

# The generation of carcinogenic etheno-DNA adducts in the liver of patients with nonalcoholic fatty liver disease

Kirsten-Berit Linhart<sup>1,2</sup>, Katharina Glassen<sup>1,2</sup>, Teresa Peccerella<sup>1,2</sup>, Rüdiger Waldherr<sup>1,2</sup>, Heinz Linhart<sup>1,2</sup>, Helmut Bartsch<sup>3</sup>, Helmut K. Seitz<sup>1,2</sup>

<sup>1</sup>Centre of Alcohol Research, University of Heidelberg, Heidelberg, Germany; <sup>2</sup>Department of Medicine (Gastroenterology & Hepatology), Salem Medical Centre, Heidelberg, Germany; <sup>3</sup>Division of Toxicology and Cancer Risk Factors, Germany Cancer Research Centre (DKFZ), Heidelberg, Germany

*Correspondence to:* Helmut K. Seitz, MD, AGAF. Professor of Medicine, Department of Medicine, Salem Medical Centre, Gastroenterology and Alcohol Research, University of Heidelberg, Heidelberg, Germany. Email: [helmut\\_karl.seitz@urz.uni-heidelberg.de](mailto:helmut_karl.seitz@urz.uni-heidelberg.de).

**Background:** Nonalcoholic fatty liver disease (NAFLD), in particular its more aggressive form nonalcoholic steatohepatitis (NASH) is increasingly observed as a cause of end stage liver disease and hepatocellular carcinoma (HCC). Reactive oxygen species (ROS) are an important factor in the pathogenesis of HCC. ROS can react with polyunsaturated fatty acids derived from membrane phospholipids resulting in the production of reactive aldehydes as lipid oxidation (LPO) byproducts, such as 4-hydroxynonenal (4 HNE). 4 HNE can react with DNA to form mutagenic exocyclic etheno-DNA adducts. ROS is induced by inflammatory processes, but also by induction of cytochrome P450 2E1 (CYP2E1), as seen with chronic alcohol consumption.

**Methods:** Immunohistochemical detection of CYP2E1, 4 HNE and hepatic exocyclic etheno-DNA adducts was performed on liver sections from 39 patients with NAFLD. Spearman rank correlation was calculated to examine possible correlations.

**Results:** Exocyclic etheno-DNA adducts were detected and correlated significantly with 4 HNE, but not with CYP2E1.

**Conclusions:** This is the first description of highly carcinogenic exocyclic etheno-DNA adducts in NAFLD patients. We could show that exocyclic etheno-DNA adducts significantly correlated with lipid peroxidation product 4 HNE, but not with CYP2E1, implying that in NAFLD ROS generation with consecutive DNA damage is rather inflammation driven through various cytokines than by induction of CYP2E1.

**Keywords:** Nonalcoholic fatty liver disease (NAFLD); etheno-DNA adducts; hepatocellular carcinoma (HCC)

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## Introduction

Nonalcoholic fatty liver disease (NAFLD) is a significant cause of chronic liver disease and its prevalence is increasing worldwide. NAFLD is directly associated with the metabolic syndrome and obesity (1-3) and it is estimated that NAFLD affects approximately 90% of morbidly obese patients in the United States (4). In addition, to metabolic syndrome and obesity, insulin resistance and diabetes mellitus (DM) type 2 have been established as risk factors for NAFLD (5-10).

NAFLD is subdivided into nonalcoholic fatty liver (NAFL), in which hepatic steatosis is observed without significant inflammation and nonalcoholic steatohepatitis (NASH), in which hepatic steatosis is associated with inflammation. NAFLD, in particular the more aggressive form NASH, is increasingly observed as a cause of end stage liver disease and hepatocellular carcinoma (HCC), the most deleterious complication of cirrhosis. Obesity, DM and advanced fibrosis increase the risk of NASH progressing to cirrhosis and subsequently to HCC, however, the causal

link between NASH and the development of HCC is still largely unclear.

Oxidative stress is considered an important factor in the pathogenesis of cancer including HCC (11) and reactive oxygen species (ROS) with consecutive DNA damage are induced by inflammatory processes. For example various cytokines can induce nitric oxide synthetase (iNOS), xantine oxidase (XO), myeloperoxidase (MPO) and NADPH oxidase (12,13). ROS can also be generated through other mechanisms, such as the induction of cytochrome P450 2E1 (CYP2E1), as demonstrated in the context of alcohol consumption (14,15).

