Hepatic exposure of metformin in patients with non-alcoholic fatty liver disease

Sundelin, Elias Immanuel Ordell; Gormsen, Lars Christian; Heebøll, Sara; Vendelbo, Mikkel Holm; Jakobsen, Steen; Munk, Ole Lajord; Feddersen, Søren; Brøsen, Kim; Hamilton-Dutoit, Stephen Jacques; Pedersen, Steen Bønløkke; Grønbaek, Henning; Jessen, Niels

Published in:
British Journal of Clinical Pharmacology

DOI:
10.1111/bcp.13962

Publication date:
2019

Document version:
Accepted manuscript

Citation for published version (APA):

Go to publication entry in University of Southern Denmark's Research Portal

Terms of use
This work is brought to you by the University of Southern Denmark. Unless otherwise specified it has been shared according to the terms for self-archiving. If no other license is stated, these terms apply:

• You may download this work for personal use only.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim. Please direct all enquiries to puresupport@bib.sdu.dk

Download date: 05. Jun. 2021
Hepatic exposure of metformin in patients with non-alcoholic fatty liver disease

Short running title: Hepatic metformin exposure in NAFLD

Elias Immanuel Ordell Sundelin1, Lars Christian Gormsen2, Sara Heebøll3, Mikkel Holm Vendelbo2,4, Steen Jakobsen2, Ole Lajord Munk2, Søren Feddersen5,6, Kim Brøsen5,7, Stephen Jacques Hamilton-Dutoit8, Steen Bønløkke Pedersen9,10, Henning Grønbæk3, Niels Jessen1,4,10,11

1. Research Laboratory for Biochemical Pathology, Department of Clinical Medicine, Aarhus University Hospital, Denmark
2. Department of Nuclear Medicine & PET Center, Aarhus University Hospital, Denmark.
3. Department of Gastroenterology & Hepatology, Aarhus University Hospital, Denmark.
4. Department of Biomedicine, Aarhus University, Denmark
5. Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark
6. Department of Clinical Research, University of Southern Denmark, Odense, Denmark
7. Department of Public Health, Clinical Pharmacology, University of Southern Denmark, Denmark
8. Department of Pathology, Aarhus University Hospital, Denmark.
9. Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Denmark
10. Steno Diabetes Center Aarhus, Aarhus University Hospital, Denmark
11. Department of Clinical Pharmacology, Aarhus University Hospital, Denmark.

Submitting author: Elias Immanuel Ordell Sundelin, elias.sundelin@clin.au.dk
Corresponding author: Niels Jessen, niels.jessen@biomed.au.dk
Word count: 3299
The authors confirm that the PI for this paper is M.D., Ph. D Elias Sundelin and that he had direct clinical responsibility for patients.

**Keywords:** organic cation transporters, pharmacokinetics, metformin, non-alcoholic fatty liver disease

Metformin: [http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=4779](http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=4779)

OCT: [http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=196](http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=196)

MATE: [http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=236#1216](http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=236#1216)

**WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT**

- The antidiabetic drug metformin is positively charge at physiological pH and demands facilitated transport for cellular uptake. The liver is a target organ for metformin action. Whether non-alcoholic fatty liver disease, a common disease, among type 2 diabetes patients, affect metformin transport is however unknown.

**WHAT THIS STUDY ADDS**

- 11C-metformin PET/CT was used to evaluate metformin pharmacokinetics dynamically in the liver in patients with non-alcoholic fatty liver disease
- There is no correlations between organic cation transporter mRNA expression and hepatic metformin exposure
- Metformin distribution to the liver is not impaired by inflammation or fibrosis while hepatic function is preserved
Abstract

**Aim:** Metformin is first line treatment of type 2 diabetes mellitus and reduces cardiovascular events in patients with insulin resistance and type 2 diabetes. Target tissue for metformin action is suggested to be the liver, where metformin distribution depends on facilitated transport by polyspecific transmembrane organic cation transporters (OCTs). Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the western world with strong associations to insulin resistance and the metabolic syndrome but if NAFLD affects metformin biodistribution to the liver is not known. In this study, the primary aim was to investigate *in vivo* hepatic uptake of metformin dynamically in humans with variable degrees of liver affection. As secondary aim, we wished to correlate hepatic metformin distribution with OCT gene transcription determined in diagnostic liver biopsies.

**Methods:** 18 patients with biopsy-proven NAFLD were investigated using 11C- metformin PET/CT technique. Gene transcripts of OCTs were determined by real time PCR.

**Results:** We observed similar hepatic volume of distribution of metformin between patients with simple steatosis and NASH (Vd 2.38 ± 0.56 vs 2.10 ± 0.39, p=0.3). There was no association between hepatic exposure to metformin and the degree of inflammation or fibrosis, and no clear correlation between metformin distribution and OCT gene transcription.

