

## Current and future applications of *in vitro* magnetic resonance spectroscopy in hepatobiliary disease

I Jane Cox, Amar Sharif, Jeremy FL Cobbold, Howard C Thomas, Simon D Taylor-Robinson

I Jane Cox, Imaging Sciences Department, Division of Clinical Sciences, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0HS, United Kingdom  
Amar Sharif, Jeremy FL Cobbold, Howard C Thomas, Simon D Taylor-Robinson, Department of Medicine, Division of Medicine, Imperial College London, St Mary's Campus, London W2, United Kingdom

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Correspondence to: Dr. SD Taylor-Robinson, Reader in Medicine, Division of Medicine, Imperial College London, London W2 1NY, United Kingdom. s.taylor-robinson@imperial.ac.uk  
Telephone: +44-20-83833266 Fax: +44-20-87493436  
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### Abstract

Nuclear magnetic resonance spectroscopy allows the study of cellular biochemistry and metabolism, both in the whole body *in vivo* and at higher magnetic field strengths *in vitro*. Since the technique is non-invasive and non-selective, magnetic resonance spectroscopy methodologies have been widely applied in biochemistry and medicine. *In vitro* magnetic resonance spectroscopy studies of cells, body fluids and tissues have been used in medical biochemistry to investigate pathophysiological processes and more recently, the technique has been used by physicians to determine disease abnormalities *in vivo*. This highlighted topic illustrates the potential of *in vitro* magnetic resonance spectroscopy in studying the hepatobiliary system. The role of *in vitro* proton and phosphorus magnetic resonance spectroscopy in the study of malignant and non-malignant liver disease and bile composition studies are discussed, particularly with reference to correlative *in vivo* whole-body magnetic resonance spectroscopy applications. In summary, magnetic resonance spectroscopy techniques can provide non-invasive biochemical information on disease severity and pointers to underlying pathophysiological processes. Magnetic resonance spectroscopy holds potential promise as a screening tool for disease biomarkers, as well as assessing therapeutic response.

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**Key words:** Magnetic resonance spectroscopy; Liver; Hepatobiliary disease; Membrane metabolism

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### INTRODUCTION

The nuclear magnetic resonance (NMR) phenomenon was first reported in 1946<sup>[1]</sup> and has since been widely used by the scientific and medical communities. The 2003 Nobel Prize for Medicine was jointly awarded to Professor Sir Peter Mansfield and Professor Paul Lauterbur for their work on clinical NMR, and this serves to underline the impact on the medical community of the utility of this technique with all its diverse applications ([www.nobel.se/medicine/laureates](http://www.nobel.se/medicine/laureates)). In this highlight article, we explain the background to NMR and discuss various clinical studies that are relevant to the liver and the biliary system, with particular emphasis on *in vitro* applications that have analyzed body fluids, such as bile, or tissue obtained from liver biopsies.

NMR is a non-invasive and non-selective technique, allowing the study of molecular composition and structure. NMR forms the basis of magnetic resonance imaging (MRI) methodologies, which have been developed using whole body magnets. Spatial localization using magnetic field gradients has enabled very detailed images of the human body to be obtained. *In vivo* NMR spectroscopy studies can be performed as an adjunct to clinical MRI, as part of the same examination. Longitudinal studies can be readily undertaken using both of these MR applications. The term magnetic resonance spectroscopy (MRS) is used in this highlight topic to denote *in vivo* and *ex vivo* clinical NMR spectroscopy studies, and also encompasses *in vitro* NMR spectroscopy, performed in the laboratory at very high magnet field strengths.

### AN OVERVIEW OF MR SPECTROSCOPY

Although a detailed knowledge of the physical principles that govern NMR is not essential for appreciating the scope of NMR applications, it is often helpful to have some understanding of some of the basic concepts that underlie these methodologies. The NMR technique

exploits the behavior of atomic nuclei in an externally applied magnetic field. Magnetic resonance occurs because of the quantum mechanical property of “spin”, which is intrinsic to certain atomic nuclei. Examples of such nuclei of clinical relevance include hydrogen-1 ( $^1\text{H}$ ), carbon-13 ( $^{13}\text{C}$ ), fluorine-19 ( $^{19}\text{F}$ ), phosphorus-31 ( $^{31}\text{P}$ ) and chlorine-35 ( $^{35}\text{Cl}$ ). Such nuclei can be imagined to act like small bar magnets in a magnetic field by ‘lining up’ with or against the field, and can be excited by irradiation with non-ionizing radiofrequency (rf) energy. During relaxation following excitation, rf signals are generated which contain information regarding the magnetic environment experienced by each nucleus, and therefore about the molecules in which they exist. The resulting signals, the “free induction decay”, are detected by a receiver coil and can be expressed as a frequency spectrum by the mathematical process of Fourier transformation. The relative frequency position of a metabolite signal, its chemical shift, is dependent on the locally experienced magnetic field and therefore the local chemical environment. Consequently, each nucleus type within a molecule has a characteristic chemical shift. Hydrogen-1 ( $^1\text{H}$ ) and phosphorus-31 ( $^{31}\text{P}$ ) are the two nuclei most commonly used for biological studies, as they are ubiquitous in nature ( $^1\text{H} = 99.985\%$ ,  $^{31}\text{P} = 100\%$  natural abundance).

