. It is important to note that current thresholds of AST and ALT were established during a time, when viral and alcoholic hepatitis were the most prevalent etiologies. As can be derived from the presented cohort, NAFLD has surpassed the prevalence of these causes for liver disease by far. One possible candidate to complement the classic serum markers would be CK-18, which is most ubiquitously expressed in normal epithelia and cancer cells [28]. CK-18 as endothelial cell death marker seems to be very valuable in assessing disease severity, as both M30 and M65 correlate well with stages of liver diseases or extent of acute injury [17]. To identify a specific liver disease these markers exhibit a too strong overlap for different etiologies. Still, M30 was significantly higher in patients with any type of liver disease compared to patients with gastrointestinal conditions, suggesting M30 indeed as liver specific marker [29]. Since the gold standard to identify NAFLD is still histopathological examination, it is of utmost importance to identify and find consent on relevant biomarkers clearly indicating on the one hand NAFLD (even if present together with other liver diseases) and on the other hand serve as progression marker for advanced stages as NASH. One promising candidate is adiponectin, which was found significantly altered in the present study. This adipocyte derived cytokine is crucial for insulin sensitivity, in particular in hepatocytes [30]. Other markers for NAFLD could be HbA1c and HDL, which were also significantly different when patients were grouped by steatosis (independent of etiology). Although these non-invasive markers are not specific for liver related injury alone they could be a starting point to refine diagnostic procedures for NAFLD. As soon as more easily available objective criteria are known to identify NAFLD and predict it's severity, a better understanding of the disease development in humans will ensue. This will open new options for research and at some point also for therapeutic approaches.

Body composition is probably the most important macroscopic cause for metabolic alterations leading to MS. In the present study BIA was used to determine body composition, utilizing a three compartment model. Surprisingly we found not only elevated fat mass in NAFLD or steatotic patients, but also all other compartments (fat free mass and the subcompartments ECM and BCM). This could be explained by a homogenous distribution of higher body mass into all compartments in metabolic disease. Though, this would contradict the current hypothesis that in particular increased fat mass is associated to metabolic risk. Another possible explanation could be that ectopic fat deposits, i. e. in the liver or in muscle, would contribute to elevated FFM and in particular BCM. Indeed, this seems to be the case in the presented cohort, where we found prominently increased BCM in steatosis and NAFLD. Previous works have suggested ectopic fat deposition in the liver – liver steatosis – as important fat deposit for development of IR [31, 32]. Taken together with the here presented findings, steatosis of the liver might contribute not only to metabolic alterations but also to increased FFM in body composition analysis. This needs to be taken into account, when body composition is determined.

In summary we show here in a real life outpatient cohort, that hepatic steatosis diagnosed by ultrasound indicates a relevant risk for progressive metabolic disease, as MS and CVD. Despite a prominent difference in metabolic profile between steatotic and non-steatotic patients, classic serum liver parameters were similar and largely not elevated above current thresholds. Liver steatosis might contribute to changes in body composition analysis. Moreover, our findings hint to liver steatosis as early event in metabolic diseases and development of CVD. Finding of liver

steatosis during ultrasound diagnosis should be considered as initiation point for detailed diagnostics on metabolic health and preventive measures. In our opinion, clinicians should regard steatosis not as hepatic implication of metabolic alterations, but as an additional (if not fundamental) metabolic risk factor.

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Adipocyte cell size, free fatty acids and apolipoproteins are associated with non-alcoholic liver injury progression in severely obese patients

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ABSTRACT

Purpose. Obesity is a modern pandemic with continuous expansion and represents an independent risk factor for non-alcoholic fatty liver disease (NAFLD), the most common liver disease in westernized countries. The crosstalk between adipose tissue and the liver is key to the development of NAFLD.

Procedures. Therefore, in an observational study blood, visceral adipose tissue and liver tissue were obtained from 93 severely obese patients with a mean age of 43 years and mean BMI of 52 kg/m^2 at the time of weight loss surgery. In a subset of patients a follow-up blood sample was obtained 6 weeks after surgery to assess acute effects of weight loss. In addition to routine parameters of liver injury, serum samples were analyzed for leptin, adiponectin, free fatty acids (FFAs), and several apolipoproteins.

Main findings. The diameter of visceral adipocytes correlated to liver injury, serum markers of inflammation and serum adipokine levels. Liver injury assessed by serology (ALT, AST) and histology (NAFLD activity score, NAS) was independent of the BMI. However, serum levels of triglycerides and Apolipoprotein CIII (ApoCIII) were associated with NAS. Serum levels and composition of FFAs, especially long chain FFAs, also correlated with NAS.

Abbreviations: ALT, alanine aminotransferase; ApoAI, apolipoprotein AI resp. ApoAII, ApoCII, ApoCIII; AST, aspartate aminotransferase; CRP, C-reactive protein; DGLA, dihomo-gamma-linolenic acid; FFA, free fatty acid; GGT, gamma glutamyltransferase; IL-6, interleukin-6; INR, international normalized ratio; LDH, lactate dehydrogenase; Lp(A), lipoprotein A; LPL, lipoprotein lipase; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activitiy score; NASH, non-alcoholic steatohepatitis; PTT, partial thromboplastin time; VLDL, very low density lipoprotein; WAT, white adipose tissue; vWAT, visceral WAT.

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Analysis of serum samples six weeks after surgery revealed beneficial changes in serum triglycerides, levels of ApoCIII and several FFAs.

Conclusions. In severely obese patients beneficial effects on liver injury can been observed as early as six weeks after bariatric surgery. These effects may be explained by the observed changes in adipose tissue and lipid metabolism. Collectively, these findings underline the importance of the link between adipose tissue and the liver.

1. Introduction

The prevalence of obesity in children, adolescents, and adults in the United States has increased over several decades [1]. At the beginning of the 21st century, more than 30% of the U.S. population was obese, and globally there are at least 400 million obese adults in low-, middle- and high-income countries [2]. Obese individuals are at high risk for non-alcoholic fatty liver disease (NAFLD) and its progressive form, non-alcoholic steatohepatitis (NASH). Nowadays, the prevalence of NAFLD and NASH in severely obese persons (body mass index (BMI) > 35 kg/m^2) is approximately 70% and 30%, respectively [3]. Indeed, NAFLD and NASH have become increasingly common causes of chronic liver disease in the developed world, with NASH projected to be the leading cause of liver transplantation in the United States by 2020 [4]. Disease progression is accelerated in patients with features of the metabolic syndrome, such as insulin resistance, type II diabetes, hypertension, and dyslipidemia [5]. The metabolic syndrome is often reflected as a chronic inflammatory state of visceral white adipose tissue (vWAT) in the visceral fat. In addition to its storage capacity for lipids, white adipose tissue (WAT) is considered to be an endocrine organ that secretes tissue specific cytokines, known as adipokines. These cytokines are responsible for the inflammatory environment associated with central obesity and its complications, including NAFLD and NASH [6]. Recently, interactions between oncogenic pathways linking obesity and chronic liver disease showed why the hepatocellular carcinoma is one of the few malignancies with rising incidence in developed countries [7]. In addition, vWAT also releases free fatty acids (FFAs), which play a pivotal role in the development of NAFLD [8]. Under fasting conditions lipolysis in vWAT contributes to 82% of the FFA pool, while in feeding periods 62% of FFAs originate from the vWAT [9]. Currently, the best treatment for NAFLD and NASH is weight reduction. Options for weight loss include life-style modifications such as optimization of nutrition and increased physical activity, and in some cases, medications and invasive procedures. The prevalence of severe obesity continues to increase and dietary interventions and drug therapy alone in patients with BMI > 35 kg/m^2 have shown limited success. As a result, bariatric surgery has become one of the most common surgical procedures performed in the United States [8]. Bariatric surgery has proven to be safe, and outcomes include substantial and sustained weight loss in most individuals, significant improvements in quality of life, significant reduction or remission of most obesity associated diseases, and increased lifespan [10]. Recent studies displayed improvement in major comorbitities including hyperuricemia and reduced cardiovascular disease risk in severely obese patients who have undergone weight loss surgery [11,12]. However, the underlying pathophysiological mechanisms involved in the improvement of global status of these patients are still unknown. Indeed, we recently showed that bariatric surgery has a beneficial effect on liver cell death as quantified by serum levels of M30 (caspase-cleaved cytokeratin-18 fragments) and M65 (total cytokeratin-18) in patients with NASH. Moreover, adiponectin levels, as markers for visceral adipose tissue integrity and inflammation, increased continuously in NASH patients after bariatric surgery [13].

In the present study we therefore aimed at addressing the interplay between visceral adipose tissue and non-alcoholic fatty liver disease in a cohort of severely obese patients undergoing bariatric surgery. We also examined the short-term effects of bariatric surgery on serum levels of free fatty acids and apolipoproteins as known modulators of non-alcoholic fatty liver disease.

2. Methods

2.1. Study design

Severely obese patients from our institution with the indication for weight loss surgery were included according to National Institutes of Health (NIH) guidelines (BMI ≥ 40 kg/m^{2}or ≥ 35 kg/m^2, plus comorbidities) [14]. Additional inclusion criteria were: (I) complete anthropometric patient data, (II) routine laboratory data present, (III) liver sample as well as visceral adipose tissue sample available. Subjects reporting excess alcohol consumption (>20 g/day in males or >10 g/day in females) or those with other known causes of secondary fatty liver disease (e.g. viral hepatitis, metabolic liver disease, toxic liver disease) were excluded. We identified a total of 93 obese patients (72 female and 21 male) meeting the aforementioned criteria in our cohort (see Table 1). The surgeon's procedure of choice – adjustable gastric band, Roux-Y gastric bypass surgery, or sleeve gastrectomy – was based on clinical experience, as well as the current guidelines as adapted to the patient's clinical conditions and comorbidities.

This observational study was approved by the ethics committee (Institutional Review Board) at the University Hospital Essen. Patients volunteering were informed about intraoperative risks of wedge liver biopsy and provided written informed consent.

2.2. Sample preparation and analyses

The degree of NAFLD was quantified using wedge liver biopsies taken during surgery and stained with hematoxylin and eosin, according to the NAFLD activity score (NAS) by an experienced pathologist (H. B) [15]. In representative slides the mean adipocyte diameter was calculated using multiple (>50) individual adipocyte diameter measurements (Image J, NIH, USA). The remaining samples were scored by two independent observers (A.W. and M.S.) and discordant cases were reevaluated and discussed using a conference microscope. Full length CK18 (M65),

Table 1 – Principal characterization of study population (n = 93) and serum parameters.

Gender		male	n = 21	22%	female	n = 72	78%
surgery	gastric bypass	36%			46%		
	sleeve	64%			49%		
	gastric banding	0%			5%		
		median	min	max	median	min	max
age at surgery	[years]	45	35	63	42	23	65
Size	[cm]	179	165	191	168	153	188
Weight	[kg]	176	114	220	142	95	234
BMI	[kg/m^2]	52	38.5	72	50	34.5	78
AST	[U/l]	35	14	102	27	11	117
ALT	[U/l]	29	15	50	25	15	98
GGT	[U/l]	38	12	180	29	14	136
LDH	[U/l]	244	140	472	227	89	397
bilirubin	[mg/dl]	0.55	0,26	1.17	0.48	0.18	2.21
Quick	[%]	100	83	118	100	43	119
INR		1.00	0.90	1.12	0.96	0.85	1.56
PTT	s	29	25	37	28	24	42
hemoglobin	[g/dl]	15.2	8.4	18.3	13.6	10.1	15.8
hematocrit	[%]	45.2	27.1	54.6	41.0	32.0	46.7
erythrocytes	[µl]	5.15	3.07	5.97	4.76	3.59	5.59
thrombocytes	[nl]	245	152	425	293	164	531
leucocytes	[nl]	7.6	4.3	12.0	7.85	3.9	13.3
creatinine	[mg/dl]	0.82	0.69	4.48	0.73	0.48	2.99
total cholesterol	[mg/dl]	209	96	253	198	138	307
LDL	[mg/dl]	133	64	180	132	76	222
HDL	[mg/dl]	39	24	59	48	27	87

BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyltransferase; LDH: lactate dehydrogenase; INR: international normalized ratio; PTT: partial thromboplastin time; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

released from dying epithelial cells, was quantified in patients' sera (Tecomedical Group, Sissach, Switzerland). Serum levels of adiponectin (Human Adiponectin/Acrp30 Quantikine ELISA Kit, R&D, Minneapolis, MN), C-reactive protein (CRP) (IBL International, Hamburg, Germany), and leptin (R&D, Minneapolis, MN, US) were determined using specific ELISA assays on day of surgery and six weeks after surgery. All procedures were conducted according to the manufacturers' instructions.

For lipid analysis, total fatty acids (free and esterifed) were extracted according to Bligh & Dyer [16]. Derivatization and data acquisition by GC-MS were performed as described in detail previously [17]. Briefly, lipids were dried and fatty acids hydrolyzed in methanolic NaOH. Synthesis of methyl esters was catalyzed by BF_3 A Trace-DSQ. GC-MS equipped with a TR-FAME 30 m column running on helium as a carrier gas was used for analysis. The mass spectrometer was operated in electron impact (EI) mode and fatty acids were detected in full scan of m/z 80–400.

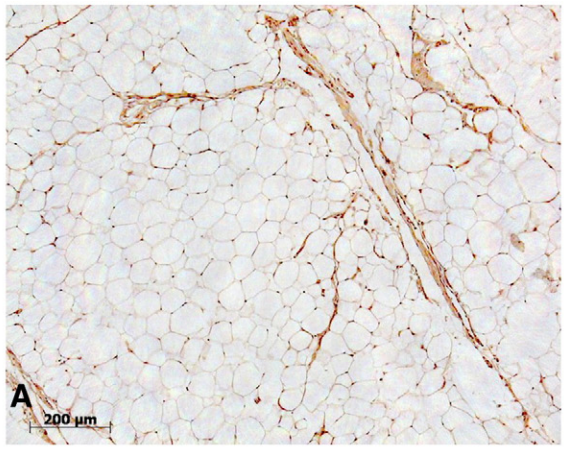
Serum cholesterol, triglycerides (DiaSys Diagnostic Systems, Holzheim, Germany) and non-esterified fatty acids (Wako Chemicals, Neuss, Germany) were measured enzymatically. ApoAI, ApoB (DiaSys Diagnostic Systems, Holzheim, Germany), ApoAII, ApoCII, ApoCIII, ApoE (Kamiya Biomedical, Seattle, USA) and lipoprotein(a) (Wako Chemicals, Neuss, Germany) were determined by immunoturbidimetry. Lipoprotein analyses were performed using an Olympus AU640 analyzer (Olympus Diagnostika, Hamburg, Germany).

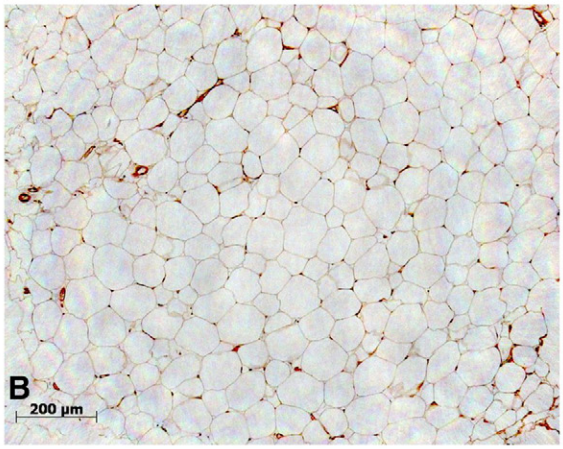
Real-time PCR analysis to assess mRNA level of NLRP3 was performed using total RNA isolated from liver tissue and analyzed as previously described [18]. The sequences of primers used are as follows: NLRP3 FWD 5′-GAT CTT CGC TGC GAT CAA CAG-3′, REV 5′-CGT GCA TTA TCT GAA CCC CAC-3′; Reference gene: HPRT FWD 5′GAC.CAG.TCA.ACA.GGG.GAC.AT-3′, REV 5′-CTT.GCG.ACC.TTG.ACC.ATC.TT-3′.

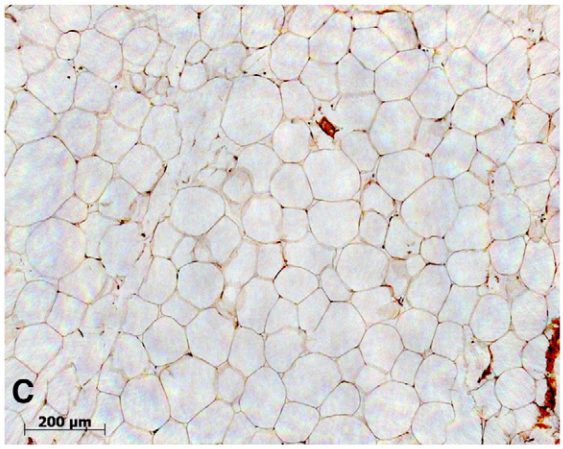
2.3. *Data analysis*

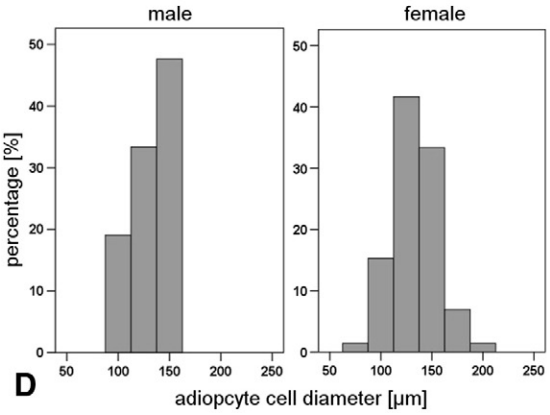
Analyses were performed with SPSS software package (version 19.0.0; SPSS, Chicago, IL, USA) and Graph Pad (version 5.03; Graph Pad, Graph Pad Software, CA, USA). The significance level was set at $\alpha = 5\%$ for all comparisons. Differences

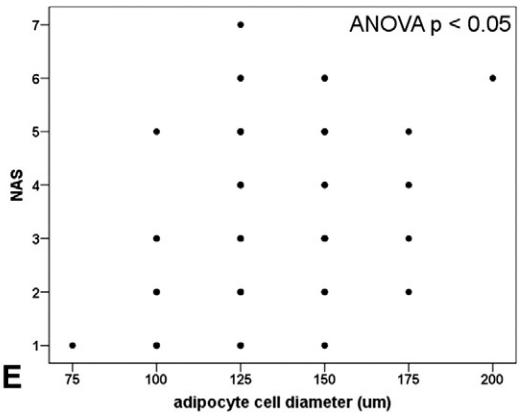
Fig. 1 – Visceral adipocyte diameter correlates with liver injury. Biopsies taken from the visceral adipose tissue were routinely stained and quantified for adipose cell diameter. Samples were grouped by their average adipocyte cell diameter and ranged from 75 µm to 200 µm. Samples with an average diameter of 100 µm (A), 150 µm (B), and 200 µm (C) were observed in men (n = 21) and women (n = 72) without significant gender specific differences (D). We found smaller adipocyte diameter preferentially in patients with lower NAS and larger adipocyte diameter in patients with higher NAS ($p < 0.05$, ANOVA). Adipocyte cell diameter was significantly correlated to serum levels of ALT ($r = 0.312$, $p < 0.05$), CRP ($r = 0.272$, $p < 0.05$), and leptin ($r = 0.304$, $p < 0.05$).