**Conclusion:** Metformin is distributed to the liver in patients with NAFLD and the distribution is not impaired by inflammation or fibrosis. The findings implicate that metformin action in liver in patients with NAFLD may be preserved.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen 1 A</td>
<td>COL1A</td>
</tr>
<tr>
<td>Computed tomography</td>
<td>CT</td>
</tr>
<tr>
<td>Glycosylated hemoglobin A1C</td>
<td>HbA1c</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>HCC</td>
</tr>
<tr>
<td>Monocyte chemoattractant protein-1</td>
<td>MCP-1</td>
</tr>
<tr>
<td>Multidrug and toxin extrusion 1</td>
<td>MATE1</td>
</tr>
<tr>
<td>Non-alcoholic fatty liver disease</td>
<td>NAFLD</td>
</tr>
<tr>
<td>Non-alcoholic steatohepatitis</td>
<td>NASH</td>
</tr>
<tr>
<td>Organic cation transporter</td>
<td>OCT</td>
</tr>
<tr>
<td>Real-time polymerase chain reaction</td>
<td>qPCR</td>
</tr>
<tr>
<td>Region of interest</td>
<td>ROI</td>
</tr>
<tr>
<td>Standardized uptake values</td>
<td>SUVs</td>
</tr>
<tr>
<td>Sterol regulatory element-binding protein 1c</td>
<td>SREBP1c</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>T2DM</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Volume of distribution</td>
<td>Vd</td>
</tr>
<tr>
<td>Volume of interest</td>
<td>VOI</td>
</tr>
</tbody>
</table>
Introduction

Metformin is first line treatment in type 2 diabetes mellitus (T2DM) and reduce cardiovascular events in subjects with insulin resistance and T2DM. Metformin is positively charged under physiological pH, and hepatic distribution is, therefore, facilitated by polyspecific transmembrane transporters known as organic cation transporter (OCT) 1 and 3, and by multidrug and toxin extrusion 1 (MATE1). Hepatic uptake of metformin is significantly diminished in OCT1 knockout mice, suggesting OCT1 is important in metformin transport into the liver. Conversely, pharmacological inhibition of MATE1 results in accumulation of metformin in the liver, suggesting that MATE1 is involved in hepatic elimination of metformin. Non-alcoholic fatty liver disease (NAFLD) is a growing health concern and is now the most common liver disease in the western world. NAFLD is strongly associated with insulin resistance and the metabolic syndrome. Simple steatosis may progress to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC). Separation between NASH and simple steatosis typically requires a diagnostic biopsy, and these are routinely performed at hepatological departments. Growing consensus suggests that NASH is a disease that requires pharmacological treatment. However, an ideal drug for the treatment of NASH is yet to be determined. It is estimated that approximately 70% of patients with T2DM have NAFLD. A long-term follow-up study on patients with NAFLD, concluded that NAFLD patients have increased mortality rates compared with a reference population. The cause of death was cardiovascular disease in 43% of the cases and only 4% died due to cirrhosis. Thus, stratified treatments aimed towards reducing the risk of cardiovascular disease in patients with NAFLD seem highly warranted. The effects of metformin in type 2 diabetes are, at least partly, attributed the action of metformin in the liver where it reduces hepatic glucose production. In addition to effects on glucose metabolism, metformin ameliorates histopathological NASH features in mice. In
clinical studies, metformin has been shown to reduce mortality in diabetic patients with cirrhosis, and to diminish the risk of developing HCC. In patients with NASH, metformin treatment is associated with a reduction in body weight, waist circumference, fasting blood glucose, and glycosylated hemoglobin type A1c (HbA1c) and with an increase in levels of high density lipoproteins and adiponectin. Animal studies have shown that NASH affects biodistribution of metformin in vivo. Plasma concentrations of metformin were 4.8-fold higher in obese mice treated with methionine- and choline-deficient diet compared to controls, suggested to be caused by altered expression of OCT2 and MATE1 in the kidneys. However, gene expression of organic cation transporters and MATE1 is highly organ and species diverse. Translating to humans, considerations must be given to differences in transporter expression, blood volume, metabolism, and how the disease model of NASH relates to NASH in humans. However, alterations of plasma concentrations of metformin may have implications for the glucose-lowering effects, and for the toxicity of metformin. Hepatic metformin distribution in these patients can also give valuable insight into factors that may determine pharmacokinetic properties of metformin because liver tissue from diagnostic procedures can be available from these patients.
Changes in organic anion transporter function has been reported in patients with NASH causing alterations in both hepatic exposure and plasma concentrations to certain substances.\textsuperscript{18} Changes in gene expression and glycosylation were proposed as being responsible for the pharmacokinetic changes. Similar changes in liver handling of metformin may affect the therapeutic and/or potential toxic effects of the drug.