ROS can react with polyunsaturated fatty acids derived from membrane phospholipids resulting in the production of reactive aldehydes as lipid oxidation (LPO) byproducts. The most abundant LPO byproducts are 4-hydroxynonenal (4 HNE) and malondialdehyde (MDA). These LPO byproducts react with DNA either directly or through bifunctional intermediates to form various mutagenic exocyclic etheno-DNA adducts. For example, LPO byproducts derived from  $\gamma$ -linoleic acid, such as 4 HNE, react with the DNA bases A,C and G to yield inter alia the unsubstituted etheno-DNA adducts 1,N<sup>6</sup>-etheno-2'-deoxyadenosine ( $\epsilon$ dA), 3,N<sup>4</sup>-etheno-2'-deoxycytidine ( $\epsilon$ dC), 1,N<sup>2</sup>-etheno-2'-deoxyguanosine (1,N<sup>2</sup> $\epsilon$ dG), and N<sup>2</sup>,3-etheno-2'-deoxyguanosine (N<sup>2</sup>,3 $\epsilon$ dG). In addition, DNA can also be modified directly by ROS to 8-nitrodG and 8-Oxo-dG (16).

Exocyclic etheno-DNA adducts are chemically stable and exhibit strong mutagenic properties, resulting in various types of base pair substitution mutations and other types of genetic damage in all organisms that have been tested so far (17,18). exocyclic etheno-DNA adducts can lead to AT GC transversion and AT TA and AT CG transitions (19,20). Incorporation of a single exocyclic etheno-DNA adducts in either DNA strand of HeLa cells showed a similar miscoding frequency and was more mutagenic than 8-oxo-dG (21). In addition, individual etheno adducts are poorly repaired in certain cells and tissues, which further increases their biological relevance (22). Importantly, exocyclic etheno-DNA adducts are preferably formed in codon 249 of TP 53, resulting in inactivation of the tumor suppressor p53 and secondary cellular resistance to apoptosis and growth advantage (23). Formation of  $\epsilon$ dA and  $\epsilon$ dC *in vivo* by vinyl chloride, a confirmed human liver carcinogen, further supports the concept that etheno-DNA adducts play a causal role in the initiation and progression

of HCC (24).

The aim of our study therefore was to investigate, if highly carcinogenic exocyclic etheno-DNA adducts are formed in livers of patients with NAFLD and if the generation of exocyclic etheno-DNA adducts correlate with the induction of CYP2E1, as it has been demonstrated in alcoholic liver disease (ALD).

## Methods

### *Human liver species and metabolic data*

Human liver fine-needle biopsy samples were obtained from 39 patients with NAFLD, 29 with NASH, 10 with NAFL, for diagnostic purposes at Salem Medical Center, University of Heidelberg, Germany. The liver specimens were fixed in formaldehyde for further testing.

Histologically normal liver sections from three healthy adult subjects were analysed as controls for exocyclic etheno-DNA adducts background staining intensity. These biopsies were originally taken to rule out hepatic tumours. The study was approved in accordance with the declaration of Helsinki by the Ethical Committee of the University of Heidelberg, Germany. All biopsies were assessed by histopathologists experienced in liver pathology and diagnosed as NASH or NAFL. Both investigators were blinded for immunohistopathology examinations related to the present study. Hepatic steatosis, inflammation and fibrosis were assessed using the scoring system of Kleiner *et al.* (25).

BMI was calculated as the individual's body mass in kilogram divided by the square of their height in meter. Eleven patients were treated for DM, aspartate aminotransferase (AST), alaninamino transferase (ALT) and gamma glutamyltransferase (GGT) were determined using standards procedures.

### *Immunohistochemical detection of etheno-DNA adducts*

Staining was performed on liver tissue sections using the method developed in our laboratory (26-29). Paraffin-fixed slides were dipped in phosphate buffered saline (PBS) for 10 min and then placed in 0.3% H<sub>2</sub>O<sub>2</sub> in absolute methanol for 10 min to quench endogenous peroxidase. Slides were incubated with proteinase K (20 mg/mL) (Roche, Mannheim, Germany) in double distilled H<sub>2</sub>O at room temperature for 10 min to remove histone and non-histone proteins from DNA increasing antibody accessibility. After