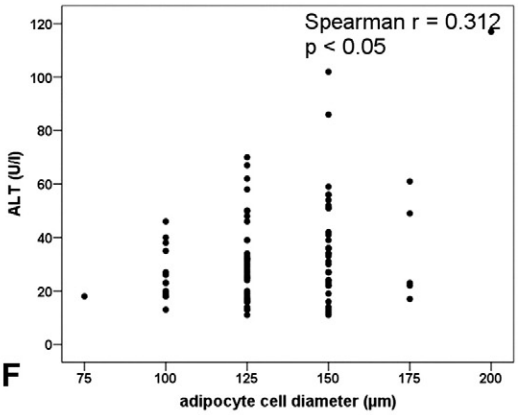

A
200 µm

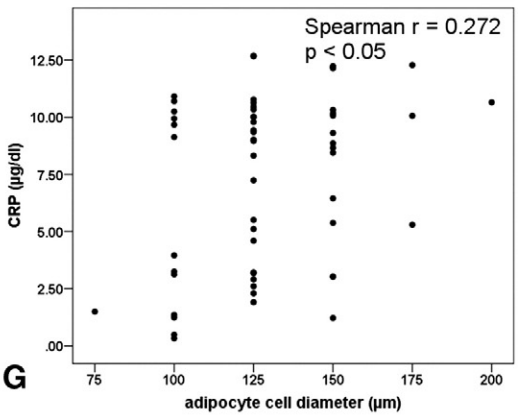

B
200 µm

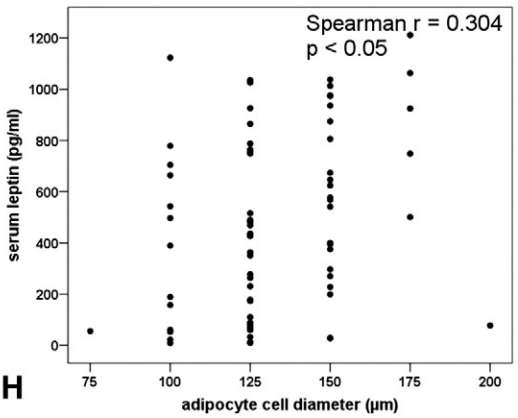

C
200 µm


male
female
percentage [%]
adiopcyte cell diameter [µm]
D


ANOVA p < 0.05
NAS
adipocyte cell diameter (um)
E


Spearman r = 0.312
p < 0.05
ALT (U/l)
adipocyte cell diameter (µm)
F


Spearman r = 0.272
p < 0.05
CRP (µg/dl)
adipocyte cell diameter (µm)
G


Spearman r = 0.304
p < 0.05
serum leptin (pg/ml)
adipocyte cell diameter (µm)
H

between three or more groups were compared by ANOVA. Differences between two groups of data with a nonnormal distribution were compared by the Mann–Whitney test. Linear correlations between two variables were described by Pearson's rank correlation coefficient or Spearman's rank correlation coefficient (rho). Wilcoxon rank-sum tests were used for comparison of continuous variables. Unless otherwise stated, data are expressed as mean ± SD or as median and range/quartiles for continuous variables and as an absolute number or percentage for categorical variables.

3. Results

3.1. General characteristics

Table 1 summarizes characteristics of 93 severely obese patients (21 male/72 female) undergoing bariatric surgery. Men had a median age of 45 years (range 35 to 63 years) and women had a median age of 42 years (range 23 to 65 years) at the time of surgery. Bodyweight of male patients ranged from 114 kg to 220 kg (median 176 kg) and between 95 and 234 kg (median 142 kg) for female patients. The BMI ranged from 38.5 kg/m^2 to 72 kg/m^2 in men and 34.5 kg/m^2 to 78 kg/m^2 in women. A Roux-en-Y gastric bypass was performed in 36% of the male patients and 46% of female patients. Sleeve gastrectomy was done in 64% of male patients and 49% of female patients. Adjustable gastric banding was implanted in 5% of female patients.

3.2. Adipocyte cell diameter is independent of gender and correlates with NAFLD Activity Score (NAS), transaminase levels, leptin levels, and CRP values

Within our cohort we found a wide range of average diameter among adipocytes in the samples of the visceral adipose tissue obtained during weight loss surgery. Recently among healthy subjects a mean adipocyte diameter of 70 μm was demonstrated [19]. The average diameter of adipocytes in tissue samples of visceral fat tissue was 125 μm (range 75 μm to 200 μm) with homogenous distribution within individual patients (Fig. 1A to C). No gender specific differences in adipocyte cell size or adipose tissue cell infiltration were observed (Fig. 1D). Interestingly, we found lower NAS in patients with small adipocyte diameter and higher NAS in patients with larger adipocyte diameter (Fig. 1E). In line with this, serum levels of ALT and C-reactive protein (CRP) – a global inflammatory marker – were also significantly correlated with the mean adipocyte diameter (Fig. 1F). As expected, the diameter of visceral adipocytes was positively correlated with serum levels of leptin (Fig. 1H).

3.3. In patients with severe obesity NAFLD Activity Score (NAS) is independent of BMI but correlated to inflammation and liver injury

Liver serum parameters including ALT, AST, GGT, bilirubin and coagulation tests, as well as thrombocyte counts remained in the normal range for most patients and were only mildly elevated in others (Table 1). Patients in the presented cohort represented the full spectrum of NAFLD with NAS ranging from 1 to 7 (NAS 0: 0%; NAS 1: 15%; NAS 2: 20%; NAS 3: 25%; NAS 4: 14%; NAS 5: 18%, NAS 6: 7%, NAS 7: 1%). Fig. 2 depicts a patient liver sample with a NAS of 1 and an adipocyte cell size of 125 μm, and one patient with a NAS of 6 and 200 μm visceral adipocyte diameter (Fig. 2A). We observed neither gender specific differences in NAS nor associations with the BMI and NAS (Fig. 2B). However, we found significantly increased serum levels of CRP (Fig. 2C), ALT (Fig. 2D), and AST (Fig. 2E), in patients with NAS > 4 (being definite NASH) when compared to subjects with NAS ≤ 4 (being simple steatosis & borderline NASH). Serum levels of M65 (Fig. 2F), an epithelial cell death marker, were also increased in patients with NAS >4 ($p = 0.06$; Mann–Whitney test).

3.4. The NAFLD activity score is associated with serum levels of triglycerides, ApoCIII and levels of long chain free fatty acids

Given that these obese patients had hypercholesterolemia and hypertriglyceridemia, we next determined their apolipoprotein contents and studied the composition of the lipoprotein particles in relation to their liver status (Table 2). Correlations between ApoCIII and triglycerides (Pearson's rank correlation coefficient $r = 0.852$), as well as between ApoAI and high density lipoprotein (HDL) ($r = 513$), were used as an internal validation of serum lipid analysis ($p < 0.05$ for both). We found that serum levels of triglycerides, as well as levels of ApoCIII, were significantly increased in patients with NAS > 4 when compared to subjects with NAS ≤ 4 (Fig. 3A–B).

Since flux of potentially lipotoxic free FAs resulting from WAT lipolysis under conditions of insulin resistance may determine liver injury in NASH [7], we next analyzed the total FA composition of serum and triglyceride particles in a representative sub-cohort of 24 patients (7 male/17 female) (Table 3). Values of FAs are given as a ratio to an internal standard control. Serum levels of palmitic acid (16:0), palmitoleic acid (16:1n7), stearic acid (18:0), oleic acid (18:1n9), and dihomo-gamma-linolenic acid (20:3n6) (DGLA) significantly increased in patients with NAS > 4 when compared to subjects with NAS ≤ 4 (Fig. 3C-D).

3.5. Patients with increased NAS exhibit greater benefits in serum lipid component changes

To assess acute weight loss effects on serum levels of triglycerides and apolipoproteins in severely obese patients, blood samples were drawn 6 weeks after surgery and analyzed. Notably, serum levels of TG and ApoCIII, which inhibits lipoprotein lipase and may delay hepatic uptake of triglyceride-rich particles, were significantly reduced at follow-up after bariatric surgery while levels of other apolipoproteins did not change significantly (Fig. 4A–B). Patients with NAS > 4 showed especially reduced levels as soon as six weeks after surgery. We also assessed the serum levels of free fatty acids and found significantly reduced levels of potentially lipotoxic stearic acid (18:0) and DGLA (20:3n6) ($p < 0.05$) (Fig. 4C–D). In line with measurements in TG and ApoCIII, patients with NAS > 4 exhibited greater benefits when compared to patients with NAS < 4.

3.6. Low serum adiponectin level and high NLRP3 mRNA are associated with NAS

As we observed an increase in several apolipoproteins and multiple FFAs in patients with greater NAS, we next analyzed serum levels of adiponectin, a major regulator of fatty acid

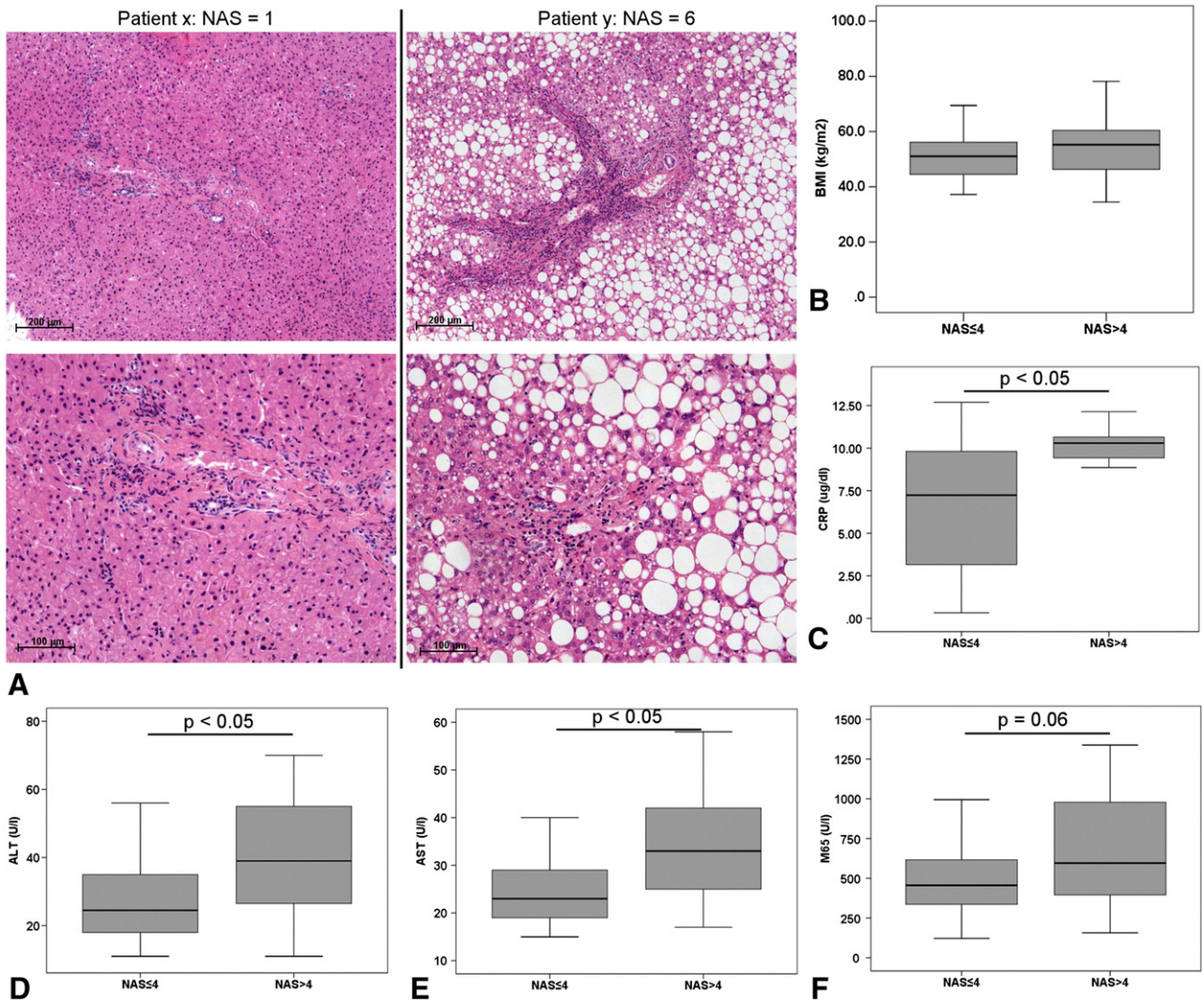


Fig. 2 – Bariatric surgery cohort displays whole spectrum of NAFLD. **Liver biopsies taken from patients undergoing bariatric surgery showed the whole spectrum of NAFLD (A). Representative liver histology of a patient with NAS of 1 (10× in upper panels; 20× in bottom panels) who had an adipocyte cell size of 125 µm, and a patient with NAS of 6 and 200 µm sized visceral adipocytes are shown. The BMI was independent of NAFLD activity score (B). Serum levels of CRP (C), ALT (D), and AST (E) were significantly increased in patients with NAS > 4 (being definite NASH) when compared to subjects with NAS ≤ 4 (being simple steatosis & borderline NASH). Serum levels of M65 (F), an epithelial cell death marker, were also increased in patients with NAS >4 (p = 0.06; Mann–Whitney test).**

Table 2 – Composition of apolipoproteins in serum samples.

		before surgery, n = 49			six weeks after surgery, n = 25		
		median	min	max	median	min	max
ApoAI	mg/dl	123	74	177	139	76	190
ApoAII	mg/dl	30	20	40	31	18	40
ApoB	mg/dl	83	51	190	86	55	191
ApoCII	mg/dl	4	2	12	4	2	18
ApoCIII	mg/dl	11	4	27	8	4	26
ApoE	mg/dl	10	3	20	10	5	17
Lp(a)	mg/dl	9	1	47	9	1	41

oxidation in the liver, and mRNA level of NLRP3, the key protein in the NLRP3 inflammasome. We found significantly reduced adiponectin serum levels in patients with a NAS score greater than 4 when compared to those with a NAS equal to or less than 4 (Fig. 5A). Hepatic NLRP3 mRNA levels were significantly higher in patients with NAS greater than 4 (Fig. 5B). Notably those mRNA levels were associated with serum levels of M65, as well as with serum levels of the long chain free fatty acid DGLA (Fig. 5C-D).

4. Discussion

The key findings of the presented study, as summarized in Fig. 6, include (i) adipocyte cell diameter is independent of

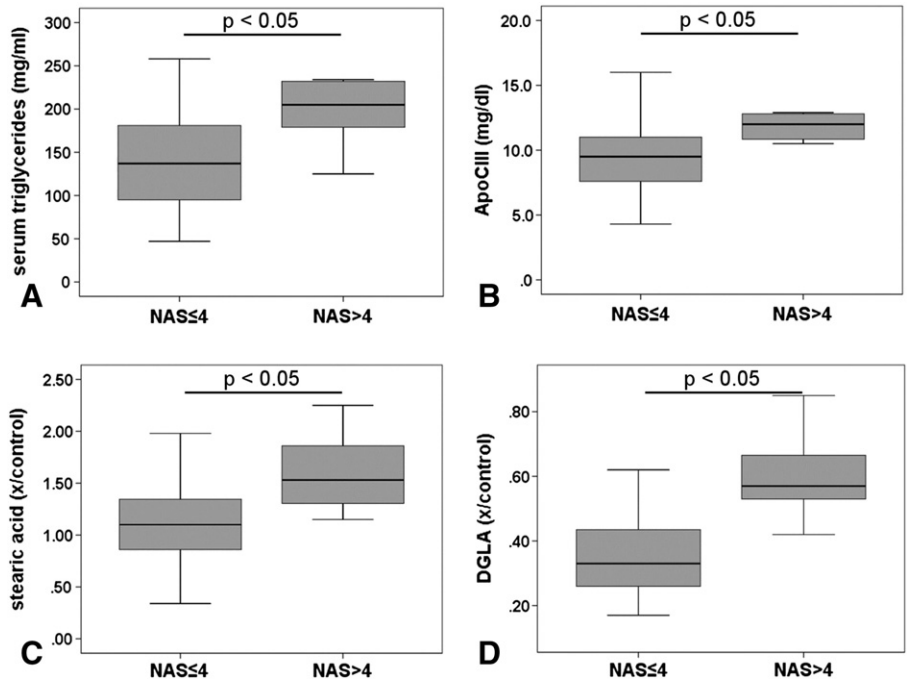


Fig. 3 – Analysis of serum lipid components. **Serum levels of triglycerides, as well as levels of ApoCIII, were significantly increased in patients with NAS > 4 when compared to subjects with NAS ≤ 4 (49 patients: 13 male/36 female) (A, B). Serum levels of long chain free fatty acids like stearic acid (18:0) and dihomo-gamma-linolenic acid (20:3n6) (DGLA) were significantly increased in patients with NAS > 4 when compared to subjects with NAS ≤ 4 undergoing bariatric surgery (24 randomly chosen patients: 7 male/17 female) (C, D).**

BMI and gender; (ii) NAS, transaminases levels, leptin levels, and CRP values are associated with visceral adipocyte cell diameter; (iii) serum and histological liver injury are associated with altered apolipoprotein serum levels; (iv) serum triglycerides and free fatty acids are correlated with liver injury and (v) effective bariatric surgery improves levels of potentially lipotoxic free fatty acids.