We have recently established methods to determine metformin distribution in humans \textit{in vivo} by carbon\textsuperscript{11}-labeling of generic metformin.\textsuperscript{19,20} Using these techniques in healthy volunteers, we showed that metformin is distributed to the liver and that genetic polymorphisms in SLC22A1, the gene encoding OCT1, reduce hepatic distribution of the drug.\textsuperscript{19} The safety and reproducibility of the tracer studies so far performed, allows the technique’s use in patients. Therefore, we investigated hepatic metformin distribution in patients with biopsy validated simple steatosis or NASH, to test the hypothesis that exposure to metformin is reduced in patients with NASH compared to patients with NAFLD. Secondary endpoints were correlation between transporter expression and hepatic distribution, the hypothesis being that the OCT1/MATE1 gene expression ratio defines hepatic metformin distribution.

\textbf{Materials and Methods}

\textit{Patients}

A total of 18 patients were examined. 18 patients with biopsy proven NAFLD were recruited from the Department of Hepatology and Gastroenterology, Aarhus University Hospital, Denmark. 14 patients had completed an intervention study with resveratrol, with a negative outcome, before entering this study.\textsuperscript{21} Resveratrol did not affect gene expression of
transporter genes involved in facilitating metformin transport in the liver, nor did it have any histopathological effect no NASH or steatosis. ²¹

NAFLD was defined according to the American Association for the Study of Liver Diseases Practice Guidelines. ²² Diabetes mellitus was defined according to the American Diabetes Association’s “Diagnosis and Classification of Diabetes Mellitus”. ²³ Patients with chronic heart failure (ejection fraction < 60 %) or, severe chronic kidney disease (estimated glomerular filtration rate < 60ml/min), as well as pregnant or breast feeding women, were excluded from the study. The patients list of medication were screened for potential drug-drug interactions with metformin based on a previous review on drug-drug interactions with metformin. ²⁴ Drugs, known to interact with metformin pharmacokinetics or pharmacodynamics, were discontinued for a minimum of 5 times the drug half-life prior to the study. None of the patients were treated with vitamin E or glitazones prior to the study. The study protocol was approved by The Central Denmark Ethical Committees (No. 1-10-72-327-14) and a written informed consent was signed by all patients before participating in the study. All participants fasted for a minimum of 6 hours before tracer injection.

Preparation of ¹¹C-metformin

¹¹C-metformin was prepared as previously described ²⁵ and contained 0.02-0.4 µg/ml metformin dissolved in aqueous (NH₄)₂HPO₄ (100 mM, pH 5). The largest injected volume was 20ml, which was estimated to a maximum injected dose metformin of 8 µg. In comparison, therapeutic doses of metformin range from 500-1000 mg up to twice daily, and metformin in tracer doses is not associated with therapeutic effects.

¹¹C-metformin positron emission tomography and computer tomography
An arterial cannula was placed in the radial artery for taking blood samples measuring plasma activity of the tracer. Another cannula was placed in the cubital vein for administration of \( ^{11} \text{C} \)-metformin. For attenuation correction and anatomical localization, a low dose computed tomography (CT) scan was performed from lower chest to lower abdomen covering the liver and kidneys. \( ^{11} \text{C} \)-metformin was injected as a bolus (102-222MBq) followed by a 90 min dynamic PET scan using a Biograph 64 PET/CT system (Siemens, Erlangen, Germany). 43 manual blood samples were collected during the PET scan. Blood and plasma activity was measured in a cell counter (Cobra II; Packard Instruments Co., Meriden, Connecticut), and cross-calibrated to the scanner.

**Regions of interest, volumes of interest and Volume of distribution**

For the liver, a half-moon shaped region of interest (ROI) was drawn in the upper right lobe of the liver on seven slides based on anatomical localization using images derived from the CT scan. The seven ROIs composed a volume of interest (VOI) in which metformin activity was measured. This procedure has previously been described.\(^{20}\) In brief, dynamic PET time-activity curves were analyzed using plasma input functions and a linear approach (Logan) yielding a volume of distribution (Vd) in the liver, a procedure described by Gormsen et al in more detail.\(^{20}\) All kinetic analyses were performed using PMOD software (PMOD, Zurich, Switzerland). Standardized uptake values (SUVs) were calculated as SUV = concentration [kBq/mL] × (body weight [g]/injected dose [kBq]).