washing with PBS, slides were treated with 20 µg/mL RNase (Roche, Mannheim, Germany) (heated for 10 min at 80 °C to inactivate DNase) at 37 °C for 1 hour to prevent antibody binding to RNA adducts and then washed in PBS. To denature DNA, cells were treated with 4N HCl for 5 min at room temperature and subsequently rinsed in double distilled water and PBS. The pH was neutralized with 50 mM Trisbase buffer, pH 7.4, for 5 min at room temperature. Non-specific binding sites were blocked with 8% bovine serum albumin (BSA), 2% normal horse serum, 0.05 % Tween and 0.05% Triton X-100 for 20 min at 37 °C. Slides were incubated at 4 °C overnight with the primary monoclonal antibody EM-A-1 against εdA (provided by Drs. P. Lorenz and M. Rajewsky, University of Essen, Essen, Germany), at a dilution of 1:20 and 2% normal horse serum to block nonspecific binding. After washing with PBS, the antibody detection was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol [incubation with secondary antibody: horse anti-mouse IgG (H + L) 1:400 for 40 min at room temperature]. Diaminobenzidine (DAB) was used as a chromogen to visualize the reaction. The reaction was stopped after 5 min with H<sub>2</sub>O. Slides were counterstained with 4',6'-diamidino-2-phenylindole and mounted with Roti-Histokitt II (Carl Roth, Karlsruhe, Germany). All slides were subjected to the same standard conditions. Negative controls were performed by omitting the primary antibody.

#### ***Immunohistochemical staining of CYP2E1 and protein bound 4 HNE***

Paraffin-embedded liver biopsy samples were cut into six micrometer sections and placed on 3-aminopropyl-triethoxysilan-coated glass slides. Sections were treated with 0.5% H<sub>2</sub>O<sub>2</sub> in absolute methanol for 10 min to quench endogenous peroxidase activity. Thereafter, sections were incubated at room temperature for 2 hours with the primary antibody (rabbit anti-human CYP2E1, 1:400, Chemicon, Hofheim, Germany) or with rabbit anti-4 HNE (1:250, Alexis, Lörrach, Germany) and 5% normal goat serum to block nonspecific binding. Vectastain Elite ABC (Vector Laboratories; Burlingame, CA, USA) was used for detection according to the manufacturer's protocol. Staining was developed by incubating the sections for 5 min in diaminobezidine (DAB). Sections were counterstained with hematoxylin and mounted with Roti-Histokitt II (Carl Roth, Karlsruhe, Germany). Negative controls were

performed by omitting the primary antibody.

#### ***Imaging and semi-quantitative analyses of etheno-DNA adducts, CYP2E1 expression and protein-bound 4 HNE***

After immunohistochemical staining for εdA, CYP2E1 and 4 HNE, the blinded slides were independently scored by a second investigator. Representative pictures were taken at a magnification of 100×/400× with a SPOT FLEX system (Model 15.2 64 Mp, shifting pixel, DIAGNOSTIC Instruments, Inc., Sterling Heights, Michigan, USA, SPOT VERSION 4.6.4.69) and analyzed using Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>).

Staining intensity for CYP2E1 and 4 HNE was assessed according to the scale devised by Tsutsumi *et al.* (30), whereby 3+, 2+, 1+ and 0 denote intense, moderate, slight and no specific immunostaining, respectively. Staining intensity of εdA was estimated and recorded as 1 to 4. In addition, the frequency of nuclei positively stained for εdA was calculated as % of stained cell nuclei over a total number of cells counted. Finally, an intensity score (staining intensity x% of stained cell nuclei) was calculated.

#### ***Statistics***

Spearman rank correlation was calculated to examine possible correlation between characteristics. A value of P<0.05 was used as level of significance.

#### **Results**

*Table 1* shows the metabolic characteristic of our patients. In order to investigate the association of steatosis with DM we correlated the degree of steatosis in our liver biopsies with the presence of DM in our patients. As shown in *Table 2*, we found a significant positive correlation between the degree of hepatocellular steatosis and the presence of DM. In addition, hepatic steatosis correlated significantly with the degree of lobular inflammation and fibrosis. We also found a significant correlation between DM and lobular inflammation, as well as DM and fibrosis. Further, we analyzed CYP2E1, 4 HNE and εdA content in liver biopsies from our patients with NAFLD using immunohistochemistry. εdA was detected in a broad range of intensity (*Figure 1*), but unlike in ALD εdA did not correlate significantly with CYP2E1. However, we found a significant correlation between 4 HNE and εdA (*Table 2*).