The observation that cell diameter of adipocytes within the visceral adipose tissue of severely obese patients is correlated neither with their bodyweight nor with BMI was made regardless of patients' gender or age and is in line with a recent study by O'Connell [20]. More recently, studies have shown functional differences in large and small adipocytes from the same subjects, including altered gene expression profiles [21]. We were able to confirm that the levels of adipocyte hormone leptin are dependent on mean adipocyte size [22]. Adipocyte size has also been shown to influence adipokine secretion, with increasing adipocyte size resulting in a shift toward a dominance of pro-inflammatory adipokines [22,23]. Circulating levels of CRP, an acute-phase protein mainly synthesized in the liver [24], are known to be elevated in obese subjects and the levels are directly correlated with the amount of body fat and visceral obesity [25]. We were able to confirm that serum CRP levels are positively correlated with NAS [26], while other studies did not find such an association [27]. Another study reported that adipose tissue macrophage number and production of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), were positively correlated with larger adipocytes [28]. Omental macrophage accumulation has also

Table 3 – Composition of free fatty acids in serum.

n = 24		before surgery			six weeks after surgery			p value
		median	min	max	median	min	max	
palmitic acid (16:0)	x/control	4.71	2.20	7.99	4.32	2.96	6.05	n.s.
palmitoleic acid (16:1n7)	x/control	0.29	0.14	0.76	0.23	0.12	0.55	n.s.
stearic acid (18:0)	x/control	1.20	0.34	2.25	0.93	0.44	1.65	p < 0.05
oleic acid (18:1n9)	x/control	3.22	1.80	5.41	3.09	2.32	4.34	n.s.
trans vaccenic acid (18:1n11)	x/control	0.27	0.15	0.61	0.30	0.18	0.46	n.s.
linoleic acid (18:2n6)	x/control	3.31	1.58	4.54	2.99	1.51	4.72	n.s.
dihomo-gamma-linolenic acid (20:3n6)	x/control	0.42	0.17	0.85	0.24	0.14	0.60	p < 0.05
arachidonic acid (20:4n6)	x/control	1.61	0.99	2.64	1.60	1.01	3.02	n.s.
clupanodic acid (22:5n3)	x/control	0.08	0.03	0.19	0.07	0.04	0.14	n.s.
cervonic acid (22:6n3)	x/control	0.33	0.22	0.71	0.32	0.17	1.03	n.s.

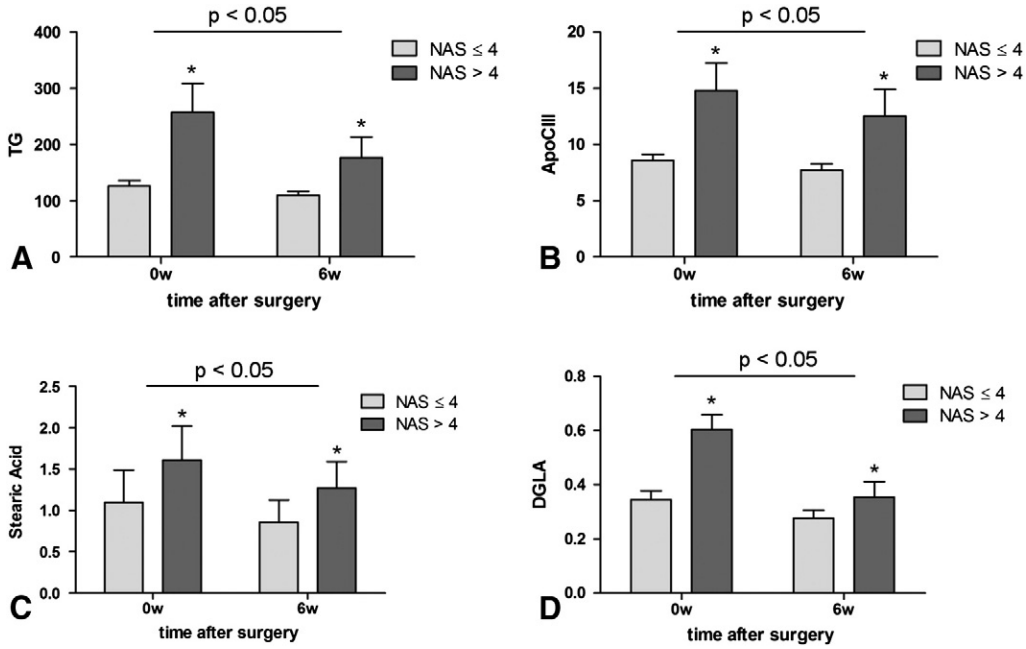


Fig. 4 – Short term follow-up of serum lipid components. **Analysis of blood samples drawn 6 weeks after bariatric surgery revealed significantly decreased serum triglyceride levels (A). Patients with NAS > 4 showed markedly reduced levels as soon as six weeks after surgery. A follow-up analysis of apolipoprotein levels was done six weeks after surgery in 25 patients (7 male/18 female) and showed that ApoCIII serum levels were significantly reduced (B). Serum levels for long chain free fatty acids like stearic acid and dihomo-gamma-linolenic acid (DGLA) were also significantly lower between the 2 time points (C,D). (* = p <0.05 when NAS ≤4 are compared to NAS > 4 at indicated time points).**

been associated with liver damage independent of the degree of insulin resistance [29]. Finally, secretion of the proinflammatory adipocytokines, leptin, IL-6, IL-8, and monocyte chemoattractant protein-1, from cultured adipocytes correlated positively with cell size [22]. In summary, this could suggest an inflammatory adipo- or cytokine profile due to

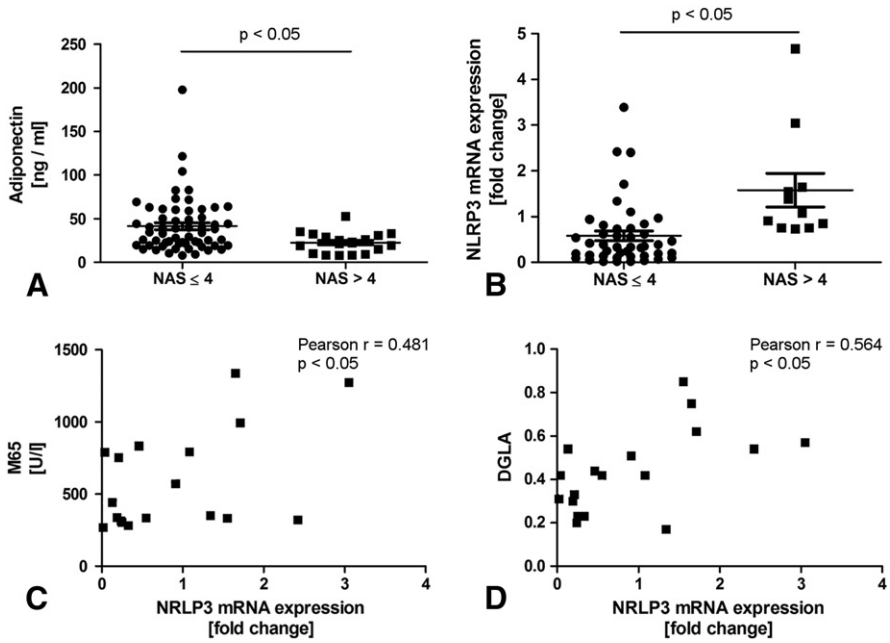


Fig. 5 – Adiponectin and NLRP3 mRNA levels correlate with NASH. **We were able to demonstrate that adiponectin levels are significantly lower in patients with NAS greater than 4 (A). Notably, mRNA levels of NLRP3 were significantly increased in the liver samples of patients with NAS greater than 4 when compared to patients with values less than or equal to 4 (B). Those mRNA levels were significantly correlated with a serum marker of total cell death (M65) (C) and to serum levels of long chain fatty acids as DGLA (D).**

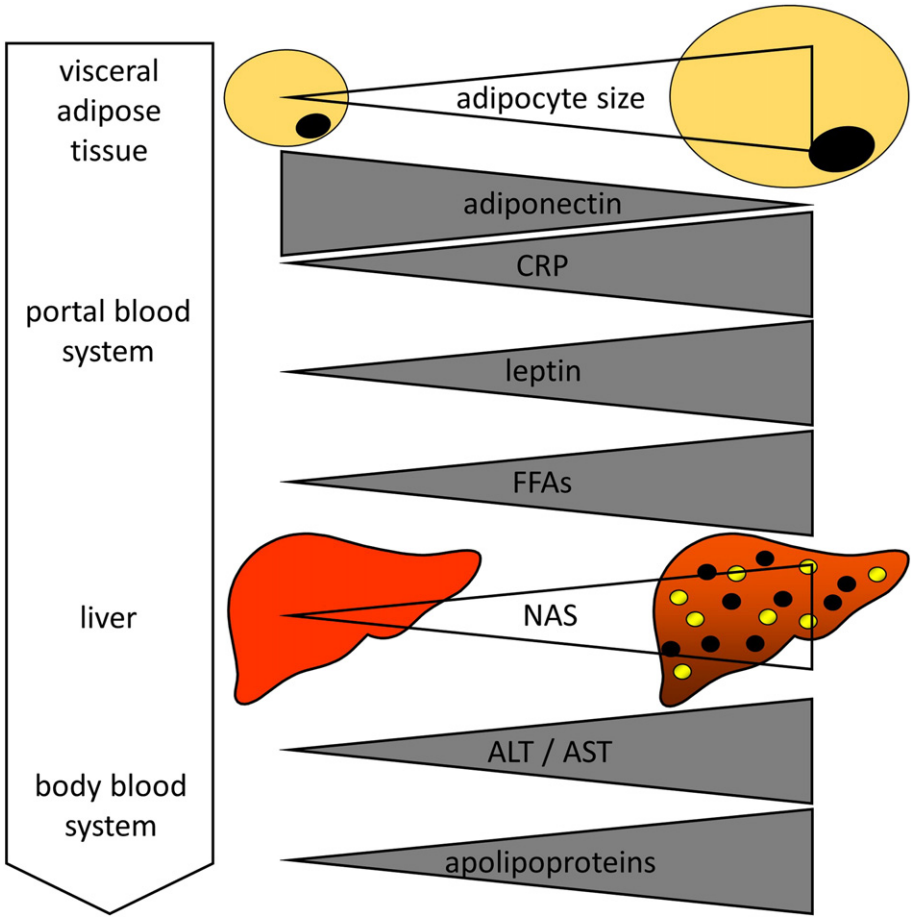


Fig. 6 – Visceral adipocyte diameter correlates with liver injury. Increasing diameter in visceral adipocytes is associated with reduced adiponectin and increased leptin levels in the portal blood system. Both factors, along with increased levels of c-reactive protein (CRP) and free fatty acids (FFAs), are associated with increased liver disease when assessed by the NAFLD activity score (NAS). The liver secretes serum transaminases (ALT/AST) and apolipoproteins in response to injury that enter the blood system and thus the whole body. Bariatric surgery positively influences all mentioned serum parameters (gray objects).

increased omental adipocyte size as an important factor in the 'second hit' of the 'two hit' hypothesis of steatohepatitis [30].

In the present manuscript we show that the serum concentrations of several apolipoproteins, especially ApoCIII, are associated with serological and histological markers of liver injury. Apolipoprotein (apo) C-III, a small protein that resides on the surface of several lipoproteins [31], provokes inflammatory and atherogenic responses in cells that are involved in atherosclerosis [32]. Genetic variants in ApoCIII have recently been implicated in the pathogenesis of NASH, in addition to variants of adiponutrin/PNPLA3 [33,34]. It is known that the plasma concentration of ApoCIII in VLDL and LDL, or the concentration of LDL itself, can predict the risk of cardiovascular disease, or progression of coronary atherosclerosis, independently of standard lipid risk factors [35,36]. In the present study, patients demonstrated decreased TG levels in association with a lowering of their ApoCIII levels as early as 6 weeks after bariatric surgery. This finding may have important implications for moderating the elevated cardiovascular risk notoriously associated with NAFLD/NASH.

Visceral adipocytes exhibit several differences when compared with subcutaneous adipocytes: (a) higher basal lipolysis, (b) distinct sensitivity to catecholamines, (c) poor sensitivity to insulin, and (d) direct access to the portal vein [37]. These features may be further evident when visceral adipocytes become hypertrophic [38]. Adipocytes tend to flood the portal circulation with FFAs at metabolically inappropriate times when FFAs are unlikely to be oxidized and more likely to induce hepatic injury. Our patients' sera also revealed high FFA levels, a common feature of the metabolic syndrome [39]. This overflow hypothesis proposes that as the size of an adipocyte increases, it will eventually reach a limit at which point it will be unable to store further lipid [40]. Subsequently, visceral fat cell hypertrophy leads to detrimental metabolic effects, possibly because of close anatomic proximity to hepatic and visceral immune cell populations [41]. In the present study we are the first to show that bariatric surgery has a beneficial influence on serum FFA levels. We found significantly reduced levels of stearic acid and DGLA six weeks after surgery. Stearic and palmitic acids originate from both diet and *de novo* lipogenesis, whereas linoleic acid is not produced in the human body. Since DGLA is the precursor used to produce arachidonic acid, it stands to reason that the lowering of DGLA could result in

less inflammation. The reduction in the serum fatty acid levels is likely a reflection of fewer and less FFA-secreting adipocytes generating an environment for decreased WAT inflammation.

We recently showed that the NLRP3 inflammasome is important in the development of liver inflammation [18]. Moreover, in a murine model of NASH using both NLRP3 gain of function and loss of function mice, we were able to uncover a crucial role for the NLRP3 inflammasome in the development of steatohepatitis and fibrosis [42]. In the present study we demonstrated that patients with NAS greater than 4 exhibit increased NLRP3 mRNA levels and that those levels are associated with liver cell death and increased serum levels of long chain free fatty acids.

The strength of our study is the comprehensive analysis of the interaction between adipose tissue metabolism and non-alcoholic fatty liver disease. To the best of our knowledge, this is the first study that integrates the aforementioned tissue readouts with changes in apolipoproteins and free fatty acids. Our study has the limitation of being a short term observational study and can therefore not address associations in long-term changes with regard to the surgical procedure. Mechanistic differences between various bariatric surgery techniques are known. However, it was not possible to discriminate these techniques, as separation of patients according to surgical procedures would lead to very small groups. A limitation of most studies, including the presented, is the fact that they do not have follow-up liver biopsies to assess histologic changes after surgery. While this study was performed without healthy controls, which is another limitation, the major findings relate to changes after bariatric surgery. A similar control setting with comparable baseline parameters or healthy individuals is currently not available. However, previous data clearly demonstrate the ability of bariatric surgery to achieve profound weight loss, normalize hyperlipidemia, resolve hyperglycemia, and improve NAFLD [43,44].

5. Conclusions

In summary, our data provide a coordinated and comprehensive assessment of adipocyte cell size, liver histology, and serum parameters of lipid metabolism in morbidly obese patients undergoing bariatric surgery. Adipocyte cell size and serum values of free fatty acids, as well as apolipoproteins, were significantly associated with liver injury. Following bariatric surgery, we observed elevations in adiponectin, reduced liver cell death markers, and beneficial changes in the composition of free fatty acids and apolipoproteins.

Author Contributions

Study concept and design: AW, MS, MT, AC;
Acquisition of data: AW, MS, TC, TS, HS, HK, HB;
Analysis and interpretation of data: AW, LPB, JPS, HS;
Drafting of the manuscript: AW, LPB, TC;
Critical revision of the manuscript for important intellectual content: JPS, HB, AF, MT, AC;
Statistical analysis: AW, LPB;
Obtained funding: GG, AF, MT, AC;
Administrative, technical, or material support: HS, HK, HB, AF;
Study supervision: AF, MT, AC.
All authors approved the final draft submitted.

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Disclosure statement

No conflicts of interest to disclose.

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Non-alcoholic fatty liver disease progresses to hepatocellular carcinoma in the absence of apparent cirrhosis

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Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in developed countries, and accumulating evidence suggests it as the hepatic manifestation of the metabolic syndrome (MS). Although the published prevalence of hepatocellular carcinoma (HCC) is low in NAFLD/NASH patients, most of these data have been derived from areas endemic for viral hepatitis. We recruited 162 adults with HCC between February 2007 and March 2008, investigated the underlying etiologies and determined the prevalence of the MS and related features within each group. Patients with NAFLD/NASH-associated HCC exhibited a higher prevalence of metabolic features (Type 2 diabetes mellitus, hypertension, dyslipidemia, coronary artery disease) compared to non-NAFLD/NASH-HCC. Intriguingly, a significant number (41.7%; $p < 0.005$) of individuals with NAFLD/NASH-HCC had no evidence of cirrhosis. Patients with alcohol-induced liver disease also displayed many features (14/19, 73.7%) of the MS, although, in contrast to NAFLD/NASH-HCC, alcohol-associated HCC was highly associated with cirrhosis (95.0%; $p = 0.064$). NAFLD/NASH as the hepatic entity of the MS may itself pose a risk factor for HCC, even in the absence of cirrhosis. The MS may also promote development of HCC among those with alcoholic liver disease. Increased awareness of liver manifestations in the MS may instigate early interventions against developing HCC.