**Liver biopsy procedure**
Using a sterile procedure, local anesthesia, and ultrasound guidance, liver biopsies were taken by an experienced hepatologist or radiologist. Biopsies where split in two, one part being used for clinical histological examination, while the other was frozen in liquid nitrogen within 30 seconds, and used for real-time polymerase chain reaction (qPCR). Histological examination of liver biopsies was performed by an experienced hepatopathologist blinded for PCR and PET data. The biopsies were scored for steatosis, hepatocyte ballooning, inflammation and fibrosis according to the criteria proposed by the NASH-Clinical Research Network. Simple steatosis was differentiated from NASH according to the FLIP Pathology Consortium algorithm.

**Gene transcription analysis**

Total RNA was extracted with TRIzol (Gibco BRL, Life Technologies, Roskilde, Denmark). RNA was quantified using a NanoDrop 8000 Spectrophotometer (Thermo Scientific Pierce, Waltham, Maine). Integrity of the RNA was checked using Experion RNA Analyzer from BioRad. Two biopsies were excluded due to low RNA Integrity Number (<4). The cDNA was synthesized using a Verso cDNA kit (cat# Ab-1453, Thermo Fischer Scientific) with random hexamer primers. PCR-reactions were performed in duplicate using LightCycler SYBR Green master mix (Roche Applied Science) in a LightCycler 480 (Roche Applied Science). The following cycling conditions were used: One step at 95 °C for 3 min., then 95 °C for 10 sec., 60 °C for 20 sec. and 72 °C for 10 sec and finally a melting curve analysis was performed. The increase in fluorescence was measured in real time during the extension step.
The relative gene expression was estimated using the default “Advanced Relative Quantification” mode of the software version LCS 480 1.5.1.62 (Roche Applied Science) and specificity of the amplification was checked by melting curve analysis.

The following primer pairs were designed using QuantPrime:

**OCT1:** TAATGGACCACATCGCTCAA and AGCCCTGATAGACACAGA 190bp

**MCP1:** AGCCAGATGCAATCAATGCC and GTCTTGAAGATCACAGCTTCTTTGG 133bp

**MATE1:** TCGGCTTATCTTCTGCCTGT and CTGGGTAAGCTGGACACAT 197bp

**OCT3:** GGAGTTTCGCTCTGTTCAGG and GGAATGTGGACTGCCAAGTT 216bp

**SREBP1C:** GCCATGGATTGCACTTTCAAGAC and TGGGTCAAATAGGCCAGGGAAG 78 bp

**COL1A:** TGCGATGACGTGATCTGTGACG and TTTCTTGGTCGGTGGGTGACTCTG 112bp

**TGFβ:** GGACACCAACTATTGCTTCAGCTC and AAGTTGGCAGTAGTTGCCCCTGG 123bp

Several housekeeping genes were tested but S18 had equal expression between the groups and was therefore selected. The housekeeping gene, S18, was amplified using:

**S18:** TGGATACCGCAGCTAGGAAT and AACTACGACGGTATCTGATC 229 bp.

The expression level of the housekeeping gene was similar between all groups and interventions. All primers were from DNA Technology (Risskov, Denmark). A similar set-up
was used for negative controls, except that the reverse transcriptase was omitted and no PCR products were detected under these conditions.

**Genotyping**

Genotyping was performed as previously described.\(^{29}\) In short, in *SLC22A1* encoding OCT1, rs72552763 (M420del) and rs34130495 (G401S) were genotyped by Sanger sequencing while rs12208357 (R61C) and rs34059508 (G465R) were genotyped using predesigned TaqMan SNP genotyping assays on a StepOne Plus real-time instrument (Applied Biosystems - Thermo Fisher Scientific, Foster City, California) according to manufactures protocol. All variants were in Hardy-Weinberg equilibrium.

**Statistics**

Normal distributed data is presented as mean ± SD, and as median and range for non-normally distributed data, if not stated otherwise. Comparison of means was evaluated with Student’s t-test. Non-parametric analysis was performed using Wilcoxon rank sum test. Linear regression was performed for correlation analysis with goodness of fit displayed as R-squared ($r^2$). A two-tailed p-value <0.05 was considered statistically significant. A power calculation using $\alpha=0.05$, $\beta=0.8$ and hepatic Vd 2.21 ± 0.47 among healthy subjects previously examined, was performed. Calculations yielded a sample size of 18. All statistical analyses were performed using STATA 13.1 (Statacorp, College Station, Texas).