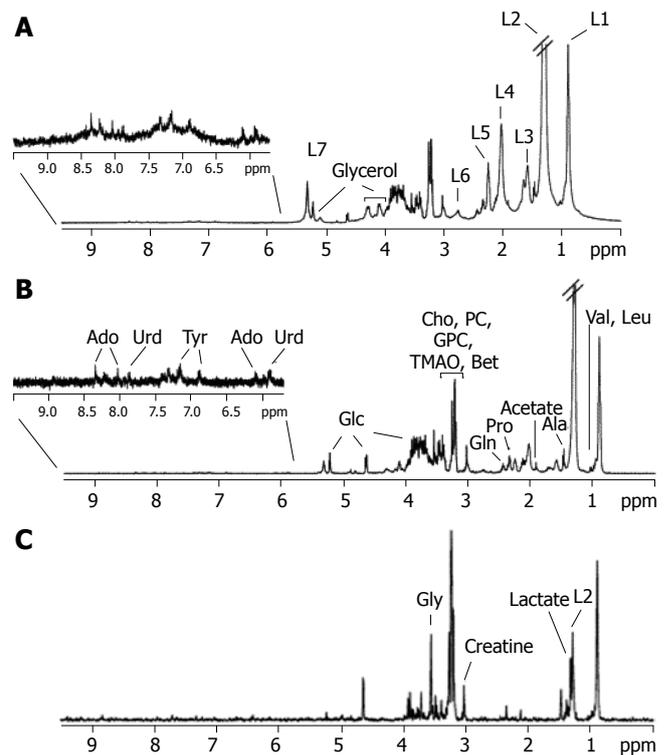
#### Metabolic significance of clinical MR spectroscopy

Jacobson and colleagues<sup>[2]</sup> used MRS to examine the effects of hydration of DNA in 1954. Current investigations include both *in vitro* MRS studies on tissue samples or body fluids and *in vivo* whole-body MRS studies.

*In vitro* MRS studies include the analysis of body fluids (such as plasma, urine or bile), extracts of tissue, or small biopsy-sized specimens of intact tissue. *In vivo* MRS is more difficult to perform and is characterized by poorer resolution of metabolites than *in vitro* MRS. This is due to factors such as the lower magnetic field strengths used for *in vivo* MRS clinical studies and also to the effects of magnetic susceptibility and patient motion. The typical magnetic field strength is 1.5-3.0 Tesla (T) for clinical MRS studies and 11.7-18.8 T for *in vitro* MRS studies.

*In vitro* MRS can detect and characterize a range of metabolic components simultaneously, even if their chemical identities are unknown at the time of analysis. *In vitro* MRS studies therefore provide a comprehensive metabolic profile of the low molecular weight components in biofluids and tissues, reflecting levels of endogenous metabolites involved in key cellular pathways, which indicate the physiological and pathophysiological status. The technique can also provide a profile of exogenous agents, including xenobiotics and their metabolites, and give an indication as to their effects on endogenous compounds<sup>[3]</sup>. For such reasons, *in vitro* MRS has become one of the most successful and popular techniques for biofluid analysis over the past 10 years<sup>[4]</sup>.

It is relevant to consider *in vitro* MRS applications to the liver in the context of metabolite findings from both *in vivo*  $^{31}\text{P}$  and  $^1\text{H}$  MRS studies of patients with liver disease, who have been examined using whole body MRI/MRS

