Brief report:

Oxidised and reduced glutathione and related thiols in human gallbladder bile.

Wilbert HM Peters¹, Annie van Schaik¹, Joost Peters², Harry van Goor².

Depts. of Gastroenterology¹ and General Surgery², Radboud University Medical Center, Nijmegen, the Netherlands.

Short Title: Thiols in human bile

Keywords: gallbladder, bile, glutathione, cysteine, homocysteine, oxidative stress.

Corresponding author:

Wilbert HM Peters, PhD

Dept of Gastroenterology,

Radboud University Medical Center

P.O. Box 9101, 6500 HB

Nijmegen, the Netherlands,

E-mail: w.peters@mdl.umcn.nl
SUMMARY.

**Objective:** Endogenous thiols such as glutathione, cysteine and cysteinyl-glycine are very important in the protection against chemical or oxidative damage. Synthesis of glutathione is mainly performed in the liver, and other tissues or organs are supplied with glutathione via sinusoidal efflux into the blood. However, also canalicular efflux may occur and thiols may be present in human gallbladder bile, the exact function here is greatly unknown. So far, no extensive data on thiol levels in human gallbladder bile have been published.

**Research design and Methods:** Gallbladder bile was obtained from 30 patients after surgery for pancreatic or duodenal cancer, chronic pancreatitis or cholecystolithiasis, and was analysed for glutathione and related thiols by high performance liquid chromatography.

**Results:** A more than 100-fold inter-individual variety in thiol levels was found, cysteine being by far the most common thiol present. In contrast to earlier data on rodent bile, human bile did contain far less glutathione, whereas most thiols present were in the oxidized state.

**Conclusions:** Composition of human gallbladder bile with respect to thiols is completely different as suggested in literature mainly based on animal studies. Most thiols are in the oxidised state, indicating much chemical or oxidative stress in the patients studied here.
INTRODUCTION.

Glutathione is a tripeptide composed of glutamate, cysteine and glycine that exists in a reduced (Glu) and oxidised (oGlu) form. Oxidised glutathione may occur in part as protein bound glutathione, whereas reduced glutathione is not bound to proteins. The most widely recognized function of reduced glutathione is its defence against toxic compounds, both of endogenous and exogenous origin. Another important role of glutathione however, is its reservoir function for cysteine, an important amino acid. Because glutathione is more stable and resistant against oxidation than cysteine, it serves as a preferred form for storage and transport of cysteine (1).

Glutathione is synthesized mainly in the liver and following sinusoidal efflux from liver into blood plasma, glutathione is transported, hydrolysed and taken up in tissues rich in γ-glutamyl-transpeptidase (GGT) such as kidney and lung. Thus, blood plasma serves as a transport medium for glutathione (and cysteine) from liver to the various other tissues that are consumers of glutathione and cysteine (2).

In contrast to the sinusoidal efflux of glutathione from the liver, the role of the canalicular efflux into bile and intestine is less well understood (1). Numerous studies on content and composition of glutathione and its metabolites in bile have been performed in bile duct canulated animals, demonstrating that glutathione content may be very high, depending on species (3). Furthermore it was shown that glutathione may be rapidly oxidised and hydrolysed in bile (1). Interestingly, it was also demonstrated that the biliary concentration of glutathione was lower than, but directly proportional to the intrahepatic concentration (4). Furthermore, adjustment of toxins to rats did increase the biliary excretion of oxidised glutathione, thus indicating that biliary levels of oxidised glutathione may be a good measure of oxidative or chemical stress (4). This supports the main role of glutathione in
detoxification. Animal studies have also suggested a role of glutathione as an osmotic driving force for the bile acid independent bile flow (1). In addition, glutathione or its related thiols such as cysteine and cysteinylglycine excreted via bile, may be taken up by the (small) intestinal epithelium, and it was suggested that the “bilio-entero-hepatic cycle” of glutathione and its related thiols functions as an extra reservoir, making more “hepatic” glutathione available for sinusoidal efflux, when needed (1).

Thus, simultaneous measurement of total and oxidised glutathione and related thiol levels in bile may give a good indication of the thiol status of the liver and of the degree of oxidative/chemical stress of an individual. However, hardly any data on content of glutathione and other thiols in human bile can be found in literature. Therefore we performed a pilot study on the levels and composition of total and oxidised glutathione and related thiols in human gallbladder bile, obtained at surgery for pancreatic- or duodenal adenoma/carcinoma, chronic pancreatitis or cholecystolithiasis.
MATERIALS AND METHODS.

Patients.