**Nomenclature of Targets and Ligands**

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY.
Results

Steatosis and inflammation

Using the FLIP algorithm, ten patients were categorized as having simple steatosis and eight patients as having NASH (Table 1). The two groups were significantly different in mRNA expression of monocyte chemoattractant protein-1 (MCP-1), TGFβ, collagen 1 A (COL1A), and sterol regulatory element-binding protein-1c (SREBP-1c) with higher expression in NASH compared with simple steatosis (Fig. 1 A-C). Basic characteristics of the studied subjects can be seen in Table 2. Alanine aminotransferase and HAlc levels were significantly higher among patients with NASH compared with simple steatosis; T2DM was more prevalent among NASH patients. All other measured parameters were equally distributed between the groups. Four subjects had prescriptions of medication which may interact with metformin pharmacokinetics. The combination of metformin and proton pump inhibitors were prescribed to three subjects and one subject was taken proton pump inhibitor only. Proton pump inhibitors and metformin were discontinued for two and three days prior to investigations respectively.

Hepatic metformin distribution is similar in patients with simple steatosis and with steatohepatitis

There was no significant difference in hepatic exposure of metformin between patients with simple steatosis (Vd 2.38 ± 0.56) and NASH (Vd 2.10 ±0.39) p= 0.30. Hepatic Vd’s in both groups were within the range previously found in eight healthy volunteers (Fig. 2A). Maximum standardized uptake values (SUVmax) in the liver (8.55 ± 1.62 vs 7.64 ± 2.39)
g/ml and T_{max} (159 ± 61 vs 142 ± 80) seconds were also comparable between the groups (Fig 2B).

**OCT1, OCT3, and MATE1 expression in simple steatosis and NASH**

By simple pooling of liver biopsies from all subjects, transporter mRNA expression was analyzed. OCT1 mRNA levels in the liver biopsies was 4-fold higher compared to MATE1 and 14-fold higher compared to OCT3. Analysis of mRNA expression of OCT1 and MATE1, revealed significantly higher expression in patients with NASH (n=8) compared with simple steatosis (n=8) (Fig. 3). There was a trend towards higher expression of OCT3 in patients with NASH compared to simple steatosis (p=0.06).

**No correlation between mRNA expression of OCT1, OCT3, nor MATE1 and hepatic exposure to metformin**

We tested for a correlation between transporter expression and hepatic metformin Vd by linear regression analysis. We found no correlation between hepatic metformin Vd and expression of OCT1, OCT3 nor MATE1 (Fig. 4). However, there was a positive correlation between OCT1 and MATE1 expression (p<0.05 R^2=0.3), OCT1 and OCT3 expression (p<0.05 R^2=0.5), and OCT3 and MATE1 expression (p<0.05 R^2=0.56) (supplemental information).

**Effect of genetic polymorphism on hepatic exposure to metformin**
A previous study has demonstrated an effect of genotype on hepatic exposure to metformin using $^{11}$C-metformin PET/CT. Several other studies have demonstrated pharmacokinetic and pharmacodynamics effects of OCT1 variants R61C, G401S, M420del and G465R. The entire cohort was therefore genotyped for the OCT1 variants R61C, G401S, M420del and G465R. Two subjects were heterozygotes for the R61C variant and three were heterozygotes for the M420del variant. No patient carried the variants G401S or G465R. Hepatic Vd of metformin was slightly lower among the five heterozygote carriers compared with carriers of the wild type allele (2.02 ± 0.40 vs 2.32 ± 0.52), but in this limited number of subjects the reduction in hepatic Vd did not reach statistical significance. We cannot preclude that some of the subjects carry variants in SLC47A1, however the number is expected to be low.

**Discussion**

Metformin, the most commonly used antidiabetic drug, is suggested to target the liver for its glucose-lowering effects. In this study, we for the first time demonstrate, hepatic metformin distribution in NAFLD patients using a newly developed $^{11}$C-metformin PET/CT technique. Our data demonstrate that hepatic distribution of metformin is similar among patients with steatosis and the more severe condition, NASH. Based on our power calculation, differences in hepatic metformin exposure less than 30% between groups cannot be ruled out. A direct comparison with subjects without liver disease were not made although the pharmacokinetic properties of metformin among NAFLD patients in this study were comparable to observations in healthy subjects under similar conditions. Together, these results suggest that metformin action in the liver is not negatively affected by inflammation and fibrosis in patients with NAFLD, a common condition among patients with T2DM.
Hepatic distribution of metformin depends on the function of specific organic cation transporter proteins, and these polyspecific transporters mediate transport of substrates in lipid metabolism, such as carnitine and acylcarnitine.\textsuperscript{36} The combination of liver biopsies and \textsuperscript{11}C-metformin dynamic PET-scan allowed for a comparison between function and gene expression of selected organic cation transporters. OCT1 is highly expressed in the liver\textsuperscript{17,37}, and is of significance in hepatic uptake of metformin.\textsuperscript{1} In animal models of NASH, gene expression of OCT1 is increased\textsuperscript{16} and higher OCT1 expression has been observed in patients with NASH compared with healthy controls.\textsuperscript{38} Our data support previous findings with significantly higher OCT1 gene expression in patients with NASH compared with simple steatosis. Direct comparisons with OCT1 expression in healthy subjects cannot be made in study design, since liver biopsies can only be taken on clinical indication. However, the above could indicate that increased OCT1 expression is an early feature of hepatic steatosis. Importantly, we found no correlation between OCT1 function, determined by \textsuperscript{11}C-metformin dynamic PET-scan, and OCT1 gene expression. This suggests that alterations in OCT gene expression reflect protein turn-over more than protein abundance.