**Table 1** Patients metabolic characteristics

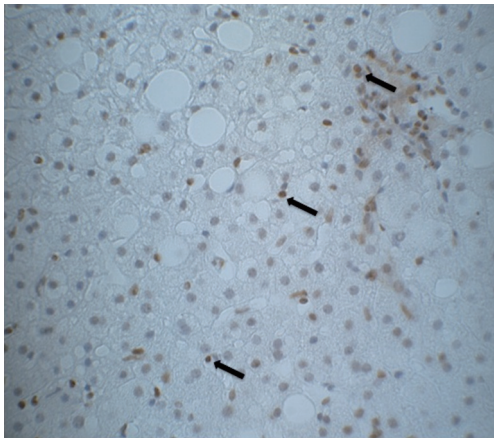
Patient No.	Sex	BMI (kg/m <sup>2</sup> )	Diabetes mellitus	GOT (U/L)	GPT (U/L)	GGT (U/L)
1	M	31.14	No	36.64	77.34	30
2	F	27.92	No	27.76	35.48	159
3	M	24.46	No	NA	NA	NA
4	M	28.41	No	38.86	42.76	408
5	F	22.04	Yes	83.26	177.44	657
6	M	31.17	Yes	47.74	80.98	264
7	F	33.06	No	38.86	44.58	204
8	F	26.42	No	125.44	115.56	42
9	F	37.65	Yes	NA	NA	NA
10	M	29.32	No	291.94	477.74	363
11	F	24.8	No	103.24	101	375
12	F	39.92	Yes	60	82	80
13	F	27.64	No	NA	NA	142
14	M	NA	Yes	27.17	39.12	54
15	F	28.13	Yes	74.38	91.9	486
16	M	27.08	No	45.52	166.52	18
17	F	38.1	No	98	131.94	1281
18	M	24.22	No	68	159	35
19	M	27.78	No	51	73	40
20	F	33.91	Yes	NA	NA	NA
21	M	33.33	No	52	97	44
22	M	21.95	No	142	311	143
23	F	27.55	Yes	107.68	146.5	357
24	F	25.86	No	37	99	235
25	M	24.62	No	69.94	157.42	360
26	M	30.69	No	54.4	102.82	93
27	F	26.44	Yes	69	141	483
28	M	28.37	Yes	40	105	126
29	M	25.31	No	56.62	64.6	78
30	F	NA	No	83.26	70.06	696
31	F	29.41	No	45.52	71.88	330
32	M	34.02	No	136.54	221.12	384
33	F	NA	Yes	23.32	28.2	165
34	M	23.99	No	61.06	70.06	549
35	M	22.47	No	75	83	122
36	F	24.86	No	NA	NA	NA
37	M	NA	No	40	60	263
38	F	NA	No	45	53	156
39	F	33.39	No	33	65	72

NA, not available because of lack of material; GGT, gamma glutamyltransferase.

**Table 2** Correlations of patients with NAFLD

Variables	εdA	4 HNE	CYP2E1	Steatosis	Inflammation	Fibrosis	DM	AST	ALT	GGT
εdA	1	0.466**	-0.140	0.790	0.145	-0.370	-0.220	-0.133	-0.193	-0.154
4 HNE	-	1	-0.171	0.036	0.068	-0.072	-0.200	0.052	-0.166	0.780
CYP2E1	-	-	1	0.188	0.206	0.303	-0.009	0.103	-0.530	0.220
Steatosis	-	-	-	1	0.582**	0.671**	0.331*	-0.760	0.028	-0.250
Inflammation	-	-	-	-	1	0.668**	0.449**	0.129	0.160	-0.320
Fibrosis	-	-	-	-	-	1	0.460**	-0.920	-0.110	-0.269
DM	-	-	-	-	-	-	1	-0.650	0.170	0.168
AST	-	-	-	-	-	-	-	1	0.741**	0.387*
ALT	-	-	-	-	-	-	-	-	1	0.107
GGT	-	-	-	-	-	-	-	-	-	1

Data represent Spearman correlation coefficient, \*, P<0.05; \*\*, P<0.01; NAFLD, nonalcoholic fatty liver disease; εdA, etheno-2'-deoxyadenosine; 4 HNE, 4-hydroxynonenal; CYP2E1, cytochrome P450 2E1; DM, diabetes mellitus; AST, aspartate aminotransferase; ALT, alaninamino transferase; GGT, gamma glutamyltransferase.



**Figure 1** Immunohistology of εdA in one liver biopsy from a patients with NASH, magnification 40x. εdA, etheno-2'-deoxyadenosine; NASH, nonalcoholic steatohepatitis.

