

# Characterisation of ANIT-induced hepatobiliary injury *in vivo* using $^{31}\text{P}$ MRS and *in vitro* $^1\text{H}$ MAS MRS

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**Introduction** A number of human diseases affecting the biliary tree can be modelled in rats by the hepatobiliary toxin, alpha-naphthylisothiocyanate (ANIT). In this study the hepatobiliary dysfunction caused by chronic ANIT feeding was investigated using *in vivo* hepatic  $^{31}\text{P}$  MRS. This modality allows the non-invasive study of certain metabolites including those with a central role in cell membrane metabolism and cellular energetics<sup>1</sup>, namely phosphomonoesters (PME) and phosphodiester (PDE), thus giving an insight into the metabolic state of the hepatocytes and cholangiocytes involved in ANIT-induced injury.  $^1\text{H}$  magic angle spinning (MAS) MRS of liver samples was also performed to obtain supporting data on metabolites involved in plasma membrane synthesis and degradation, this included total choline (tCho), phosphatidylcholine (PtC), phosphocholine (PC), glycerophosphocholine (GPC) and hepatic lipid. Characterisation of ANIT-induced hepatic injury was validated by clinical chemistry and histology.

## Protocol

48 male Sprague Dawley rats (160-170g, Charles River UK Ltd) were obtained and divided into six equal groups. Three groups were fed a diet supplemented with 0.05%, 0.04% or 0.025% ANIT for 14 days (high dose (HD), medium dose (MD), and low dose (LD) groups respectively). Controls were pair-fed on a normal diet according to the food intake of the ANIT-fed animals, and referred to as PFC\_HD, PFC\_MD and PFC\_LD groups respectively. At baseline, animals were fasted and then anaesthetised for MRS study using a 4.7T Varian system with dual surface coil tuned to  $^{31}\text{P}$  and  $^1\text{H}$  frequencies. MR spectra were acquired using a simple pulse-acquire sequence. 544  $^{31}\text{P}$  MRS data collects were averaged for each rat at a TR of 5s. This was repeated at 1 and 2 weeks after the provision of the supplemented diet. After the final scan, animals were sacrificed and serum and livers harvested. For each animal, liver samples were fixed in formalin and frozen for subsequent histological and MAS analysis, respectively. For  $^1\text{H}$  MAS MRS, tissue (~17mg) was thawed and CPMG performed on a 500MHz JEOL ECP system with  $\tau=1\text{ms}$ ,  $N=40$ ,  $TE=80\text{ms}$ ,  $TR=5\text{s}$  at a spin rate of 4000Hz at 4°C. Hepatobiliary damage was assessed by histological analysis of haematoxylin and eosin stained liver sections, prepared from formalin-fixed samples. Sera underwent routine clinical chemistry assessment.