The use of metformin to prevent progressive liver injury in NAFLD/NASH has been heavily debated.\textsuperscript{15,39} Many studies have shown an improvement in liver enzymes while taking metformin, but effects on histological endpoints have been less convincing.\textsuperscript{40} Our data does not give evidence to support the efficacy of metformin in NASH, but the distribution of \textsuperscript{11}C-metformin demonstrates that metformin can be transported into hepatocytes even during NASH with fibrosis. The most important clinical effect of metformin in NAFLD/NASH may not necessarily be due to effects on liver injury, but may instead be due to the beneficial cardiovascular effects of metformin treatment. Such effects have not been tested in a large placebo-controlled randomized study with NAFLD/NASH patients, but a systematic review by Musso et al. revealed major beneficial effects of metformin on cardiovascular parameters.
in patients with NASH.\textsuperscript{15} Our study demonstrates that the liver of patients with NASH and simple steatosis is exposed to metformin. Therefore, the preserved biodistribution of metformin supports NASH patients being sensitive to the drug. If metformin effects in the liver mediate long term beneficial effects on cardiovascular endpoints, then treating NASH patients with metformin appear feasible.\textsuperscript{41,42}

Hepatic triglyceride levels in patients with NASH correlate positively to OCT1 mRNA expression.\textsuperscript{36} In accordance with this observation, we find a strong positive correlation between mRNA expression of a key protein in lipogenesis, SREBP1c, and OCT1 expression R\textsuperscript{2} = 0.4 p<0.05, suggesting that high OCT1 expression is a result of lipid accumulation in the liver per se. This is in line with studies in OCT1-/ mice, which demonstrate reduced intrahepatic fat accumulation compared with wild-type mice.\textsuperscript{36}

Hepatic mRNA levels of chemoattractant chemokine MCP-1 were higher in patients with NASH compared with simple steatosis. This is probably due to release by monocytes upon inflammatory stimulation.\textsuperscript{43} Metformin has been suggested to have anti-inflammatory effects, and metformin treatment can attenuate mRNA levels of MCP-1.\textsuperscript{44-46} We found a positive correlation between OCT1 and MCP-1 mRNA expression (r\textsuperscript{2} = 0.27, p<0.05), but in order to test for causality a randomized placebo-control setup with multiple liver biopsies will be needed. Similar to MCP-1 expression, TGFβ was also positively correlated to mRNA expression of OCT1 (r\textsuperscript{2} = 0.39, p<0.05). Fibrosis formation, measured as mRNA levels of TGFβ, is suppressed after metformin treatment in a mouse model of liver-fibrosis.\textsuperscript{47} This could indicate a connection between fibrosis formation, OCT1 expression and response to metformin treatment, although this needs to be further investigated in a different design.

A subgroup of the patients in this study, all with NASH, also had T2DM. The abundance of T2DM among patients with NAFLD reflects the fact that 14 of the patients were recruited
from a previous project where T2DM was an exclusion criteria. The prevalence of T2DM in our study, is however within the range of what has previously been reported.\textsuperscript{48} Out of the four patients with diabetes, two were diagnosed concurrently with the NASH diagnosis, and the remaining two were diagnosed 4 months and 5 years prior to the NASH diagnosis, respectively. Their HbA1c levels at the time of investigation were 74, 73, 64 and 52 mmol/mol. This group only consisted of four subjects, but these subjects represent the first diabetic patients ever to be investigated using $^{11}$C-metformin. The hepatic metformin exposure in these patients was similar to non-diabetic subjects (Vd 1.91 ± 0.09) compared to (Vd 2.33 ± 0.14) among non-diabetic patients. Due to the sparse number of individuals with NASH and diabetes statistical analysis was not performed comparing hepatic metformin Vd. However, the hepatic exposure was comparable to data from healthy subjects from a previous study.\textsuperscript{19} Hepatic distribution of metformin appears, therefore, to be stable across conditions that are central for its clinical use.