## Discussion

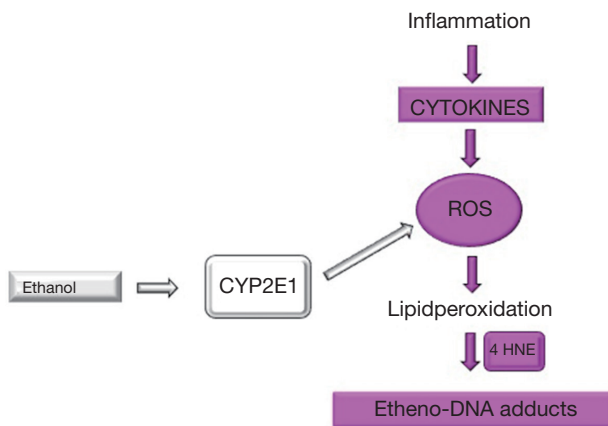
Hepatic steatosis is a manifestation of triglyceride accumulation in hepatocytes, mostly derived from fatty acids stored in adipose tissue and fatty acids synthesized by hepatocytes through *de novo* lipogenesis. Insulin resistance with consecutive increase of lipolysis in adipose tissue and excess importation of free fatty acid (FFA) to the liver is considered a major factor in the pathogenesis of hepatic steatosis. If lipid accumulation overwhelms the capacity of protective oxidative metabolic pathways in the liver, inflammatory cytokines may subsequently be induced (31), resulting in progression of NAFL to NASH. Consistent with

this, we found a significant correlation between the presence of DM and the degree of steatosis and inflammation. Further we found a significant correlation between the degree of hepatocellular steatosis and lobular inflammation.

The most important finding of our study, however, is the observation of highly carcinogenic exocyclic etheno DNA lesions in the liver of patient with NAFLD. Previous studies had shown increased levels of carcinogenic etheno-DNA adducts that are formed by the reaction with the major LPO product 4 HNE with nucleobases in hepatocytes of patients with ALD (28). In this context CYP2E1, induced by chronic alcohol consumption, significantly correlated with 4 HNE and εdA in liver biopsies from ALD patients, implying that CYP2E1 is an important factor in ethanol-mediated carcinogenesis (32). CYP2E1 is also induced in NAFLD (33-35). Although this induction is less pronounced as in ALD, it also has severe consequences with respect to the generation of oxidative stress. Indeed inflammation driven oxidative stress maybe an important mechanism in the progression of NAFLD.

Our data show clearly that εdA correlated significantly with the lipid peroxidation product 4 HNE, but not with CYP2E1. An explanation for that could be that the inflammatory process in our NAFLD patients resulting in ROS via cytokine driven inflammation predominates ROS generation by CYP2E1. Similar observations were made in children with NASH, where, in hepatic biopsies from NASH children only a borderline significance of εdA with CYP2E1 was found (36). It was speculated that most of ROS formation was rather inflammation driven than generated through CYP2E1, since it has been reported that





**Figure 2** Simplified pathophysiology of ROS and etheno-DNA adduct formation. In NASH, inflammation driven cytokine secretion results in ROS generation, which leads to lipidperoxidation with the occurrence of lipidperoxidation products such as 4 HNE. These adducts react with DNA bases to form exocyclic etheno-DNA adducts. Chronic alcohol consumption results in the induction of CYP2E1, which is involved in ethanol oxidation through the microsomal ethanol oxidizing pathway. During this reaction ROS is generated without inflammation. ROS, reactive oxygen species; NASH, nonalcoholic steatohepatitis; 4 HNE, 4-hydroxynonenal; CYP2E1, cytochrome P450 2E1.

high prevalence of oxidative stress in children with NAFLD is associated with an increased severity of steatohepatitis.

In an epidemiological retrospective study it was clearly shown that NASH patients had an increased risk to develop hepatocellular cancer (37). And this was further enhanced, when alcohol was taken even in social quantities. In both situations, in NASH and following alcohol consumption, exocyclic etheno-DNA adducts occur and regardless of the course of formation of exocyclic etheno-DNA adducts, it is clear that these adducts are highly carcinogenic and are associated with an increased risk for hepatocarcinogenesis (37). Therefore, it was recommended patients with NAFLD should avoid alcohol of any amount (38).

In summary, this is the first description of highly carcinogenic etheno-DNA adducts in NAFLD patients. We could show that  $\epsilon$ DA significantly correlated with lipid peroxidation product 4 HNE, but not with CYP2E1, implying that in NAFLD ROS generation with consecutive DNA damage is rather inflammation driven through various cytokines than by induction of CYP2E1. This context is illustrated in *Figure 2*.

It needs to be determined, whether these adduct early in the course of the disease may act as biological markers to predict a

risk for development of hepatocellular cancer later in life.

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*Disclosure:* The authors declare no conflict of interest.

*Ethical Statement:* The study was approved in accordance with the declaration of Helsinki by the Ethical Committee of the University of Heidelberg, Germany.

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