Introduction

The most common cause of chronic liver disease (CLD) among children and adults in the western world is non-alcoholic fatty liver disease (NAFLD).[1] NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH).[2] While NAFL can be quite unobtrusive, NASH is a serious condition, with nearly a quarter of affected patients developing cirrhosis, which, in turn, increases the risk of subsequent progression to hepatocellular carcinoma (HCC).[3] Recent studies, however, suggest that HCC could also arise in the steatotic liver without evidence of underlying cirrhosis,[4] although this has not been proven yet.[5]

NAFLD is strongly associated with diseases of affluence such as obesity, Type II diabetes mellitus (T2DM) or insulin resistance, hypertension, and dyslipidemia and has been regarded as the liver manifestation of the metabolic syndrome (MS).[6] Indeed, 75% of obese individuals have hepatic steatosis.[7,8] The prevalence of the MS is rapidly increasing, due to hyperalimentation and a sedentary life style,[9] and is reflected by the increasing prevalence of NAFLD and associated complications.[10] NAFLD affects a third of adults, while NASH occurs in about 3% of the adult population in the western world.[11,12]

HCC is the fifth most common tumor worldwide with increasing incidence and third leading cause of cancer-related death.[13,14] Although most HCC occur in the setting of cirrhosis, up to 20% of affected patients have no underlying cirrhosis.[15] Thus cirrhosis may not always be essential for development of HCC. Chronic viral hepatitis such as hepatitis B virus (HBV) or hepatitis C virus (HCV),[16] and alcoholic liver disease[17] are well-recognized causes of cirrhosis, while autoimmune hepatitis (AIH), primary biliary cirrhosis and NASH[18] have, hitherto, occurred less commonly and thus have not been recognized as primary drivers for HCC.

HCC remains most commonly seen in Asia and Africa, with nearly 80% of the estimated 500,000 new cases each year occurring in these areas.[13] Therefore, a large body of published data and studies has originated from Asia. Tumors

Epidemiology

Key words: hepatocellular carcinoma, NASH, NAFLD, metabolic syndrome, BMI

Abbreviations: AIH: auto-immune hepatitis; BMI: body mass index; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; T2DM: type 2 diabetes mellitus; Additional Supporting Information may be found in the online version of this article.

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Table 1. Identification of cirrhosis and non-cirrhosis in NASH and cryptogenic patients

	NASH		Cryptogenic	
Factor	No cirrhosis ($n = 17$)	Cirrhosis ($n = 19$)	No cirrhosis ($n = 14$)	Cirrhosis ($n = 9$)
Histologically confirmed	$n = 10$	$n = 5$	$n = 9$	$n = 2$
Age at time of diagnosis (mean ± SEM)	69.5 ± 2.78	67.9 ± 1.41	68.18 ± 2.33	68.31 ± 2.45
Obesity	15 (93.8%)	18 (94.7%)	4 (33.3%)	1 (11.1%)
Type II diabetes	11 (64.7%)	12 (63.2%)	0 (0%)	3 (33.3%)
Ascites[1]	3 (17.6%)	5 (26.3%)	2 (14.3%)	3 (33.3%)
Thrombocytopenia[1]	1 (5.9%)	11 (57.9%)	1 (7.1%)	4 (44.4%)
splenomegaly[1]	4 (23.5%)	16 (84.2%)	2 (14.3%)	5 (55.6%)

[1]All patients presenting with one or more of these symptoms were labeled as non-cirrhotic, when histological confirmation could be obtained or no radiological signs of cirrhosis were shown and portal hypertension was allegeable by portal vein thrombosis.

developing in Asian patients appear more aggressive and Asian patients are often younger than Caucasian patients at the time of first diagnosis.[19,20] While in China and Taiwan, the incidence of HCC is declining due to improved public health campaigns such as HBV vaccinations,[13] it is on the rise in North America and Europe. In USA, the age-adjusted prevalence has doubled in the past two decades,[21] this could be attributed to environmental factors, such as obesity, a high-calorie diet, diabetes mellitus and the associated MS, NAFLD/NASH.

Differences in liver disease etiologies among cultural and geographically diverse populations suggest that the primary causes of HCC would be non-identical. For example, over 30% of the Western populations have NAFLD, whereas only 15% of the population is similarly affected in Asia. Thus, studies performed in one population may have limited informative value in a different population and will need to be conducted separately.[11] Therefore, the aims of our study were (i) to evaluate the underlying etiologies of HCC in a central European patient collective, (ii) to investigate whether NAFLD is associated with metabolic features and (iii) whether HCC could arise in non-cirrhotic livers.

Material and Methods

Patient Characteristics

All adult patients (age ≥ 18 years) with HCC, who were referred to the Department of Gastroenterology and Hepatology at our institution between February 2007 and March 2008 ($n = 162$), were retrospectively identified and reviewed. Exclusion criteria were two or more concurrent liver diseases, which could each cause cirrhosis and/or HCC ($n = 12$). HCC was identified radiologically in all patients. Radiological diagnosis was defined according to American Association for the Study of Liver Diseases practice guidelines on the management of HCC.[5,22] According to these guidelines, radiological diagnosis of HCC is defined as either (i) the presence of a hepatic lesion >2 cm in diameter with typical vascular pattern for HCC on one dynamic imaging technique and alpha-fetoprotein >200 ng/ml or (ii) the presence of a lesion 1–2 cm in diameter with typical vascular pattern for HCC on two dynamic imaging techniques. Histological confirmation was obtained in 64 (42.7%) patients.

Demographic data (age, sex and race), details of metabolic traits, including the body mass index (BMI), fasting serum glucose, triglycerides, high-density lipoprotein, as well as detailed medical history of hypertension, hyperlipidemia (defined as total serum cholesterol >200 mg/dl or ongoing use of statins) and diabetes (including related therapies) were recorded. Clinical data from the time of HCC diagnosis or first hospital visit were used to calculate the model for end stage liver disease score.

Cirrhosis was diagnosed or excluded histologically where clinically possible. Since biopsies could not be performed in the remaining patients, radiological evidence of cirrhosis in the presence of portal hypertension was established. In particular, we looked for clinical and diagnostic signs of liver cirrhosis, such as ascites, variceal bleed, thrombocytopenia and splenomegaly by ultrasonography, gastroscopy, MRI and CT. Details regarding the observed findings are given in Table 1 and Supporting Information Table 1.

NASH was defined according to the histological features of NASH, when available or cryptogenic cirrhosis in the presence of MS and without a history of significant alcohol intake.[23] MS was defined following the National Cholesterol Education Program Adult Treatment Plan III (ATP III) guidelines, with one exception, as waist circumference trait was replaced by BMI > 28 kg/m^2 in both men and women.[24] The diagnosis of diabetes mellitus was done using criteria recommended by the American Diabetes Association and Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (fasting blood glucose values of 126 mg/dl on two separate occasions in those not receiving hypoglycemic agents or corticosteroids). Patients receiving insulin or oral antidiabetic drugs were assumed to have diabetes mellitus. Alcohol abuse was defined as consumption of more than two drinks daily or more than six drinks daily on weekends for at least 5 years,[25] implying alcoholic liver disease.

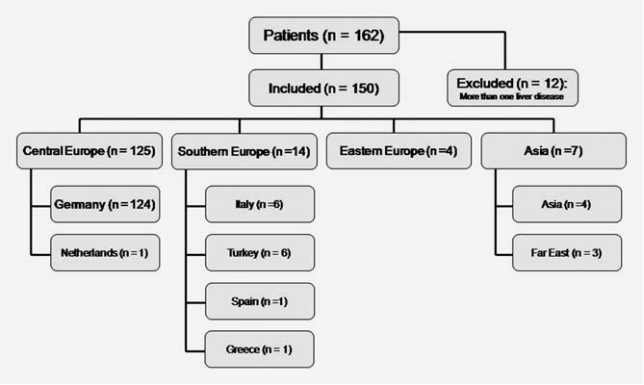

Figure 1. Distribution of patient origin. A total of 162 patients with hepatocellular carcinoma (HCC) were initially enrolled in this study, 12 were excluded, because of more than one underlying liver disease as potential cause for HCC. Thus, our study population consisted of 150 eligible patients. The largest proportion of patients originated from Central Europe ($n = 125$).

The presence of HBV and HCV was confirmed by qualitative sero-positivity for HBV DNA or HCV RNA using standard laboratory tests.

Data Analyses and Statistics

Data are given as means ± SEM for numeric variables, or as counts and percentages for categorical variables, unless stated otherwise. For statistical evaluation (i) two-sided unpaired *t*-tests; (ii) two-sided *t*-tests with Welch's correction in case of significantly different variances were performed. For categorical variables, we performed Mann-Whitney-U test (nonparametric). All calculations were done in GraphPad Prism software (GraphPad Software, San Diego, CA) and a $p < 0.05$ was considered significant.

Results

Demographic Data of Enrolled Patients

As previous studies have shown, male patients were predominant ($n = 125$, 83.3%). A total of 76.7% ($n = 115$) of the patients had histological, clinical or radiological features of cirrhosis. Incidence of cirrhosis was higher in male than female patients (M: 77.6% *vs.* F: 64%). Mean age of all patients was 64.7 ± 9.6 years (range 18.6–82.8 years). Patients with NASH and cryptogenic disease were slightly older compared to the other groups (NASH = 68.6 ± 8.4 years, cryptogenic = 68.2 ± 8.0 years, others = 62.7 ± 10.0 years; n.s.). Figure 1 displays the origin of the patients in our study. Most patients of this study were Caucasians from Western Europe ($n = 125$), mainly Germans ($n = 124$), followed by Southern Europe ($n = 14$), Asia ($n = 7$) and Eastern Europe ($n = 4$). Independent of patient origin, all patients had their main residence in Germany for at least a few years up to a few decades.

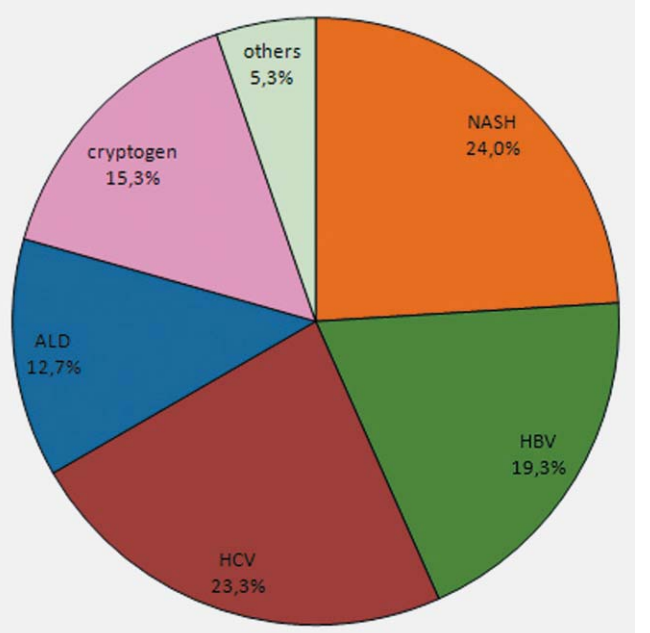

Figure 2. Etiologies of underlying liver disease for hepatocellular carcinoma (HCC). HCCs were most commonly associated with chronic viral hepatitides. The second leading cause for HCC was non-alcoholic steatohepatitis (NASH) (24%), while only 13% had alcoholic steatohepatitis.

Etiologies of HCC

Distribution of underlying etiologies (Fig. 2) and clinical parameters of the known liver diseases for HCC patients are given in Table 2. A total of 42% HCC patients had viral hepatitis (HBV—19%, HCV—23%). Surprisingly, only 13% of the HCC were associated with alcohol-induced liver disease. As anticipated, HBV, HCV and ALD were highly associated with cirrhosis (HBV—93.1%, HVC—94.3, ALD—94.7%), and contrasted other etiologies. Five percent of patients had AIH ($n = 2$), hemochromatosis ($n = 3$), primary sclerosing cholangitis ($n = 1$), and toxic liver disease ($n = 2$). Histologically or morphologically confirmed NASH was identified in 24% ($n = 36$). A comparatively high percentage of patients (15.3%) had cryptogenic disease (no liver disease was identified despite detailed laboratory and clinical investigations).

Low incidence of Cirrhosis in NASH/HCC Patients

All patients presenting with one or more of the symptoms ascites, thrombocytopenia, and splenomegaly were labeled as non-cirrhotic only when absence of cirrhosis was confirmed histologically ($n = 3$) or no radiological signs of cirrhosis could be seen and portal hypertension was allegeable by portal vein thrombosis ($n = 1$). Details regarding this classification can be found in Supporting Information Table 1.

Even though cirrhosis is a crucial risk factor for developing HCC, cirrhosis was detected in only 52.8% of patients

Table 2. Demographic data of enrolled HCC patients

Factor	Total (n = 150)	NASH (n = 36)	Cryptogen (n = 23)	HBV (n = 29)	HCV (n = 35)	Alcohol (n = 19)	Others (n = 8)
Age at time of diagnosis	64.7 ± 9.6	68.6 ± 8.4	68.2 ± 8.0	59.8 ± 11.6	64.0 ± 9.7	65.9 ± 5.7	60.2 ± 11.9
Male sex	125 (83.3%)	32 (88.9%)	18 (78.3%)	24 (82.8%)	27 (77.1%)	17 (89.4%)	7 (87.5%)
Caucasian	126 (84.0%)	35 (97.2%)	23 (100%)	16 (55.2%)	27 (77.1%)	17 (89.4%)	8 (100%)
Cirrhosis	115 (76.7%)	19 (52.8%)	9 (39.1%)	27 (93.1%)	33 (94.3%)	18 (94.7%)	6 (75.0%)
Type II diabetes	41 (61%)	23 (63.9%)	5 (21.7%)	10 (34.5%)	10 (28.6%)	11 (58.0%)	2 (25%)
BMI (kg/m^2)	26.7 ± 4.5	29.0 ± 3.7	23.8 ± 1.8	26.8 ± 4.8	25.2 ± 3.32	27.9 ± 6.5	26.5 ± 5.4
Albumin (mg/dl)	3.8 ± 0.8	3.8 ± 0.5	3.9 ± 0.4	3.8 ± 0.6	3.7 ± 0.5	3.8 ± 0.5	3.3 ± 1.1
Creatinine (mg/dl)	1.3 ± 0.9	1.4 ± 1.3	1.5 ± 1.3	1.1 ± 0.3	1.0 ± 0.1	1.2 ± 0.8	1.6 ± 1.3
Bilirubin (mg/dl)	1.4 ± 3.1	2.1 ± 6.1	0.8 ± 0.6	1.4 ± 1.3	1.2 ± 0.7	1.2 ± 0.9	1.8 ± 1.5
INR	1.2 ± 0.3	1.2 ± 0.4	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.4	1.1 ± 0.1	1.2 ± 0.1
MELD score	10.3 ± 4.4	10.9 ± 5.9	10.2 ± 4.9	10.1 ± 3.0	9.9 ± 3.3	10.1 ± 4.7	12.3 ± 2.5
AFP (ng/ml)	13,888.5 ± 68,030.0	19,576.9 ± 85,992.6	831.0 ± 2,578.2	24,216.7 ± 98,570.5	2,058.6 ± 8,612.3	8,014.8 ± 2,9095.2	47,059.7 ± 132,290.5

with NASH-associated HCC. Among the cryptogenic group, even fewer patients had cirrhosis (39.1%). In contrast, cirrhosis was seen in nearly 90% of patients with non-NASH and non-cryptogenic HCC, suggesting that NASH *per se* could promote development of HCC. Detailed data on differentiation between cirrhotic and non-cirrhotic NASH patients are given in Table 1 and Supporting Information Table 1. Incidence of obesity and T2DM was higher in cirrhotic NASH patients, than in non-cirrhotic patients, although this difference failed to reach significance.

Patients with NASH and HCC Exhibit More Features of the MS

As expected, patients with NASH were more likely to be overweight (*i.e.* BMI: NASH *vs.* HCV $p < 0.0001$; NASH *vs.* cryptogenic: $p < 0.0001$) (Fig. 3*a*). Close to 95% of NASH patients exhibited BMI > 25 kg/m^2 (Fig. 3*b*).

The prevalence of Type II diabetes mellitus was nearly two fold higher in NASH (64%) compared to other liver diseases (Fig. 3*c*). Similarly, over 70% of NASH individuals are hypertensive, compared to 50% in other HCC-groups (Fig. 3*d*), although this could be attributed to an age-bias within the NASH cohort.

Nearly half of the NASH patients had dyslipidemia (Fig. 3*e*). Interestingly, fewer than 10% of HCV patients had dyslipidemia despite recognized aberrations in lipid metabolism.[26–28] A higher frequency of coronary artery disease and heart failure were also noted among NASH patients (30.6% *vs.* 17.5%; $p = 0.0945$; Fig. 3*f*), although these findings could similarly be related to the increased age in NASH patients.

To exclude the effect of cirrhosis on the occurrence of the MS, we grouped all non-cirrhotic patients according to NASH or non-NASH etiology. Again the incidence of features of the MS was higher among NASH patients compared to the non-NASH group (Fig. 4).