A previous study has demonstrated that hepatic expression of OCT1 and OCT3 is affected by specific SNPs in $SLC22A1$ and $SLC22A3$ encoding these proteins.\textsuperscript{49} In this study, OCT1 mRNA expression was similar in patients with one reduced function allele in $SLC22A1$ compared with patients with no reduced function allele. However, these data should be interpreted cautiously due to the low number of patients included in this study and given that no prior power calculation was made for this endpoint. Among the limitations of our study is the inability to detect OCT1 protein levels in liver biopsies. However, in our hands, it has not been possible to validate specific antibodies that detect OCT1 in the minute biopsy material available from human liver samples. Furthermore, progression or reversal in NAFLD/NASH from time of biopsy and pathology evaluation to performing PET/CT may have caused misclassification of patients. However, there was no significant change in weight nor in alanine transaminase, which have been reported as predictors of liver disease progression in
NAFLD, from time of diagnosis to 11C-metformin PET/CT. All subjects had detectable distribution of metformin to the liver. Therefore, although progression or regression of liver disease cannot be excluded, it is evident that hepatic inflammation per se does not block hepatic metformin uptake.

In conclusion, this study demonstrates that hepatic exposure to metformin among patients with simple steatosis is not different from exposure in patients with NASH. The observed hepatic exposure in patients with NAFLD is within range observed in healthy subjects during previous investigations. Furthermore, we found a higher expression of OCT1 in patients with NASH compared to simple steatosis. However, there was no correlation between OCT1 or MATE1 expression and hepatic metformin distribution. Observations from this study provides a rationale for continuation metformin administration during NAFLD, but future studies are needed to evaluate the clinical impact of such treatment.

Acknowledgments
The authors would like to thank laboratory technician Lenette Egelund Pedersen for her work with mRNA extraction and PCR analysis and the grateful help and assistance by the staff at the Department of Nuclear Medicine and PET-center, Aarhus University Hospital. We would also like to thank Novo Nordisk Foundation, Danish Council for Independent Research and A. P. Møller Foundation for the financial support.

Statement of interest
The authors of this manuscript declare no conflicts of interest

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author Contributions:**

E.S wrote the manuscript, performed experiments and analyzed data; M.V, S.J, L.G, S.H, S.H.D, S.B.P performed experiments; S.F, H.G, S.B.P analyzed data, N.J, K.B. edited the manuscript; All authors reviewed the manuscript before submission.


9. Mattias Ekstedt HH, Patrik Nasr, Mats Frederikson, Per stål, Stergios Kechargias, Rolf Hultcrantz. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology. 2014.


Table 1. Results of histopathological examination of the liver biopsies using FLIP Pathology Consortium algorithm and NAS + Fibrosis score. Abbreviations: SAF, steatosis and fibrosis; NAS, non-alcoholic fatty liver disease activity score.

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Steatosis</th>
<th>Ballooning</th>
<th>Inflammation</th>
<th>Fibrosis</th>
<th>SAF score</th>
<th>SAF diagnosis</th>
<th>Kleiner (NAS) + Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1b</td>
<td>3+4+1</td>
<td>NASH</td>
<td>3+2+2=7 + 1</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1b</td>
<td>3+4+1</td>
<td>NASH</td>
<td>3+2+2=7 + 1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1b</td>
<td>3+3+1</td>
<td>NASH</td>
<td>3+2+1=6 + 1</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2+2+4</td>
<td>NASH</td>
<td>2+1+1=4 + 4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1+1+0</td>
<td>NAFLD</td>
<td>1+0+1=2 + 0</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2+1+0</td>
<td>NAFLD</td>
<td>2+0+1=3 + 0</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1+1+0</td>
<td>NAFLD</td>
<td>1+0+1=2 + 0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1a</td>
<td>3+3+1</td>
<td>NASH</td>
<td>3+1+2=6 + 1</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2+1+0</td>
<td>NAFLD</td>
<td>2+0+1=3 + 0</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2+2+0</td>
<td>NASH</td>
<td>2+1+1=4 + 0</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3+1+0</td>
<td>NAFLD</td>
<td>3+0+1=4 + 0</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2+1+0</td>
<td>NAFLD</td>
<td>2+0+1=3 + 0</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1a</td>
<td>3+2+1</td>
<td>NASH</td>
<td>3+1+1=5 + 1</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2+1+0</td>
<td>NAFLD</td>
<td>2+0+1=3 + 0</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2+0+0</td>
<td>NAFLD</td>
<td>2+0+0=2 + 0</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2+1+0</td>
<td>NAFLD</td>
<td>2+0+1=3 + 0</td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3+0+0</td>
<td>NAFLD</td>
<td>3+0+0=3 + 0</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1a</td>
<td>3+3+1</td>
<td>NASH</td>
<td>3+1+2=5 + 1</td>
</tr>
</tbody>
</table>
Table 2. Basic characteristics. All data presented as mean ± SD if not stated otherwise. P values acquired using Students t-test, comparing means between groups with simple steatosis and NASH. aReported as median and (range) bNon parametric test. Abbrevations: BMI, Body Mass Index