Gallbladder bile was sampled from 30 patients, 19 males and 11 females, mean age 55 years [range 20-80] who were operated upon for chronic pancreatitis (n=7), pancreatic cancer (n=7), duodenal cancer (n=4), duodenal adenoma (n=3), cholecystolithiasis (n=7) or other reasons (n=2) in the period between Januari 2004 and Januari 2006, at the Department of Surgery, Radboud University Medical Centre Nijmegen. In addition to the primary diagnoses mentioned above, additional cholecystitis and/or cholecystolithiasis was found present at pathology in eight of the patients.

The study was approved by the local medical ethical review committee.

Gallbladder bile was collected by puncture of the gallbladder, immediately after surgical removal. Bile was transported to the laboratory immediately after sampling and handled for assessment of total (both reduced and oxidised) non-protein bound and non-protein bound oxidised levels of cysteine (Cys and oCys), homocysteine (Hcys and oHcys), cysteinylglycine (CG and oCG) and glutathione (Glu and oGlu), essentially as described before for the assay in blood [5]. In short, for assay of total thiol levels, 100µL bile was added to 100µL ice-cold 12% perchloric acid (PCA) containing 2.0mM bathophenanthrolinedisulfonic acid (BPDS; Sigma Chemicals). For assay of oxidised thiols, another 100µL bile was added to 100µL 12% PCA containing 2.0mM BPDS and 40mM N-ethylmaleimide (NEM). After thorough mixing and centrifugation at 16,000xg for 20 min and 4°C, supernatants were collected and stored at -80 °C. Samples were analysed within one month. The excess of NEM was removed by adding 70µL KOH (2.0M) followed by 60µL HCl (0.01M in 0.3M 3-[N-morpholino]-propanesulfonic acid buffer) to 100µL of the sample. In the assay, 100µL sample or standard was reduced by adding 10µL tris(2-carboxyethyl)phospine (Fluka, Bornem, the Netherlands;
10% (w/v) in 0.9% sodium chloride/4.0mM EDTA) and incubation at room temperature for 30 min. After reduction, 100µL PCA (0.6M with 1.0mM EDTA) was added and subsequently samples were mixed and centrifuged for 5 min at 10,000xg. Clear supernatant (100µL) was incubated for 1 hour and 60°C with 240µL reaction-mixture containing 20µL NaOH (1.55M), 200µL borate buffer (125mM K₂B₄O₇·4H₂O and 4.0mM EDTA, pH 9.0), and 20µL 7-fluorobenzofurazane-4-sulfonic acid (Fluka Chemie AG, Bornem, the Netherlands; 5mg/mL in borate buffer). Of the derivatised sample 20µL was injected into the high performance liquid chromatography system. The total-to-oxidised ratio for each thiol as a measure of oxidative stress was also calculated.

Data obtained were analysed with the Chromeleon chromatography data-system (Gynkotek, München, Germany). Thiol levels were calculated using four-point calibration curves for each thiol and were expressed in µmol/L.

All statistical analyses were performed using SPSS (version 12.0; SPSS Inc. Chicago, USA). Since multiple testing was performed, a p value of less than 0.01 was considered significant.
RESULTS.

Mean levels and ranges of total and oxidised thiols glutathione, cysteine, homocysteine and cysteinyl-glycine as measured in gallbladder bile of 30 patients are given in Table 1. Mean content of cysteine (Cys) was found highest (124 µmol/L), followed by cysteinyl-glycine (CG, 19.7 µmol/L) and glutathione (Glu, 17.1 µmol/L), whereas only minor amounts of homocysteine (Hcys, 0.6 µmol/L) were present in human bile. Most thiols were found present in their oxidised forms; 100%, 86% and 81% for Hcys, CG and Cys, respectively, except for glutathione which was found for only 40% in its oxidised form. This trend was also reflected in the mean ratios of total total/oxidised thiol; Rcys, RHcys, RCG and Rglu, which were 1.5, 1.3, 1.2 and 2.3, respectively.

No associations between levels of any thiol and age, gender or primary diagnosis of the patients was found, only tendencies for higher levels of total CG (17.2 vs. 24.0 µmol/L, p=0.026) and oxidised CG (14.4 vs. 21.3 µmol/L, p=0.025) were found in male vs. female patients, respectively. When biliary thiol levels of patients without gallbladder pathology (n=15) were compared with those of patients with cholecystitis, with/or without cholecystolithiasis (n=15), tendencies for lower total glutathione (21.4 vs. 12.7 µmol/L, p=0.050) and oxidised glutathione (8.2 vs. 5.4 µmol/L, p= 0.067) levels in patients without gallbladder pathology were noticed.
DISCUSSION.