Patients with ALD and HCC Exhibit Increased Features of the MS

As in NASH-HCC, patients with alcohol-induced cirrhosis display many features of the MS. Nearly two-thirds of ALD patients have a BMI ≥ 25 kg/m^2 (Fig. 3*b*), and more than 50% had T2DM (Fig. 3*c*). Dyslipidemia and hypertension were also more common among ALD patients than non-ALD and non-NAFLD groups. Interestingly, coronary artery disease is less common in this group (Fig. 3*f*).[29,30]

Discussion

NAFLD is the leading cause of CLD in the western world.[1] NASH is the progressive form of NAFLD that can lead to liver fibrosis and cirrhosis, with subsequent complications such as HCC. Among patients with NAFLD or NASH, liver disease is the third leading cause of death,[3] while HCC represents the main cause of death in this group.[31] Improved treatment, earlier detection and better health education of the general population about MS may reduce the incidence of

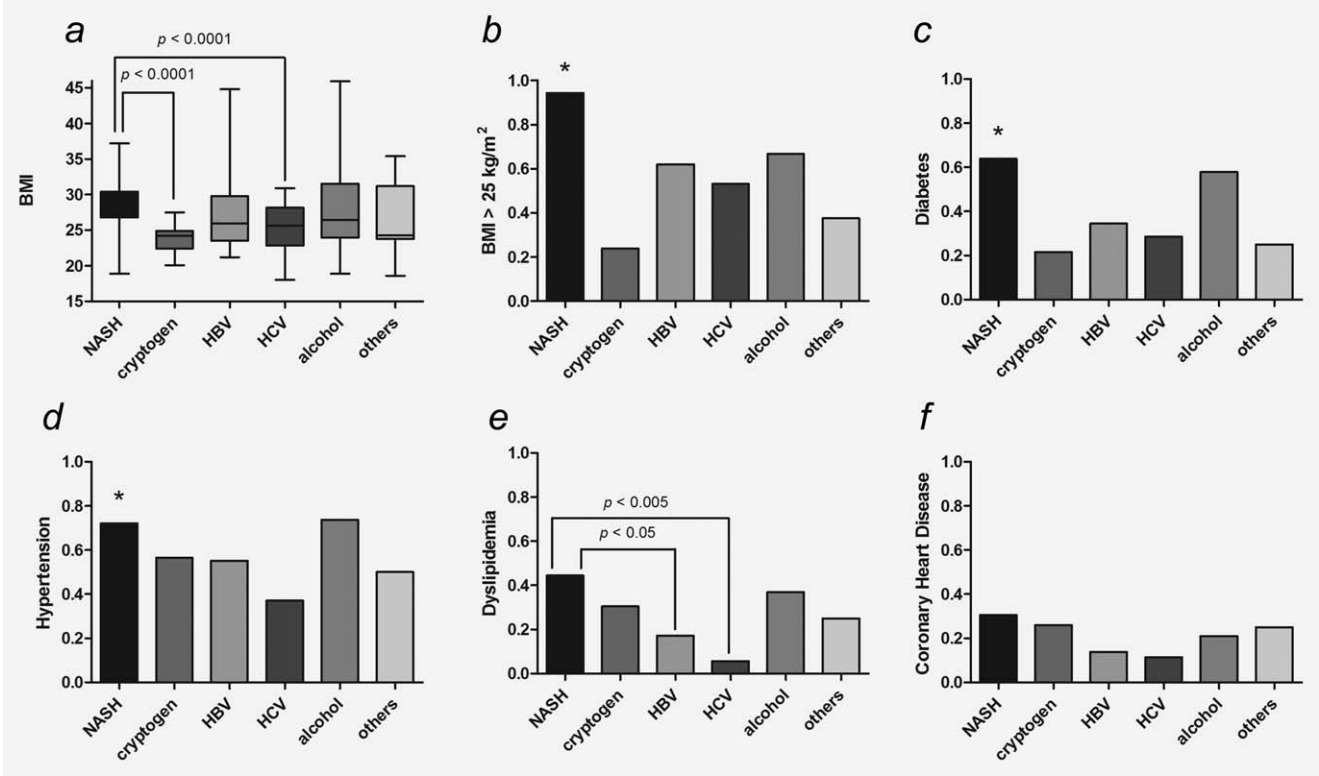

Figure 3. Incidence of metabolic syndrome (MS) within hepatocellular carcinoma (HCC) patients. (*a*) Mean body mass indices (BMIs) of patients categorized according to etiologies; (*b*) percentage of patients who were obese or overweight. (*c–f*) Features of the MS, such as diabetes mellitus Type II (*c*), hypertension (*d*), dyslipidemia (*e*) and coronary artery disease (*f*) were most prevalent among non-alcoholic fatty liver disease (NAFLD)NASH individuals. *: *p vs*. non-NASH patients < 0.05.

NASH and its related complications. Despite its prevalence and injurious sequelae, data on the actual incidence of HCC among affected NAFLD patients are lacking.

The hepatic manifestation of the MS, NAFLD/NASH, is associated with obesity and possibly results in undiagnosed liver diseases and cirrhosis.[3,32,33] As expected, we noted increased frequency of metabolic features among NASH-associated HCC. Significantly, a high prevalence of cirrhosis (>90% of the patients) was observed in non-NAFLD/NASH-HCC. In contrast, cirrhosis was observed in slightly more than 50% of NASH-HCC and 40% of cryptogenic-HCC patients, despite exhibiting more features of the MS. This is consistent with previous reports of HCC occurring in non-cirrhotic NASH.[34,35] Indeed, HCC has been reported to develop in the setting of mild fibrosis and/or chronic active steatohepatitis.[36,37] In a recent study, Paradis *et al.*[38] investigated 128 HCC patients with or without a risk factor for CLDs or the MS. The authors reported a significant number of non-cirrhotic patients in the MS group. Interestingly, the patients were recruited over a period of 12 years, while our patient cohort was recruited over 13 months, underscoring the increasing incidence of this disease entity. The collective data highlights the need for clinicians to be aware of the potential risks of the MS, and HCC development even without cirrhosis, and identifies the need for future research in this area.

Since no control group of non-cirrhotic NASH patients without HCC was available, it was not possible to investigate on the incidence of metabolic features in the development of HCC. Apart from this issue, prolonged exposure to the detrimental effects of obesity and diabetes may be risk factors for the formation of HCC on a non-cirrhotic NASH background. Despite inherent limitations of this study (*i.e.*, ethical and practical constraints in obtaining liver biopsies from every individual with HCC), it represents the first, large single-center study which confirms the potential risk of HCC in sub-groups of patients. Recent studies suggest that tumor suppressor genes such as PTEN (Phosphatase and Tensin homolog), promyelocytic leukemia and p53 play an important role in development of steatosis and that steatosis induces liver cell damage.[39,40] It follows that the loss of tumor suppression could promote formation of HCC, even without the development of cirrhosis. Dysregulated NF-κB, IL6 and TAK1 signaling have also been shown to promote HCC development,[41] while ablation of NEMO (IκB-kinase (IKK)-subunit NF-κB essential modulator) generates a NASH phenotype in mice.[42] c-jun kinase (JNK1) is crucial for development of steatohepatitis in the methionine-choline-

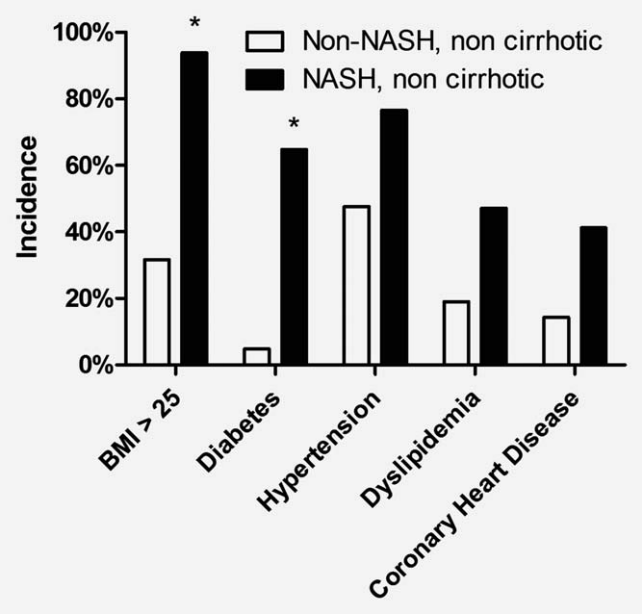

Figure 4. Incidence of metabolic syndrome (MS) within non-cirrhotic hepatocellular carcinoma (HCC) patients. Patients without cirrhosis were grouped according to NASH or non-NASH etiology. As expected, NASH patients exposed a higher incidence of obesity and diabetes. Age or mean body mass index (BMI) did not differ between the groups (not shown). *: p *vs*. non-NASH, non-cirrhotic < 0.05.

deficient diet mouse model[43] and JNK-phosphorylated Smad3 (pSmad3L) acts in an oncogenic fashion.[44] Excessive Hedgehog activity occurs in progressive NAFLD, promotes progenitor cell viability and differentiation, and may enhance HCC development.[45] The aggregate pre-clinical evidence and clinical observations highlight the close relationship between fatty liver and HCC development. Another potential mechanism relates to dysregulated bile acid metabolism in NAFLD. As bile acid accumulation has been reported to induce hepatocyte apoptosis, such dysregulation may promote HCC occurrence in the setting of hepatic steatosis. The interplay of multiple regulatory signals and mechanisms by which cancers arise are complex, and additional studies are needed to identify potential targets for therapy.

Interestingly, a large number of patients with alcohol-induced cirrhosis and HCC had features of the MS. It is tempting to speculate that the MS could function as trigger in promoting development of HCC in alcohol-induced cirrhosis. In support, we reported that FFA, resveratrol, and red wine components could promote hepatic stellate cell activation and fibrogenesis,[46] explaining the accelerated progression of liver cirrhosis in patients with the MS and excessive alcohol consumption. Unfortunately, it remains unclear, if alcohol enhances liver injury based on an underlying MS, or if alcohol consumption *per se* leads to symptoms representing the MS, which then affects the liver. More extensive prospective studies could probably clarify this connection.

It has been proposed that a proportion of individuals with cryptogenic cirrhosis exhibit histological features consistent with NASH. NASH is characterized by intracellular fat accumulation, hepatocellular ballooning, inflammation and fibrosis, but in cases of advanced NASH-associated cirrhosis, such features may not be detectable.[34] Caldwell *et al*. stated that the histopathological findings of NASH-cirrhosis differed from HCV-cirrhosis, potentially allowing the discrimination of cryptogenic-cirrhosis which might truly be NASH-related.[47] While the benefit of identifying NASH as the underlying cause of an already diagnosed HCC might in itself be limited for the patient, family members may be screened for undiagnosed NAFLD or NASH, as familial incidence constitutes a risk factor for the MS.[48] Individuals identified with having the MS could potentially be screened for NAFLD/NASH and/or early HCC using routinely available measurements of liver enzymes and ultrasonography. Moreover, as observed from this study (Supporting Information Table 1), the absence of cirrhotic features does not preclude the diagnosis of cirrhosis, while liver biopsies carry inherent risks and are subject to sampling errors.[49] Thus, the possibility of HCC developing in a—apparently or truly—non-cirrhotic NASH liver would be an even greater impetus for such surveillance.[31]

The current curative options for HCC include surgical resection and liver transplantation, both of which require early diagnosis. Unfortunately, only 5–10% of HCC patients are deemed resectable at the time of diagnosis[50]; this could possibly be improved with targeted screening in high-risk individuals. Given the rapid increase in the incidence of the MS, NAFLD and associated comorbidities, it is imperative that clinicians are appropriately educated and kept abreast of this syndrome.

Acknowledgements

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Novel Algorithm for Non-Invasive Assessment of Fibrosis in NAFLD

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Abstract

Introduction: Various conditions of liver disease and the downsides of liver biopsy call for a non-invasive option to assess liver fibrosis. A non-invasive score would be especially useful to identify patients with slow advancing fibrotic processes, as in Non-Alcoholic Fatty Liver Disease (NAFLD), which should undergo histological examination for fibrosis.

Patients/Methods: Classic liver serum parameters, hyaluronic acid (HA) and cell death markers of 126 patients undergoing bariatric surgery for morbid obesity were analyzed by machine learning techniques (logistic regression, k-nearest neighbors, linear support vector machines, rule-based systems, decision trees and random forest (RF)). Specificity, sensitivity and accuracy of the evaluated datasets to predict fibrosis were assessed.

Results: None of the single parameters (ALT, AST, M30, M60, HA) did differ significantly between patients with a fibrosis score 1 or 2. However, combining these parameters using RFs reached 79% accuracy in fibrosis prediction with a sensitivity of more than 60% and specificity of 77%. Moreover, RFs identified the cell death markers M30 and M65 as more important for the decision than the classic liver parameters.

Conclusion: On the basis of serum parameters the generation of a fibrosis scoring system seems feasible, even when only marginally fibrotic tissue is available. Prospective evaluation of novel markers, i.e. cell death parameters, should be performed to identify an optimal set of fibrosis predictors.

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Background

Non-alcoholic fatty liver disease (NAFLD) is an increasingly prevalent disease entity, affecting up to one-third of Europeans [1]. NAFLD may progress to non-alcoholic steatohepatitis (NASH) with or without fibrosis and thus predisposes to liver cirrhosis, end stage liver disease and hepatocellular carcinoma (HCC) [2]. Approximately 33% of patients with NASH develop fibrosis and 15% develop cirrhosis [3]. To date, the diagnosis of NASH is established by histological means, including inflammation, steatosis, hepatocellular injury and ballooning. According to Kleiner *et al.*, fibrosis in NAFLD is assessed in a 7-stage system, which includes detailed evaluation of perisinusoidal and periportal fibrosis [4]. However, liver biopsy is associated with a limited, but significant risk of adverse events (bleeding, infection) and a significant rate of observer- and sampling errors, specifically for assessment of fibrosis [5,6]. Given the high prevalence of NAFLD and the limitations of liver biopsies, biomarkers and surrogate parameters of fibrosis might evolve as important diagnostic means in NAFLD patients.

Hepatocyte cell death in NAFLD is an important predictor of hepatic stellate cell (HSC) activation and thus indirectly of fibrogenesis [7,8]. Recently, assessment of hepatocyte apoptosis by quantification of soluble cytokeratin 18 (CK-18) has been validated in large cohorts of NAFLD patients as a novel biomarker of disease activity [9]. As engulfment of remnants from apoptotic hepatocytes (apoptotic bodies) by non-parenchymal cells directly and indirectly activates HSCs [10,11], fibrosis was also found to correlate with serum CK-18 levels in some studies [12,13]. Another derivate marker with regard to collagen production is hyaluronic acid (HA). HA serum levels increase with progressive fibrosis [14]. In fact, some studies showed a correlation between serum HA and fibrosis stage in chronic liver diseases, including NAFLD [15,16]. However, in validation studies, individual biomarkers failed to accurately predict fibrosis [17]. Thus, diagnostic multi-panel tests, including a variety of individual parameters have recently been implied as non-invasive fibrosis tests. Most tests have been established in HCV patients and might lack validity in NAFLD patients [18,19]. Novel tests that include cell death markers, designed for fibrosis assessment in NAFLD, are emerging, but still require large-cohort validation studies [20].

Limitations of these scoring systems comprise small cohorts [21], comparison of no or low-grade cirrhosis *vs.* high-grade of cirrhosis [21,22], and inclusion of metabolic or biometric parameters increasing the effort needed to generate this score for individual patients [21,23]. To monitor early fibrogenesis and progression of disease it would be imperative to differentiate even low grades of fibrosis. This is of utmost clinical significance, as pre-existing chronic liver diseases – even in early fibrotic stages – predispose to acute-on-chronic liver failure [24]. However, currently available non-invasive tests for fibrosis fail to distinguish early fibrosis stages.

Here, we aim to introduce a novel score of non-invasive fibrosis parameters, designed specifically for NAFLD. To this end, we used machine-learning algorithms, which are widely used for prediction and classification problems in biomedical research [25]. Typically, a supervised learning strategy is used for training a machine learning algorithm with a set of training samples, for which the input parameters and associated target classes are known. In the setting presented here, these algorithms were trained to differentiate between fibrosis stage 1 and fibrosis stage 2 within a cohort of adipose NAFLD patients, utilizing serum derived parameters.

Materials and Methods

Ethics Statement

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board (Ethics Committee) at the University Hospital of Essen. All patients provided written, informed consent before enrollment.

Patients and Sample Acquisition

126 morbidly obese patients (BMI: 52.2 ± 0.7 kg/m^2; age: 45.2 ± 0.96; males/females: 28 (22.2%)/98 (77.8%)) who underwent bariatric surgery at a renowned center for bariatric surgery were included (Tab. 1). Some clinical data on this collective have been published previously in a different approach of analysis [26]. Indication for bariatric surgery was made according to NIH guidelines (BMI $\geq$40 kg/m2 or $\geq$35 kg/m2, plus co-morbidities). Subjects reporting excessive alcohol consumption (>20 g/day in males or >10 g/day in females) indicating alcoholic liver disease were excluded. Liver serum parameters (ALT, AST) were determined in the central laboratory unit of the University Hospital Essen by standardized methods. Liver biopsies were obtained during the bariatric surgery as wedge biopsy. Individual specimens were stored in 4% formalin solution (Roth, Karlsruhe, Germany) for histological examination. The fibrosis stage was assessed in a blinded fashion by a single pathologist according to Kleiner *et al.* [4].

ELISA

Sera were collected upon admission and throughout hospitalization and stored within 2 h at -20°C until testing. Individual values of clinical and standard laboratory data, overall cell death and apoptosis markers M65 and M30 (determined by the M30-Apoptosense and M65 ELISAs from Peviva; Bromma, Sweden), as well as hyaluronic acid (Corgenix, Bloomfield, CO, USA) were determined. All procedures were conducted according to the manufacturers' instructions.

Dataset and Statistics

The resulting parameters used for classification were ALT, AST, M30, M65 and HA. Samples with incomplete parameters were discarded prior to analysis. The final dataset consisted of 25 samples of fibrosis status 1 (including stages 1a, 1b, 1c; handled as positive samples) and 101 samples of fibrosis status 2 (representing negative samples). We compared all parameters of fibrosis-status 1 (1a, 1b, 1c) with the corresponding parameters of fibrosis-status 2 using Wilcoxon Signed-Rank tests.

Machine Learning

Several machine learning techniques were evaluated, namely logistic regression (logReg), k-nearest neighbors (knn), linear support vector machines (SVM), rule-based systems (RB), decision trees (DT) and random forest (RF). For the logistic regression, we used the implementation in the *stats* package of R (http://www.r-project.org) with standard settings. The k-nearest neighbor implementation in the R package *class* was used with k = 3. The SVM implementation in the package *kernlab* of R was used with the *vanilladot* kernel. For the rule-based systems we used the *Part* [27] implementation provided in the R package *RWeka*. For the DTs we used the implementation in the *rpart* package and for the RFs [28] we used the implementation in the *randomForest* package of R. In our application each RF consisted of 2000 randomly and independently grown decision trees. When using the trained RF for prediction, an unseen instance was assigned to the positive class voted for by at least 50% of the trees. The importance of each variable for the correct classification can be assessed by determining the decrease in Gini impurity [29].

Cross-validation

All machine learning methods were validated using ten-fold leave-one-out cross-validation [29] to assess for the different machine learning methods the mean prediction sensitivity, specificity, and accuracy (see formulas below) and the ability to generalize to unseen instances.