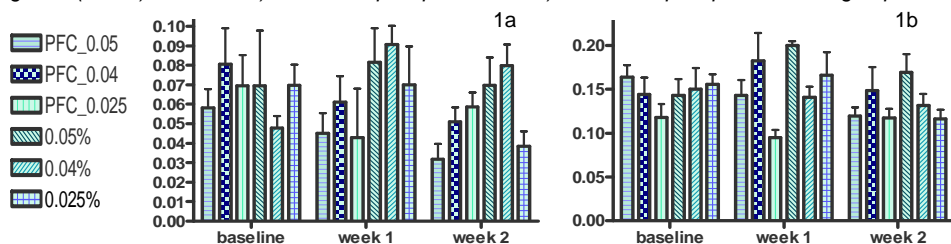
## Results

At baseline, no significant variations were seen in PDE and PME relative to the total phosphorus signal (tPh) across all groups (Figure 1). PDE/tPh significantly increased in both the HD and MD groups ( $0.08\pm 0.02$  and  $0.09\pm 0.01$ ), relative to their PFCs ( $0.06\pm 0.01$  and  $0.05\pm 0.01$ ) after 1 week on the supplemented diet. This rise was maintained at week 2, in contrast to the LD group, which exhibited a reduction in PDE/tPh relative to its PFCs. The increase in PDE/tPh expressed by the HD group was accompanied by a concomitant increase in PME/tPh; this increase was still evident at week 2 ( $0.17\pm 0.02$   $p<0.05$ ). However, this effect in PME was not seen in the MD or LD groups. By  $^1\text{H}$  MAS MRS, tCho was only shown to be significantly higher in the HD group relative to its PFC group ( $p<0.05$  Table 1). This is consistent with the increase in both PDE/tPh and PME/tPh measured for this group only *in vivo*. The increase in PDE/tPh in the HD and MD groups was also reflected *in vitro* by elevated PC+GPC ratios compared to the PFC groups (Table 1). All ANIT-fed groups showed significantly reduced hepatic lipid. Liver function tests on blood serum showed that all ANIT-fed groups had relatively higher levels of ALP and total bilirubin ( $p<0.05$ ). Albumin was significantly reduced in the HD and MD groups, again reflecting the *in vivo* increase in PDE/tPh. The increase in PME/tPh correlated with significant increases in liver transaminases ALT and AST only seen in the HD group (Table 1). Histologically a high occurrence of moderate biliary hyperplasia was displayed in the HD and MD groups, in addition to increased mitosis in the biliary epithelium and inflammation around the bile ducts. In contrast, at the lower dose only mild biliary hyperplasia and peribiliary inflammation was present.

	PFC_0.025	ANIT_0.025	PFC_0.04	ANIT_0.04	PFC_0.05	ANIT_0.05
Albumin (g/L)	13.9±0.4	12.8±0.4	15.4±0.4	11.4±0.8 *	14.6±0.5	11.5±0.7 *
ALP (iu/L)	216±7	316±13 *	261±17	392±37 *	200±10	374±17 *
ALT (iu/L)	55±7	80±11	80±13	69±10	42±4	117±19 *
AST (iu/L)	233±39	374±50	282±55	277±51	254±30	750±98 *
Cholesterol mmol/L	2.2±0.2	2.12±0.08	1.9±0.1	2.8±0.3 *	2.0±0.1	2.5±0.2
Total Bilirubin μmol/L	4.6±0.2	14.9±2.8 *	4.7±0.4	44±5 *	3.9±0.2	133±15 *
Choline	0.8±0.1	1.1±0.1	1.1±0.2	1.2±1.1	0.8±0.1	0.96±0.09 *
PC+GPC	2.7±0.1	2.9±0.4	2.8±0.3	4.2±0.4 *	3.6±0.3	4.6±0.2 *
Lipid	1.20±0.07	0.96±0.05 *	1.08±0.04	0.89±0.02 *	1.11±0.03	0.96±0.05 *

Table 1 (above): Shows the liver function test results, plus data from  $^1\text{H}$  MAS MRS \* $p<0.05$

Figure 1 (below): Ratios of a) PDE/Total phosphorus and b) PME/Total phosphorus for all groups



consequence of phospholipid biosynthesis, or GPC due to breakdown of cellular phospholipids, or both. Biliary hyperplasia was also present in the MD group, correlating with *in vivo* increases in PDE/tPh and increases in PC+GPC seen *in vitro*, however no increase in PME/tPh was detected, corresponding to a lack of hepatic injury and consequent regeneration in this group. At the LD mild inflammation around the bile ducts and mild biliary hyperplasia were apparent, however changes in cellular metabolism were not detected *in vivo* using  $^{31}\text{P}$  MRS suggesting that the concentration of the metabolites involved is too low, nor were any significant increases in PC+GPC detected *in vitro*. Interestingly hepatic lipid was reduced in all ANIT-fed animals. Consequently, this study has shown that medium doses of ANIT resulted in biliary damage, and at high doses, hepatocellular damage. This can be characterised *in vivo* using  $^{31}\text{P}$  MRS to monitor PDE/tPh and PME/tPh levels. *In vitro* MAS characterisation of biliary damage leads to increases in PC+GPC and, when hepatic damage is present, in tCho. Thus, this study demonstrates how *in vivo*  $^{31}\text{P}$  MRS, together with high resolution *in vitro*  $^1\text{H}$  MAS MRS, can be used to characterise hepatobiliary disease preclinically.

**References** 1. Menon DK et al. Hepatology, 25, 417-27, (1995) 2. Goldfarb S et al. Am. J. Pathol, 40, 685-98, (1962)