The composition of human gallbladder bile with respect to glutathione and related thiol was studied. Earlier data on biliary excretion of glutathione and other thiols and their composition in bile, were obtained almost exclusively from laboratory animals, mainly rats. Except from other species differences between humans and rats, a main difference is the absence in rats of a gallbladder as a reservoir of bile. Glutathione was found to be the main thiol present in bile of rats and mice, followed by CG and Cys, whereas in human bile Cys is by far the main thiol present, followed by CG and Glu, and Hcys being hardly detectable. These findings immediately demonstrate the immense species differences between humans and rodents. In rodents, concentrations of glutathione in bile in the order of several mmol/L were reported (1,3,4) whereas in human gallbladder bile glutathione concentrations of only up to 0.1 mmol/L were found. Possibly, due to the high levels of \( \gamma \)-glutamyl-transpeptidase in gallbladder epithelium, the glutathione excreted in the gallbladder may be rapidly degraded into CG and Cys.

Gallbladder thiol levels show considerable interindividual variation; several hundred-fold for Cys and CG whereas Glu values varied between 0-106 \( \mu \)mol/L. Animal studies suggested that biliary concentrations of thiols may reflect the concentrations in the liver. When this is also valid in humans, it would mean that hepatic concentrations are critically low in certain individuals, since very low concentrations (<2.5 \( \mu \)mol/L) of glutathione were measured in the bile of 6 out of 30 individuals.

Also in contrast to the findings in animal studies are the much higher amounts of oxidised thiols detected in human bile. Most of Cys, CG and Hcys in human bile is present in the oxidised form, whereas in animal bile, most thiols are present in their reduced state. This could be explained by the fact that healthy animals were investigated, having little oxidative
or chemical stress, whereas the human bile collected and analysed here was obtained from patients after surgery for a variety of reasons. These patients were subjected to chemical stress such as anesthetics and medication, or oxidative stress as a result of the surgical intervention or their underlying diseases, in most cases being cancer or severe inflammatory diseases.

**In conclusion**, this pilot study on thiol composition of human gallbladder bile obtained at surgery reveals that:

- Thiol composition is different from earlier published values, which were mainly based on data of rodents.
- Most thiols are in the oxidised form, indicating considerable oxidative or chemical stress.
- Large (several hundred-fold) inter-individual variation exists, indicating that thiol content and composition may reflect in part the health or disease status of the patient.
REFERENCES.


Table 1. Levels and ratios of total free and oxidised thiols in human gallbladder bile.

<table>
<thead>
<tr>
<th>Thiol</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys (µmol/L)</td>
<td>30</td>
<td>124</td>
<td>101</td>
<td>1.4-404</td>
</tr>
<tr>
<td>oCys (µmol/L)</td>
<td>30</td>
<td>101</td>
<td>105</td>
<td>0.8-488</td>
</tr>
<tr>
<td>R Cys/oCys</td>
<td>30</td>
<td>1.52</td>
<td>0.61</td>
<td>1.0-3.8</td>
</tr>
<tr>
<td>Hcys (µmol/L)</td>
<td>30</td>
<td>0.66</td>
<td>0.65</td>
<td>0.0-2.9</td>
</tr>
<tr>
<td>oHcys (µmol/L)</td>
<td>30</td>
<td>0.72</td>
<td>0.75</td>
<td>0.0-3.2</td>
</tr>
<tr>
<td>R Hcys/oHcys*</td>
<td>22</td>
<td>1.32</td>
<td>0.85</td>
<td>1.0-5.0</td>
</tr>
<tr>
<td>CG (µmol/L)</td>
<td>30</td>
<td>19.7</td>
<td>19.5</td>
<td>0.5-84.0</td>
</tr>
<tr>
<td>oCG (µmol/L)</td>
<td>30</td>
<td>16.9</td>
<td>17.6</td>
<td>1.3-81.3</td>
</tr>
<tr>
<td>R CG/oCG</td>
<td>30</td>
<td>1.24</td>
<td>0.24</td>
<td>1.0-1.8</td>
</tr>
<tr>
<td>Glu (µmol/L)</td>
<td>30</td>
<td>17.1</td>
<td>24.8</td>
<td>0.0-106</td>
</tr>
<tr>
<td>oGlu (µmol/L)</td>
<td>30</td>
<td>6.8</td>
<td>8.5</td>
<td>0.0-34.2</td>
</tr>
<tr>
<td>R Glu/oGlu *</td>
<td>25</td>
<td>2.29</td>
<td>1.15</td>
<td>1.0-5.3</td>
</tr>
</tbody>
</table>

*Note that some ratios of Hcys/oHcys and Glu/oGlu can not be calculated due to the presence of zero values.