For each test in the cross-validation, the sensitivity (SN), specificity (SP), and accuracy (AC) were calculated according to:

$$SN = \frac{TP}{TP+FN}$$

$$SP = \frac{TN}{TN+FP}$$

$$AC = \frac{TP+TN}{TP+FP+TN+FN}$$

with true positives TP, false positives FP, false negatives FN and true negatives TN. Furthermore, we calculated the Receiver Operating Characteristics (ROC) curve [30] and the corresponding area under the curve (AUC) with ROCR [31]. The ROC curve is built by plotting sensitivity *vs.* specificity for every possible cut-off between the two classes.

Permutation Test

All machine learning methods were tested for significance using a permutation test. The AUC distribution for each classifier was calculated by ten-fold leave-one-out cross-validation. 1000 (= N) random permutations of the class labels were generated and the classifiers were trained and evaluated again. Each of the resulting AUC distributions of the permutation was compared with the *real* AUC distribution using Wilcoxon Signed-Rank test. The number k of permutations for which the mean AUC had no significant differences compared to the *real* AUC was counted for each classifier. The p-value of the permutation test was calculated by

$$p = \frac{k}{N}$$

Statistical Comparison

All models were compared by applying Wilcoxon Signed-Rank test on the AUC distributions from the ten-fold leave-one-out cross-validation runs. The null hypothesis was that there are no differences between the compared classifiers.

Results

Patient Characteristics

Detailed data of the included patients can be found in table 1, comprising distribution of demographic parameters as well as standard parameters of liver damage (transaminases, bilirubin, gamma-GT and LDH). Serum parameters for liver damage were within normal range and no pathological alterations were detected. As the patient collective consisted of adipose patients, the BMI was significantly above normal ranges. Partial data of this patient cohort has been presented before in a different type of analysis in Kälsch *et al.* [26].

No Prognostic Value of Individual Parameters

We found no significant differences between ALT, AST, M30, M65 and HA of fibrosis status 1 and 2 with regard to Wilcoxon Signed-Rank test ($p = 0.55$, $p = 0.30$, $p = 0.70$, $p = 0.87$ and $p = 0.86$, respectively).

Accuracy of the New Diagnostic Algorithm for Prediction of Fibrosis Stage

Most of the classifiers were not able to accurately predict the fibrosis status (or the results were insignificant according to the permutation test). Neither the logistic regression, nor support vector machines, nor rule-based systems were able to predict the fibrosis status from the given blood parameters. However, random forests and the k-nearest-neighbor algorithms had an accuracy of about 79%. The RFs reached an AUC of 0.6704+/−0.0062 ($p = 0.008$). A cutoff of 0.22 between positive and negative samples led to a sensitivity of over 60% and a specificity of 77% (Figure 1). The knn reached a sensitivity of 30.8% with a specificity of 91.3% ($p = 0.02$), which is slightly worse compared to the RFs (sensitivity of 30.8% with specificity of 92.2%). Decision trees displayed the best performance (sensitivity of 53.9% and specificity of 94.2), but failed to reach a significance level of 5% in the permutation test ($p = 0.099$).

Furthermore, we analyzed the prediction output for each sample. The output of the RFs is shown as boxplots for each of the two classes in Figure 2. Subjects from stage 1 had higher prediction values compared to subjects from stage 2 on average.

As mentioned before, RFs are able to identify the most important parameters for the classification process. The RFs identified M30 and M65 as being slightly more important than the other variables, which is in accordance with the DTs (only M30, M65 and AST are used in the trees, see Figure 3). The estimated importance for each parameter is shown in table 2.

Table 1. Demographic and basic parameters of the investigated patient collective.

Parameter	Fibrosis Stage 1 (1a, 1b, 1c)	Fibrosis Stage 2
Age (y)[1]	45.04±2.54	45.84±0.99
Gender[1]	M 16% (n = 4)/F 84% (n = 21)	M 24% (n = 24)/F 76% (n = 77)
BMI (kg/m^2)[1]	51.96±1.59	52.25±0.81
AST[1]	27.12±2.65	29.98±1.61
ALT[1]	34.44±4.81	37.67±2.59
gamma-GT[1]	34.24±4.48	55.29±12.40
LDH	232.92±9.99	226.47±5.31
bilirubin	0.56±0.08	0.55±0.03

No significant differences were found between stage 1 and stage 2.
[1]These data have been previously shown in a different analysis of this patient cohort in Kälsch *et al.* [28].
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Discussion

Diagnosis of liver fibrosis or cirrhosis provides important clinical information. Different etiologies and conditions, affecting liver integrity and function may increase susceptibility to various toxins (e.g. drugs) or viruses and are associated with enhanced morbidity or mortality in acute liver injury [24]. Pre-existing liver disease, though, often persists in presence of marginally or only slightly elevated classic liver parameters, which would not suggest a liver biopsy to test for fibrosis. This is particularly common in obese NAFLD patients, as we have recently shown. Then again even a liver biopsy – comprising only 1/50.000th of the total liver volume – may not lead to an unambiguous judgement by a pathologist [21,32,33]. Moreover, liver biopsy is a painful invasive technique, which also confers a certain health risk, especially for patients with already reduced functional liver mass. Thus non-invasive methods that indicate progression to fibrosis or cirrhosis are needed that more reliably predict which patients should undergo a liver biopsy, or have their fibrosis status estimated if a biopsy is clinically not feasible.

A major concern for all attempts to establish such adequate scoring systems, is that as reference the gold standard has to be applied, which is biopsy and subsequent histological examination. As mentioned above, this technique contains two main sources of error that have to be considered when calculating a score for fibrosis/cirrhosis estimation [32,33].

Previous studies could clearly demonstrate that single serum parameters are not sufficient to reach the sensitivity or specificity of liver histology [19,21]. A scoring system combining different parameters seems to be the most promising approach. Knowing that cell death by apoptosis is a critical step for NASH development, integrating markers of cell death appears reasonable. Subsequent activation of HSC by hepatocyte apoptosis leads to ECM production [11,34]. In our calculations we included an importance analysis, demonstrating the highest importance for cell death parameters and hyaluronic acid, which has been linked to cirrhosis and collagen production [14]. This would fit with current theories discussing hepatocellular death and subsequent activation of non-parenchymal cells as crucial events in fibrogenesis and progression to cirrhosis in chronic, but also in acute liver injury [35].

While there have been previous attempts to establish a non-invasive method to detect fibrosis or cirrhosis, most groups have done their analyses in patients with viral hepatitis, where abundant collagen deposition and progression to cirrhosis is given. Systems which were established in NAFL/NASH cohorts often distinguish only no or low-grade fibrosis from cirrhosis [22]. This obviously does not suffice to monitor fibrosis progression in NAFLD, where

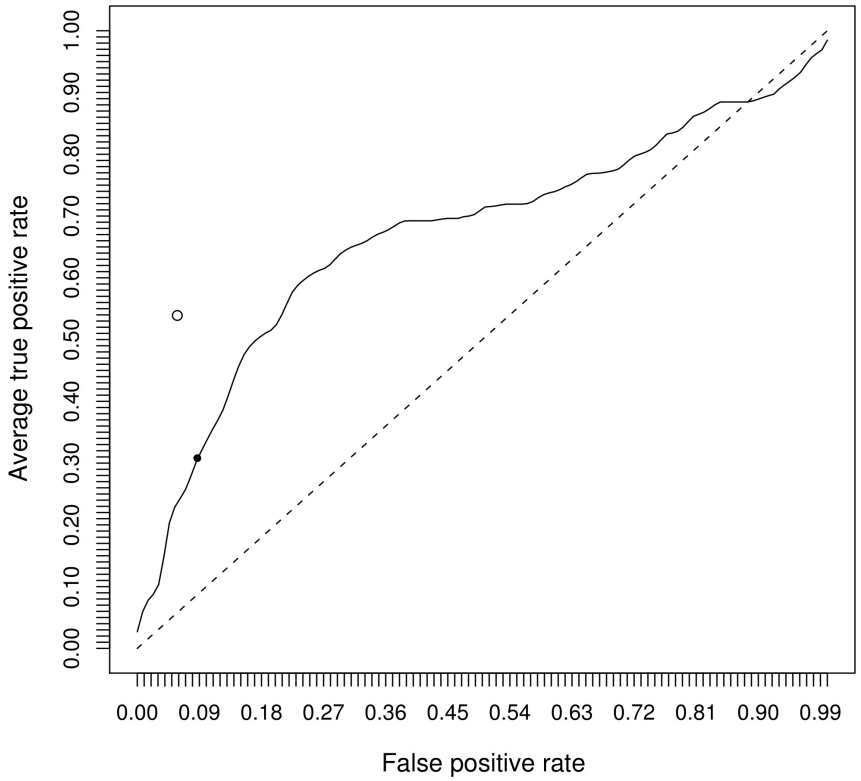

Figure 1. Prediction performance. The ROC curve of the RF is shown (p = 0.008). Black dot: performance of the 3 nn (p = 0.02); white circle: performance of the DT (p value = 0.099). The dashed line marks the performance by chance.
doi:10.1371/journal.pone.0062439.g001

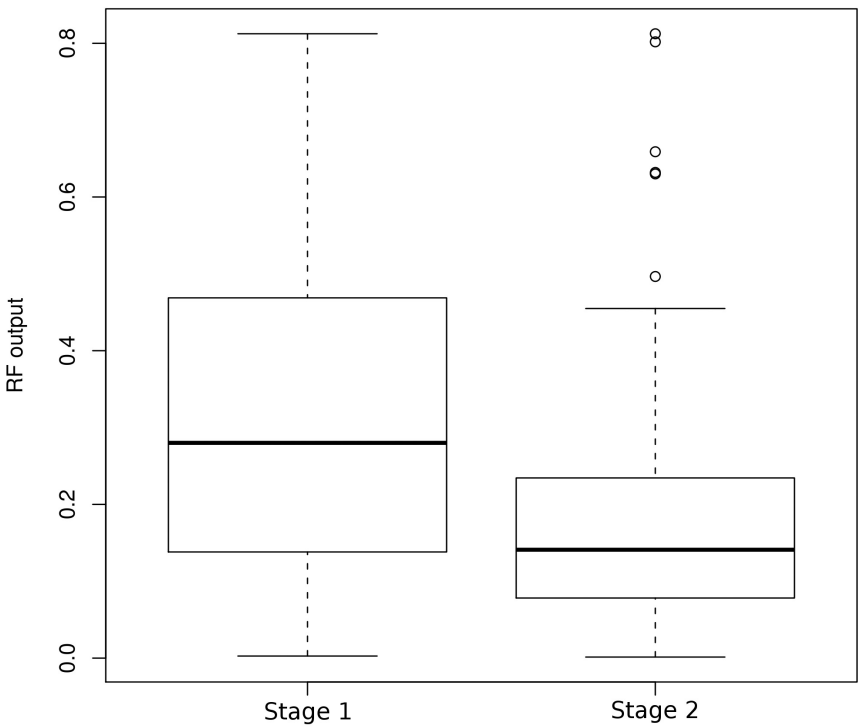

Figure 2. Boxplots of the outputs of the RFs. On the y-axis the predicted class probabilities for stage 1 and stage 2 are plotted. Generally, the RFs give higher prediction values for subjects from stage 1 compared to subjects from stage 2. The upper and the lower quartiles, the median (bold line) as well as outliers (circles) are shown.
doi:10.1371/journal.pone.0062439.g002

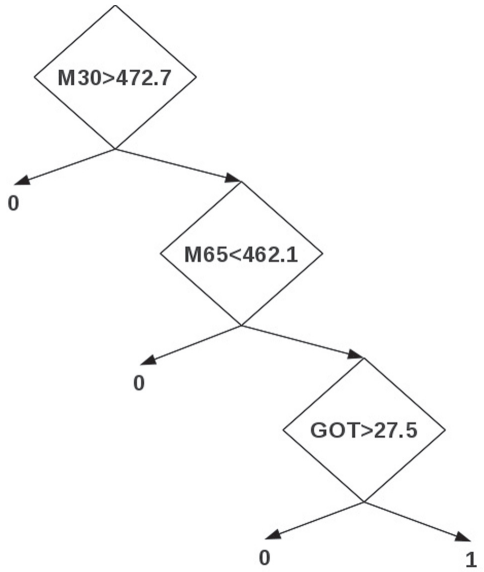

Figure 3. Decision Tree. The decision tree (DT) focuses on the parameters M30, M65 and AST (GOT), which is in partial agreement with the RFs. At the first level, the DT focuses on M30. If the M30 value for a given sample is less than 472.7, it is assigned to the negative class (0). If the M30 value is greater than 472.7, it is transferred to the next level that uses M65. If the M65 value for the given sample is greater than 462.1, it is assigned to the negative class, otherwise it is transferred to the last level. The last level in the DT focuses on GOT. If the GOT value for the given sample is less than 27.5, the sample is assigned as negative, otherwise as a positive sample (1).
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progression is rather slow compared to viral or alcoholic hepatitis. Moreover, when at some point a therapeutic intervention against fibrosis is possible, detecting established cirrhosis vs. low fibrosis may not be helpful to identify patients in need of this – hypothetical – medication. Thus, early detection of fibrosis may be crucial for a therapeutic approach to counter fibrosis progression, lowering vulnerability to additional, acute injury. A recent study by Tomeno *et al.* assessed the efficacy of real time transient elastography to detect liver fibrosis [36]. While the calculated liver fibrosis index correlated well with histological scores in chronic hepatitis, no such correlation was found for NAFLD. One could speculate that either overweight or fat deposition within the liver may interfere with elastography measurements in NAFLD. In any case, elastography has to be interpreted with care in the setting of NAFLD. Some NAFLD related fibrosis scores took the distinctiveness of this etiology into account and included biometric data [21,23], which requires additional data for each patient (height, weight). This may not be a limitation for a few individual patients, though it has to be viewed in the light of increasing numbers of overweight or obese individuals in the general population. Constraining the utilized markers to serum derived parameters reduces the clinical course of action to a single blood withdrawal. This would allow a true screening of patients without the need to take additional measurements.

Table 2. Importance analysis.

parameter	importance
ALT	6.76
AST	6.53
M30	9.90
M65	9.57
hyaluronic acid	8.28

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Another major difference to other scoring systems is the employment of non-linear machine learning techniques. For instance, the RFs allow to estimate variable importance and hence can be used to further improve prediction performance. Moreover, some of these methods provide simple rules, which can be applied in clinical settings to predict the fibrosis status of new patients and thus estimate a potential progression of the liver disease.

Limitations of our results are due to the number of patients with a complete dataset and moreover on the limited variability of fibrosis stages. As we did our investigation on a mostly obese collective with a probable NAFLD/NASH type of liver damage, the generation of fibrotic tissue is expected to be rather slow, in contrast to viral etiologies or alcohol abuse. Thus, we decided to differentiate between the two lowest fibrosis stages and trained the models accordingly. Although the dataset has the mentioned limitations, we were able to build a model that is able to discriminate between the two fibrosis classes with a reasonable sensitivity and specificity. Prospective studies recruiting a more diverse group of patients, exhibiting the full range of fibrotic stages (0–4) could increase the quality of the prediction model. Moreover, a broader range of parameters should be included to identify those parameters with the highest importance for diagnosis of fibrosis using machine learning techniques. Another possible option is to combine serum parameters with other non-invasive data, e.g. transient elastography of the liver. Additionally, it might also be possible to accurately predict the NAS status with an enlarged dataset.

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Author Contributions

Sample acquisition and data collection: LPB AC. Obtained funding: GG AC. Conceived and designed the experiments: JPS D. Heider AC. Performed the experiments: JPS D. Heider. Analyzed the data: JPS D. Heider D. Hoffman. Contributed reagents/materials/analysis tools: GG D. Hoffman. Wrote the paper: JPS D. Heider LPB AC.

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Non-Invasive Separation of Alcoholic and Non-Alcoholic Liver Disease with Predictive Modeling

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Abstract

Background & Objective: Currently, a major clinical challenge is to distinguish between chronic liver disease caused by metabolic syndrome (non-alcoholic fatty liver disease, NAFLD) from that caused by long term or excessive alcohol consumption (ALD). The etiology of severe liver disease affects treatment options and priorities for liver transplantation and organ allocation. Thus we compared physiologically similar NAFLD and ALD patients to detect biochemical differences for improved separation of these mechanistically overlapping etiologies.

Methods: In a cohort of 31 NAFLD patients with BMI below 30 and a cohort of ALD patient with (ALDC n = 51) or without cirrhosis (ALDNC n = 51) serum transaminases, cell death markers and (adipo-)cytokines were assessed. Groups were compared with One-way ANOVA and Tukey's correction. Predictive models were built by machine learning techniques.

Results: NAFLD, ALDNC or ALDC patients did not differ in demographic parameters. The ratio of alanine aminotransferase/aspartate aminotransferase - common serum parameters for liver damage - was significantly higher in the NAFLD group compared to both ALD groups (each $p<0.0001$). Adiponectin and tumor necrosis factor(TNF)-alpha were significantly lower in NAFLD than in ALDNC ($p<0.05$) or ALDC patients ($p<0.0001$). Significantly higher serum concentrations of cell death markers, hyaluronic acid, adiponectin, and TNF-alpha (each $p<0.0001$) were found in ALDC compared to ALDNC. Using machine learning techniques we were able to discern NAFLD and ALDNC (up to an AUC of 0.9118 ± 0.0056) or ALDC and ALDNC (up to an AUC of 0.9846 ± 0.0018), respectively.

Conclusions: Machine learning techniques relying on ALT/AST ratio, adipokines and cytokines distinguish NAFLD and ALD. In addition, severity of ALD may be non-invasively diagnosed *via* serum cytokine concentrations.

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Introduction

One of the major clinical challenges currently is to distinguish chronic liver disease on the basis of obesity from liver damage derived from long term or excess alcohol consumption. Both entities comprise a metabolic injury to the liver either as non-alcoholic fatty liver disease (NAFLD) or as alcoholic liver disease (ALD). Both diseases initially present as steatosis [1,2], but can progress to steatohepatitis, fibrosis and subsequently cirrhosis, the latter greatly increases the risk of hepatocellular carcinoma. The histologic changes caused by these diseases are similar enough that without close inspection of general physiology, co-morbidities and patient history, it is often difficult to determine the major damaging component in each individual case.