<table>
<thead>
<tr>
<th></th>
<th>Simple steatosis (n=10)</th>
<th>NASH (n=8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yearsa</td>
<td>46 (26-71)</td>
<td>40 (29-69)</td>
<td>0.50b</td>
</tr>
<tr>
<td>Female sex, nr. (%)</td>
<td>2 (20%)</td>
<td>3 (38%)</td>
<td>N/A</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>100.5 ± 18.0</td>
<td>103.0 ± 24.0</td>
<td>0.80</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>32.7 ± 5.5</td>
<td>34.7 ± 6.9</td>
<td>0.50</td>
</tr>
<tr>
<td>Number of patients with</td>
<td>0/10</td>
<td>4/8</td>
<td>N/A</td>
</tr>
<tr>
<td>Diabetes Mellitus Type 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic, mmHg</td>
<td>141 ± 14</td>
<td>140 ± 11</td>
<td>0.81</td>
</tr>
<tr>
<td>Diastolic, mmHg</td>
<td>91 ± 7</td>
<td>93 ± 7</td>
<td>0.65</td>
</tr>
<tr>
<td>Biochemical levels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase, U/l</td>
<td>75 ± 36</td>
<td>175 ± 76</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Alkaline phosphatase, U/l</td>
<td>96 ± 35</td>
<td>83 ± 18</td>
<td>0.36</td>
</tr>
<tr>
<td>Bilirubin µmol/l</td>
<td>11 ± 5</td>
<td>10 ± 7</td>
<td>0.61</td>
</tr>
<tr>
<td>Albumin g/l</td>
<td>42 ± 3</td>
<td>42 ± 3</td>
<td>0.97</td>
</tr>
<tr>
<td>Creatinine µmol/l</td>
<td>81 ± 13</td>
<td>68 ± 13</td>
<td>0.05</td>
</tr>
<tr>
<td>Hemoglobin mmol/l</td>
<td>9.3 ± 0.7</td>
<td>9.5 ± 0.6</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Fig. 1. Hepatic gene expression profile of fat accumulation, inflammation and fibrosis in patients with simple steatosis (n=8) and NASH (n=8). Each column represents mean ± SEM (*p<0.05, **p<0.01). (E) Liver biopsy showing moderate steatosis (NAFLD). Zone 3 liver cells (lower half, to the right) show moderate steatosis without ballooning or perisinusoidal fibrosis (Masson-trichrome stain). (F) Liver biopsy showing active non-alcoholic steatohepatitis (NASH). There is marked steatosis and in zone 3 (lower third, to the right) with numerous clusters of enlarged, ballooned hepatocytes, accompanied by inflammation and perisinusoidal fibrosis (Masson-trichrome stain). Abbreviations: NASH, non-alcoholic steatohepatitis; MCP-1, monocyte chemoattractant Protein-1; SREPB1c, sterol regulatory element-binding protein 1c; COL1A, Collagen Type 1 alpha; TGF-β, transforming growth factor beta.
Fig. 2. Distribution of metformin in patients with simple steatosis and NASH. (A) Hepatic volume of distribution, Vd, of metformin in patients with simple steatosis (n= 10) and NASH (n=8) compared to the range among eight healthy controls (dashed line). (B) Hepatic TACs after i.v. injection with 11C-metformin and 90 min PET-scan. (C) Transaxial PET co-registered with low dose CT. PET images are average 11C-metformin activity 0-40 min (representative images). Columns represent mean ± SEM. Abbreviations: TACs, time-activity curves; SUVs, Standardized uptake values; NASH, non-alcoholic steatohepatitis; PET, positron emission tomography; CT, computed tomography; sec, seconds.
Fig. 3. Hepatic mRNA expression of membrane bound transporters involved in metformin distribution. Simple steatosis (n=8) and NASH (n=8). Each column represents mean ± SEM (*p<0.05). Abbreviations: NASH, non-alcoholic steatohepatitis; OCT1 & 3, organic cation transporter 1 & 3; MATE1, multidrug and toxin extrusion 1.
Fig. 4. Correlation between hepatic volume of distribution of metformin and gene expression of transmembrane transporters (n=16). Abbreviations: OCT1 & 3, organic cation transporter 1 & 3; MATE1, multidrug and toxin extrusion 1; Vd: Volume of distribution.