Delineating the cause of fatty liver disease has a critical impact on patient care. There is no universally accepted mechanism-based therapy to halt or reverse either ALD or NAFLD, and primary therapies focus on lifestyle modifications to reduce the proximate cause of the diseases. For example, reduction or cessation of alcohol consumption is an effective hallmark of therapy for ALD, regardless of stage [3]. However, despite many psychiatric methods to support patients willing to improve their

health *via* lifestyle changes, relapses are very common. Similar limitations exist for treating NAFLD, apart from bariatric surgery. Indeed, although lifestyle changes to reduce BMI is also an effective therapy for NAFLD, this approach is confounded by difficulties in achieving effective and permanent weight loss. Furthermore, NAFLD is not recognized by all providers of medical care or in administrative boards for treatment guidelines. Due to the increasing incidence and expected further rise as predicted by increasing adolescent obesity in industrialized societies, the latter topic is of particular importance [4].

The difficulty in distinguishing between ALD and NAFLD also impacts therapies for end-stage liver disease, namely liver transplantation. Barring biopsy, it is difficult to stage the liver disease, which is critical for prioritizing care. Furthermore, many transplantation guidelines require at least 6 months abstinence from alcohol for a patient with chronic ALD to be eligible for liver transplantation. Indeed, willingness to cease consumption of alcohol is an obligatory statement for ALD patients to be at least listed for a transplant. Although less rigorous rules are generally applied to NAFLD patients, it may still be difficult to prove true NAFLD (i.e., absent alcohol consumption, especially since physical and metabolic comorbidities of NAFLD and ALD often overlap (i.e. overweight, diabetes) [5]. In the end, many NAFLD patients face the potentially incorrect diagnosis of ALD from primary health providers. This potential misdiagnosis could therefore prohibit the option of liver transplants for NAFLD-derived end-stage liver disease. The above described situation is further complicated by conflicting results indicating moderate alcohol consumption may either ameliorate or aggravate underlying NAFLD [6,7]. These issues emphasize a critical need to develop a clear and reliable clinical assay to separate predominantly non-alcoholic *vs.* alcoholic fatty liver damage.

The rate of alcohol metabolism is too rapid to use as an index, barring active inebriation at the time of presentation. Although psychiatric assessment of a patients' alcohol consumption may be a feasible option, it relies in part on self-reporting, in a patient cohort infamous for concealing or minimizing their addiction [8].

Machine learning refers to a variety of techniques dealing with pattern recognition based on models for classification and prediction of novel unseen data. Machine learning incorporates the automatic construction of models and application of these models to new data and hence is closely related to the field of data mining. Statistical methods and machine learning techniques have been widely used in biomedical research to evaluate and analyze data. In principle, machine learning techniques are based on data given as a set of attributes, which are assigned to a specific predefined class (i.e. non-alcoholic or alcoholic liver disease, as in the present study). A classification model generated by machine learning describes the mapping from a set of attributes to the corresponding class. Once generated, this model can be used to predict new unseen data, thus enabling classification relying on a set of attributes. Among other considerations this would be an initial step towards personalized therapy for a given patient. A major advantage above other statistical methods is that machine learning techniques provide a robust multivariate approach with multiple features taken into account simultaneously, without the need for variable selection.

In the present study, the focus was on discerning NAFLD and ALD patients with similar physiological and metabolic features in cohorts of patients with similar BMIs. An added goal was to attempt to distinguish between cirrhotic and non-cirrhotic ALD by serum derived variables. These variables allow quick retrieval in a clinical setting and give clear objective measurements for disease assessment. Four different machine learning techniques were applied to analyze predictive possibilities of the collected non-invasive parameters.

Material and Methods

Patients

The study protocol conformed to the revised Declaration of Helsinki (Edinburgh, 2000), was approved by the local Institutional Review Board (Ethik Kommission am Universitätsklinikum Essen; file number 09–4252), and all patients gave written informed consent to study participation prior enrollment.

NAFLD patients were enrolled in the hepatologic outpatient clinic at the University Hospital Essen from 2009–2013. Enrollment criteria were a sonographically present steatosis and absence of any known or detected chronic or acute liver disease (viral, autoimmune, toxicity). Exclusion criteria were a BMI above 30, self reported alcohol consumption above 20 g/day for women or 40 g/day for men, or an age below 18years.

ALD patients were enrolled in the LVR-Clinic at the University Hospital Essen and in the addiction therapy unit of the Fliedner Clinic, Düsseldorf. Patients were recruited during the assessment for liver transplantation [8] or during inpatient rehabilitation for chronic alcohol abuse, respectively. Enrollment criteria were a proven history of alcohol consumption. Individuals aged <18 years, patients with a history of organ transplantation, a history of malignancy within the previous five years, drug abuse within the previous year, autoimmunity, genetic disorders, and therapies with immunosuppressive and/or cytotoxic agents were excluded. ALD patients were grouped according to ultrasonographically detectable cirrhosis into patients without (ALDNC) or with (ALDC) cirrhosis.

All enrolled patients were examined physically and ultrasonographically, and a complete set of laboratory parameters was obtained *via* the Central Laboratory Unit of the University Hospital Essen or the Fliedner Clinic. Transient elastography of the liver was measured with a FibroScan system.

Biochemical assays and ELISAs

Concentrations of serum M30 (for apoptotic cell death) or M65 (overall cell death) were detected with M30 Apoptosense ELISA or Epideath ELISA (Tecomedical group, Switzerland), respectively, according to the manufacturers' instructions. Serum concentrations of hyaluronic acid, adiponectin, and TNF-alpha were assessed with Hyaluronic Acid Test Kit (Corgenix, Bloomfield, CO, USA), the Human Adiponectin/Acrp30 Quantikine ELISA Kit, and Human TNF-alpha Quantikine ELISA Kit (both R&D, Minneapolis, MN) respectively, according to manufacturers' instructions.

Statistics

All data are expressed as means ± SEM unless specified otherwise. Graphical display gives all single data points as dot plot including mean and SEM. Statistical significance ($p<0.05$) was assessed by One-way ANOVA with Tukey's correction for multiple comparisons. All statistical analyses were performed using GraphPad Prism (Version 5.00, GraphPad Software, San Diego, CA, USA).

Machine learning

Four different machine learning techniques were employed for evaluation of prognostic properties of available parameters: logistic regression, decision trees (DT), support-vector machines (SVM) and random forests (RF). Mean imputation was performed to compensate for missing values. The SVM is probably one of the

most widely used machine learning methods. In their basic form (using the implementation in the R package *kernlab* with the *vanilla kernel*, i.e. the identity function), SVMs are based on the concept of linear separation of data. Thus, they are similar to other linear classifiers, such as the logistic regression. However, SVMs also try to maximize the margin between the two classes [9]. In contrast to the other models, RFs [10] are classifier ensembles, i.e. they are built out of a set of decision trees. For calculation of the RFs the implementation in the *randomForest* package of R (www.r-project.org) was used. Each RF consisted of 2000 randomly and independently grown DTs. When using the trained RF for prediction, an unseen instance was assigned to the positive class voted for by at least 50% of the trees. In addition to a high prediction performance, RFs are able to estimate the importance of features. The importance of each variable for the correct classification was assessed by determining the decrease in Gini impurity [11]. Single DTs were evaluated using the R package *rpart*. The logistic regression model was built in R as well.

All models were validated using ten-fold leave-one-out cross-validation [11] to assess the mean prediction sensitivity, specificity, and accuracy (see formulas below) and the ability to generalize to unseen instances.

For each test in the cross-validation, the sensitivity (SN), specificity (SP), and accuracy (AC) were calculated according to:

$$SN = \frac{TP}{TP+FN}$$

$$SP = \frac{TN}{TN+FP}$$

$$AC = \frac{TP+TN}{TP+FP+TN+FN}$$

with true positives TP, false positives FP, false negatives FN and true negatives TN. Receiver Operating Characteristics (ROC) curves [12] and the corresponding area under the curve (AUC) with ROCR [13] were calculated (for SVMs, logistic regression and RFs). The ROC curve was built by plotting sensitivity vs. specificity for every possible cut-off between the two classes. For the DTs accuracy was calculated instead of the AUC.

The models were tested for significance using a permutation test. The AUC distribution (for the DTs accuracy was used) for each classifier was calculated by ten-fold leave-one-out cross-validation. 1000 (=N) random permutations of the class labels were generated and the classifiers were trained and evaluated again. Each of the resulting AUC distributions of the permutation was compared with the real AUC distribution using Wilcoxon Signed-Rank test. The number k of permutations for which the mean AUC had no significant differences compared to the real AUC was counted for each classifier. The p-value of the permutation test was calculated by

$$p = \frac{k}{N}$$

The null hypothesis was that there are no differences between the compared classifiers.

Results

Alcoholic and non-alcoholic liver disease can occur on a similar basis of patient demography

NAFLD patients were selected on the basis of a BMI below 30. Patients with alcoholic liver disease were distributed according to presence of sonographically verified cirrhosis (ALD non-cirrhotic = ALDNC; AFD cirrhotic = ALDC). Distributions of gender, age and incidence of diabetes for patients are given in **table 1**. Due to the selection of NAFLD patients, there were no statistically significant differences in BMI between the patient groups. Although gender distribution tended slightly towards a higher proportion of females in the ALDNC group, the difference did not reach significance.

Clinical liver parameters allow discrimination between NAFLD and ALD

Standard serum parameters of liver damage were collected for all patients. As previously described [14], ALT was significantly higher in NAFLD patients compared to ALD, regardless of their cirrhotic status (**Fig. 1A**). AST and γGT did not differ significantly between the groups (**Fig. 1B,D**). The ratio of ALT to AST allowed a very clear discrimination of the NAFLD cohort from ALD patients (**Fig. 1C**). Both non-cirrhotic groups (NAFLD and ALDNC) exhibited significantly lower transient elastography values, than the ALDC patients (**Fig. 1E**). Moreover, incidence of steatosis was similar in NAFLD and ALDNC patients, while not a single case of steatosis was observed in the ALDC group (**Fig. 1F**). These effects are in-line with previously published results in similar patient cohorts.

Surrogate cell death markers and TNF-alpha discriminate NAFLD and ALD with and without cirrhosis

Cytokeratin 18 served as serum marker for apoptotic cell death (caspase cleaved epitope: M30) and overall cell death (total CK-18: M65). ALDC patients exhibited significantly higher M30 and M65 levels than NAFLD or ALDNC patients, respectively (**Fig. 2A,B**). Calculating the ratio of M30 to M65 gives a rough estimate of the predominant cell death mode (apoptotic *vs.* necrotic) [15]. In the presented patient groups, this ratio was highest in NAFLD and lowest in ALDC, suggesting a stronger contribution of necrotic cell death in cirrhotic ALD (**Fig. 2C**). The difference between ALDC and NAFLD as well as ALDNC was statistically significant. Hyaluronic acid serves as surrogate marker for fibrotic liver [16,17]. As expected, highest hyaluronic acid serum concentrations were found in ALDC (**Fig. 2D**).

Serum adiponectin reduction in NAFLD is contrasted by high elevation of adiponectin concentrations in ALD patients

The adipokine adiponectin is produced by "lean" adipocytes and is decreased in obese individuals [12,13]. As prior findings of others and our own group would suggest, we found reduced adiponectin levels in NAFLD patients with "low" BMI (**Fig. 3A**). To the contrary, adiponectin in the serum of both ALD cohorts NC and in particular of ALDC patients were significantly increased. Concentrations above normal ranges (approx. 100 ng/ml) were observed. TNF-alpha is found in higher serum concentrations in obese and may contribute to a generally proinflammatory state in these individuals. In contrast to these previous findings, serum TNF-alpha was found in lowest concentrations in this cohort of NAFLD patients (near normal

Table 1. Demographic and basic health data of the investigated study groups.

	NAFLD[1]	ALDNC[2]	ALDC[3]
N	31	51	51
Gender ratio	f: m = 15: 16	f: m = 16: 35$^{\#}$	f: m = 24: 27
Age (yrs.)	45.8±2.7$^{\#\#\#}$	49.2±1.2$^{\#}$	54.9±1.1
BMI (kg/m^2)	25.6±0.6	25.3±0.6	25.3±0.9
Incidence of diabetes	2 (9.5%) n = 21	2 (3.9%)$^{\#\#}$	7 (13.7%)

[1]non-alcoholic liver disease;
[2]alcoholic liver disease without cirrhosis;
[3]alcoholic liver disease with cirrhosis;
$^{\#}p<0.05$ *vs.* ALDC;
$^{\#\#}p<0.01$ *vs.* ALDC;
$^{\#\#\#}p<0.001$ *vs.* ALDC.
doi:10.1371/journal.pone.0101444.t001

range), with higher amounts in ALDNC and highest levels in ALDC (**Fig. 3B**).

Computational models can discern alcoholic and non-alcoholic fatty liver disease

To calculate a predictive algorithm the above described parameters were introduced into four different machine learning approaches. The single DT was able to classify between ALDNC and NAFLD with a sensitivity of 74.19%, specificity of 98.04%, and an accuracy of 89.02% (the corresponding DT is shown in **Fig. 4A**). It was also possible to discern ALDC and ALDNC with a sensitivity of 94.12%, specificity of 96.08%, and an accuracy of 95.1% (**Fig. 4B**). ROC curves were plotted to assess sensitivity and specificity of the RFs (**Fig. 4C,D**). In the presented patient cohorts the RFs reached highly significant predictions with an AUC of 0.8932±0.0052 ($p<0.0001$ for NAFLD *vs.* ALDNC) and 0.9846±0.0018 ($p<0.0001$ for ALDC *vs.* ALDNC). When transient elastography measurements were excluded, to avoid confirmation bias, the AUC for ALDC *vs.* ALDNC reached 0.8971±0.0051 ($p<0.0001$). For comparison, the SVMs reached 0.9118±0.0056 ($p<0.0001$) for NAFLD *vs.* ALDNC and 0.9058±0.0035 ($p<0.0001$) for ALDC *vs.* ALDNC, respectively. The logistic regression performed slightly worse with 0.8893± 0.0000 ($p<0.0001$; NAFLD *vs.* ALDNC) 0.8816±0.0000 ($p<$ 0.0001; ALDC *vs.* ALDNC).In addition to providing highly accurate models, RFs are able to estimate the importance of each variable to the classification process. Within each RF the most important parameters for discrimination of the classes (NAFLD *vs.*

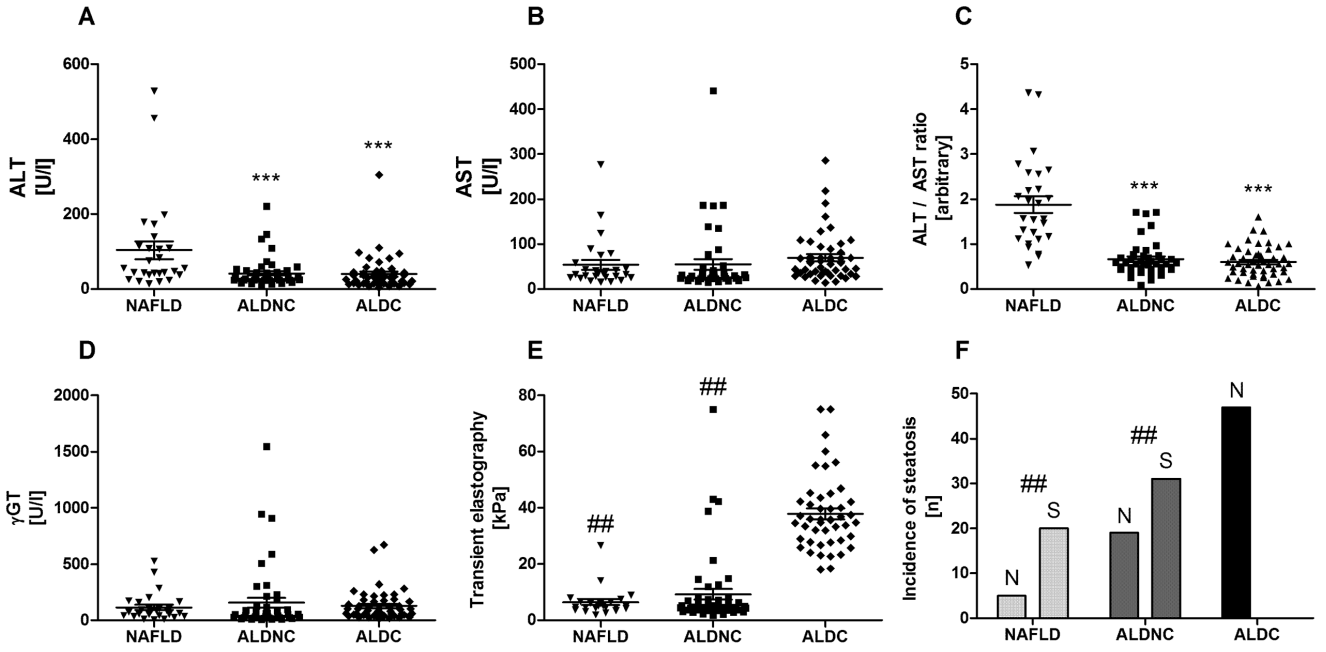

Figure 1. General liver damage parameters do not differ between NAFLD and ALD. Classic serum parameters of liver damage, transient elastography, and sonographically diagnosed steatosis were assessed in NAFLD patients and ALD patients with (ALDC) or without (ALDNC) cirrhosis. While AST (B) and γGT (D) did not differ between the groups, ALT (A) and especially the ALT/AST ratio (C) were significantly higher in NAFLD patients than in both ALD groups. In contrast transient elastography (E), as measure for fibrotic/cirrhotic alterations, and incidence of steatosis (F) were similar in NAFLD and ALDNC patients. ALDC patients exhibited significantly higher transient elastography values and lower incidence of steatosis. *** = $p<$ 0.0001 *vs.* NAFLD. ## = $p<0.01$ *vs.* ALDC.
doi:10.1371/journal.pone.0101444.g001

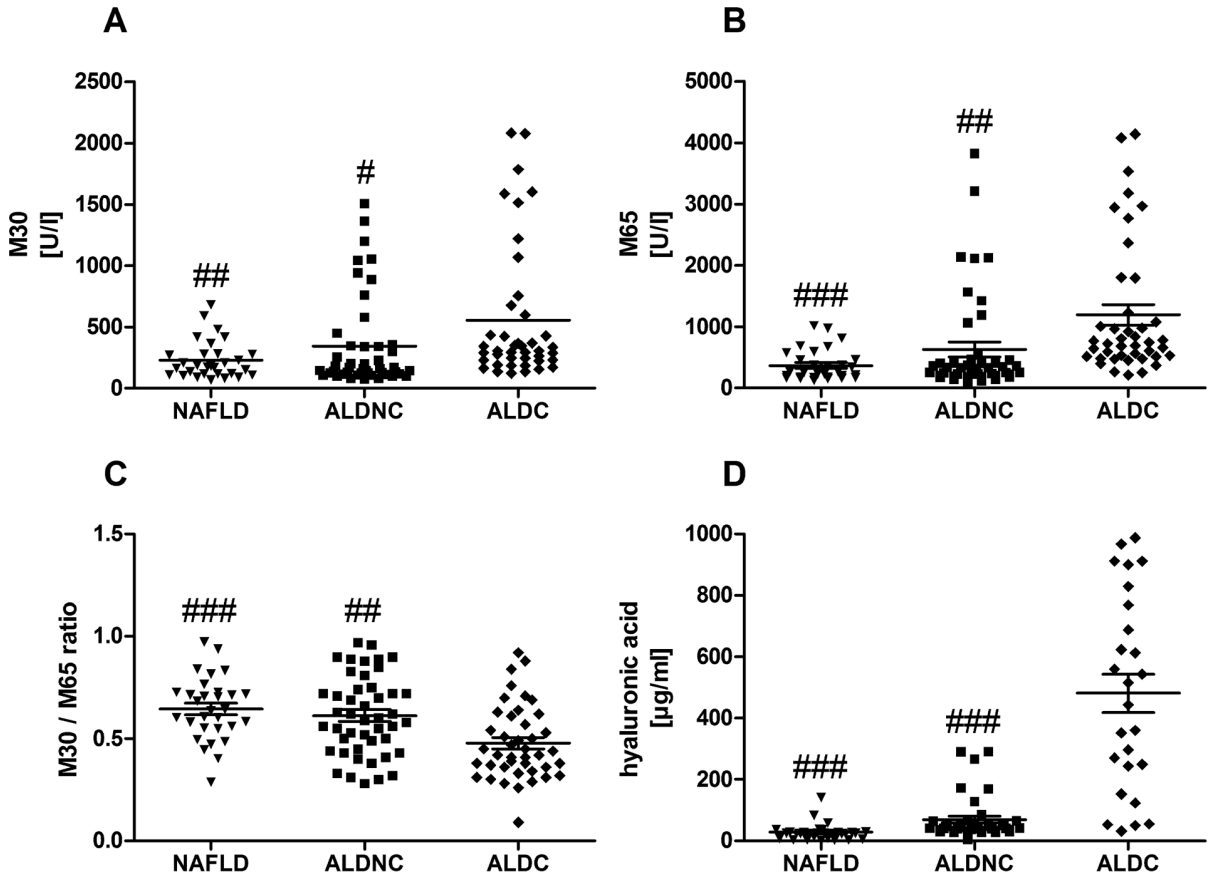

Figure 2. Elevation in serum cell death markers is specific for cirrhosis status but not for etiologies. Surrogate serum markers of apoptosis (M30, A) and general cell death (M65, B) were measured in NAFLD and ALD patients with (ALDC) or without (ALDNC) cirrhosis. Both markers were found elevated in all groups, with ALDC exhibiting significantly higher values than NAFLD or ALDNC patients. The ratio of M30/M65 (C) gives an estimate of the main cell death mode (predominantly apoptosis or necrosis). This ratio was significantly lower in ALDC compared to both non-cirrhotic groups, suggesting predominantly necrotic processes. Hyaluronic acid (D) as derivate marker for collagen production was significantly higher in ALDC patients than in the non-cirrhotic groups. #, ##, ### = $p<0.05$, 0.01 or 0.0001 *vs.* ALDC.
doi:10.1371/journal.pone.0101444.g002

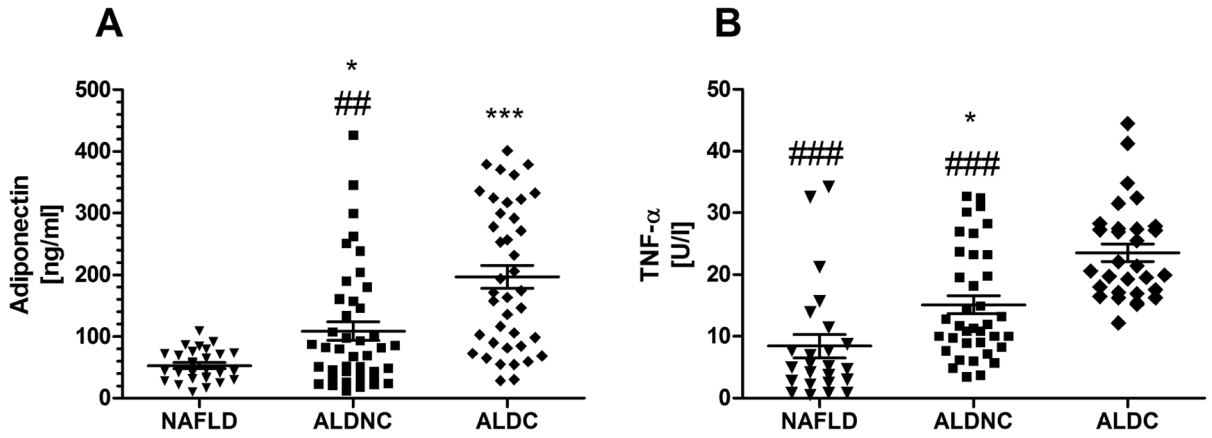

Figure 3. Adipocytokine profiles differ between NAFLD and ALD patients regardless of cirrhotic alterations. Adipokines are cytokines produced by the adipose tissue, which may affect other organ systems, including the liver. Adiponectin (A) is an anti-inflammatory and probably cell-protective adipokine, which is low in obese patients. In the NAFLD group (note: mean BMI 25.6) reduced adiponectin serum concentrations were found. Differences to both ALD groups were significant. While the ALDNC group exhibited values around normal ranges and above, the adiponectin levels in cirrhotic ALD were strongly increased and significantly different from the non-cirrhotic group. TNF-alpha (B) is a pleiotropic, generally pro-inflammatory cytokine. Surprisingly serum TNF-alpha was low in NAFLD, with significantly higher values in non-cirrhotic ALD. In ALDC patients a strong elevation of serum TNF-alpha was observed, which was significant compared to both non-cirrhotic groups. *, *** = $p<0.05$ or 0.0001 *vs.* NAFLD. ##, ### = $p<0.01$ or 0.0001 *vs.* ALDC.
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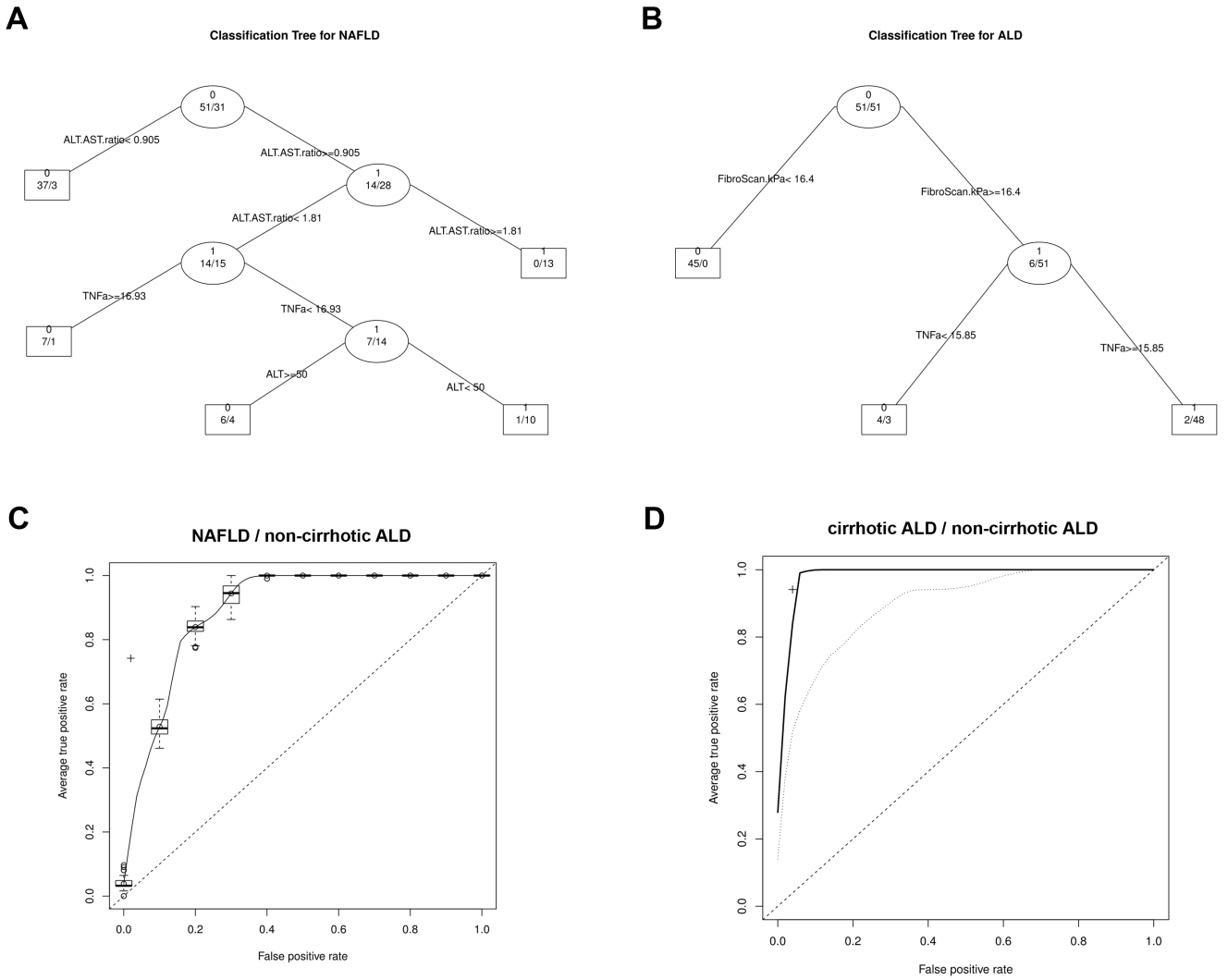

Figure 4. ROC curves for random forest group discrimination. Decision trees are shown for the classification of NAFLD *vs.* non-cirrhotic ALD (A) and cirrhotic *vs.* non-cirrhotic ALD (B), respectively. Move to the left branch when the stated condition is true, otherwise move to right branch. C: ROC curve for the RF (NAFLD *vs.* non-cirrhotic ALD); a + marks the performance of the corresponding DT; D: ROC curves for the RFs (cirrhotic *vs.* non-cirrhotic ALD), solid line: with transient elastography (FibroScan), dashed line: without transient elastography; a + marks the performance of the corresponding DT. The dotted line represents a classification by chance.
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ALDNC and ALDNC *vs.* ALC, respectively) were calculated. The corresponding results are presented in **table 2**.

Discussion

Assessment of the cause for a metabolic liver disease remains one of the current clinical difficulties. In the presented patient cohorts, a possible mode of separation between alcoholic and non-alcoholic liver disease patients *via* serum derived measurements is suggested. Separation of these causes for metabolic liver injury is important not only for conservative treatment of patients, but also crucial for the decision making processes for liver transplantation and organ allocation. The long-standing observation that NAFLD and ALD differ in the ALT/AST ratio was confirmed in our patient collective; a high ratio indicates NAFLD, while a low ratio is associated with ALD. This work also identified two new markers which could help delineate between ALD and NAFLD. These markers are the adipokine adiponectin and the cytokine TNF-alpha. Especially low adiponectin, generally associated with obesity and thus NAFLD, may be a highly valuable marker due to its specific production site (adipose tissue) and the clear distinction between a very low concentration even in NAFLD with moderately high BMI, and common concentrations in ALD in a similar BMI range.

Another important aspect of the presented findings is the difference between ALD patients with a rather mild liver injury (ALDNC) and those with end-stage cirrhotic alterations, under similar habits of alcohol consumption. Somewhat expected were higher levels of surrogate markers for cell death and collagen production. Though, again adiponectin and TNF-alpha stood out as significantly different between ALD patients with and without cirrhosis. In particular, the strong elevation of anti-inflammatory adiponectin in ALDC patients suggests a disturbed metabolic regulation in this group. Not as surprising, but still notable, is a stronger elevation of TNF-alpha in the same group. Again, one has to keep in mind that groups did not differ in the amount of alcohol consumption. This finding could imply a possible

Table 2. Importance[1] of entered parameters estimated by the RFs.

Parameter	Importance for NAFLD vs. ALDNC	Importance for ALDC vs. ALDNC	Importance for ALDC vs. ALDNC (transient elastography excluded)
Gender	0.57	0.20	0.49
Age	3.67	3.53	6.84
AST	1.65	2.63	4.84
ALT	5.15	1.18	3.00
ALT/AST ratio	10.41	1.39	3.68
M30	2.25	3.27	5.88
M65	2.65	6.21	11.96
TNF-alpha	5.6	5.52	7.65
Adiponectin	3.38	3.27	6.05
Transient elastography	2.66	23.25	-

[1]Higher values imply greater importance for the decision.

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functional involvement of adiponectin or its liver receptor ApoRII for progression of ALD to cirrhosis. Indeed, in hepatitis C virus-infected patients ApoRII expression correlates with serum adiponectin, steatosis, and liver fibrosis [18]. Increased adiponectin levels, without an actual protective effect might even indicate a crosstalk from liver to adipose tissue, initiating a compensatory mechanism [19]. Further studies are warranted to establish adiponectin as possible marker for monitoring of metabolic liver diseases. Furthermore, the current lack in mechanistical understanding of adiponectin signaling within the liver and the regulatory mechanisms in adipose tissue for adiponectin production should be targeted to evaluate this axis as drug target for ALD or NAFLD.

A crucial result of the presented work is the ability of a small set of non-invasive parameters to discern NAFLD and ALD, as shown by the calculated machine learning methods. One major advantage of the presented algorithms is the wide availability of the used parameters. Self reported consumption of alcohol is not always reliable to establish either NAFLD or ALD. From a clinical perspective it would be highly valuable to confirm or exclude ALD with high probability, without the need to rely on information given by the patient.

Similarly RFs and DTs were able to discriminate between ALDC and ALDNC with very high accuracy. This was mainly due to inclusion of transient elastography, which can detect cirrhosis reliably when ascites or other disturbing factors are absent [20,21]. Unfortunately this simple and highly informative method is not widely available, as a special ultra-sound head is needed to perform transient elastography measurements on tissue. Moreover, during patient recruitment cirrhosis was assessed by conventional ultra-sonography and transient elastography was performed as additional parameter. Though, to avoid confirmation bias from two sonographic methods, a second model to discriminate ALDC and ALDNC was calculated without transient elastography, which again yielded significant results.

RFs and DTs offer the ability to assess importance of variables used for classification in a specific model. These importance values can be used to find a minimal set of variables for the classification, thus reducing the amount of parameters which need to be determined and thus cost of a possible clinical application. Furthermore, assessment of importance enables more insights into the classification process and might even suggest underlying biological interactions, identifying interesting targets for disease monitoring or therapy. This is a clear advantage of DTs and RFs compared to other machine learning techniques that are rather black boxes, such as SVMs. It is noteworthy that in this model serum parameters of cell death and cytokines were the most important parameters for decision making. A previous approach for non-invasive fibrosis assessment in NAFLD yielded similar results [22]. While the classic liver serum parameters are still important, as seen for discerning NAFLD and ALD, additional parameters as cell death markers, cytokines and adipokines should be collected routinely to monitor disease progression or for diagnostic purposes. Broad usage of those parameters may confirm current data in larger proportions of the general population.

Limitations of the current study are the unavailability of liver tissue biopsies from the majority of patients. This unfortunately not only restricts exact pathological assessment (steatosis as well as fibrosis stages) but also excludes studies on cellular or molecular processes. For example it would be highly interesting to investigate expression of PAI-1 in the liver, as an important candidate for alcohol mediated inflammatory damage and fibrogenesis [23,24]. Differences between NAFLD and ALD or the different extent of damage in ALD might support the supposed functional involvement of PAI-1 in progression of ALD. Similarly interesting would be if expression of the adiponectin receptor ApoRII in the liver tissue correlates with severity of cirrhosis. Another limiting aspect is the relatively small number of NAFLD patients. This is partially due to the intention of comparing physiological similar patients with NAFLD and ALD. As the majority of definite NAFLD patients are obese, restriction to BMI of below 30 reduced the available number of patients. Finally, one limitation is represented by missing follow ups on the patients to assess development, progression or recession of the liver damage during disease course.

Taken together it could be shown that adipokines/cytokines may serve as markers for identification of NAFLD vs. ALD. This would enable clinicians to cross-check the information given by patients about their alcohol consumption with minor additional expenses but with high accuracy. In addition, severity of ALD may be non-invasively diagnosed *via* serum cytokine concentrations. Adiponectin or its receptors might even exhibit functional and thus therapeutic relevance in the progression of ALD to cirrhosis.

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Author Contributions

Conceived and designed the experiments: JPS NS YE AC. Performed the experiments: ÖA AK MS ML SS DH. Analyzed the data: JPS MS DH AC. Contributed reagents/materials/analysis tools: NS GG DH YE. Contributed to the writing of the manuscript: JPS KL GEA YE AC.

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