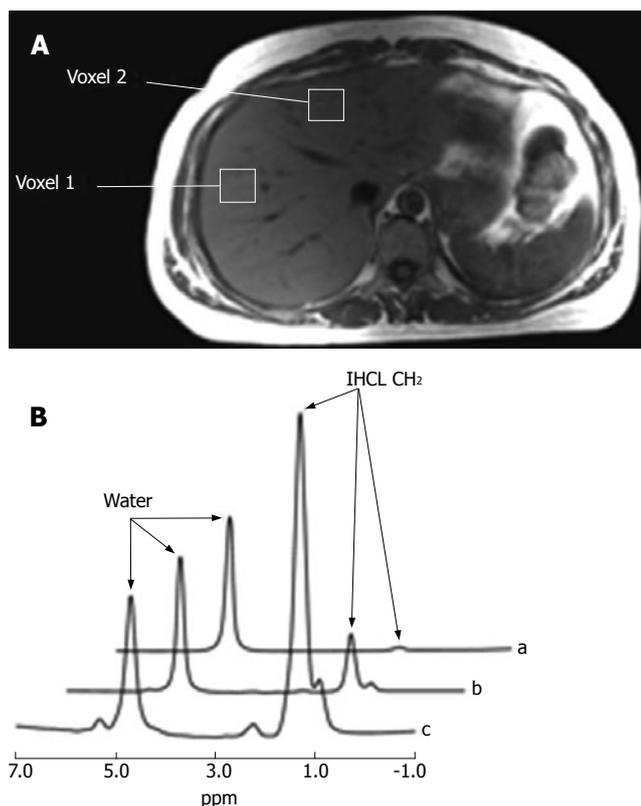


**Figure 1** 400-MHz  $^1\text{H}$  HR-MAS NMR spectra of a human liver biopsy sample (rotation rate 4 kHz). **A:** Standard 1D spectrum; **B:** Spin-echo (CPMG) spectrum; **C:** JRES f2 projection. L1: lipid  $\text{CH}_3$ ; L2: lipid  $(\text{CH}_2)_n$ ; L3: lipid  $\text{CH}_2\text{CH}_2\text{CO}$ ; L4: lipid  $\text{CH}_2\text{-CH=CH}$ ; L5: lipid  $\text{CH}_2\text{CO}$ ; L6: lipid  $\text{CH=CH-CH}_2\text{-CH=CH}$ ; L7: lipid  $\text{CH=CH}$ ; Cho: choline; PC: phosphocholine; GPC: glycerophosphocholine; TMAO: trimethylamine-N-oxide; Bet: betaine; Glc: glucose; Val: valine; Leu: leucine; Ala: alanine; Gln: glutamine; Gly: glycine; Tyr: tyrosine; Urd: uridine; Ado: adenosine. Reprinted with permission from Duarte *et al* Anal Chem 2005; 77: 5570-5578. Copyright (2005) American Chemical Society.

techniques. *In vivo* hepatic MRS studies have been primarily used in the research environment and have generally utilized  $^{31}\text{P}$  MRS<sup>[5]</sup>, because  $^1\text{H}$  MRS is technically more challenging<sup>[6,7]</sup>. *In vivo*  $^{31}\text{P}$  MRS gives information on cell turnover and energy state and has been used to grade patients with chronic liver disease<sup>[8]</sup> and to aid in diagnosis and treatment response in patients with cancer<sup>[9]</sup>. On the other hand, *in vivo*  $^1\text{H}$  MRS allows quantification of intra-hepatocellular lipid (IHCL) levels, and therefore has been utilized recently to quantify the extent of steatosis in patients with fatty liver<sup>[10,11]</sup>.

Representative hepatic  $^1\text{H}$  and  $^{31}\text{P}$  MR spectra are illustrated in Figures 1-4. *In vitro*  $^1\text{H}$  and  $^{31}\text{P}$  MR spectra of liver tissue are illustrated in Figures 1 and 3, and these can be compared and contrasted with *in vivo* hepatic  $^1\text{H}$  and  $^{31}\text{P}$  MR spectra illustrated in Figures 2 and 4. An *in vitro*  $^1\text{H}$  MR spectrum is dominated by contributions from water and IHCL. If the signal from water is suppressed using specific NMR methodologies, then more detailed contributions from IHCL can be observed along with resonances from choline-containing compounds (Cho) (Figures 1A-C). These data compare with the *in vivo* hepatic  $^1\text{H}$  MRS spectrum, which allows quantification of water and IHCL (Figure 2B).

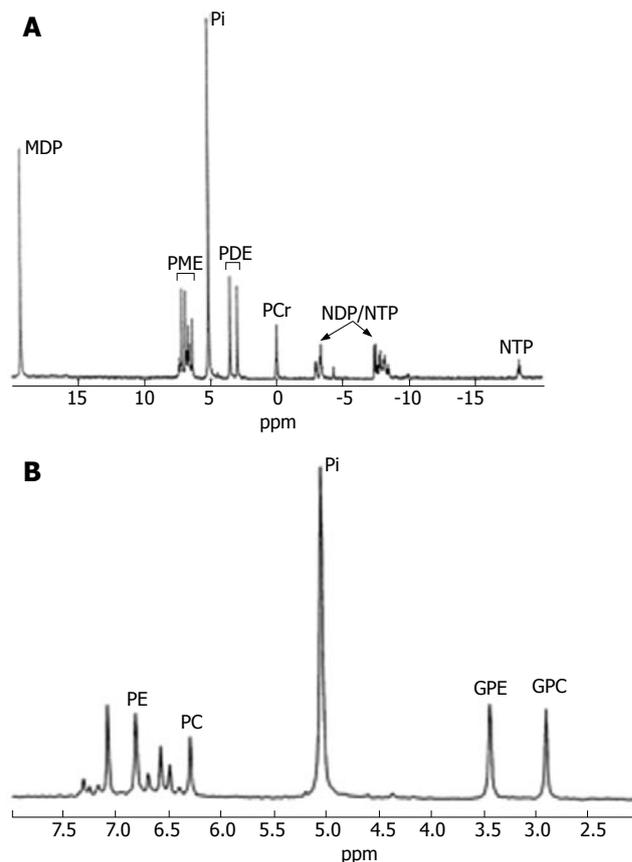
*In vitro*  $^{31}\text{P}$  MR spectroscopy of liver tissue (Figure 3) allows characterization of resonances from phospholipid



**Figure 2** **A:** A transverse image through the abdomen showing the two voxel positions used to study regional variation in hepatic fat content; **B:** Typical proton magnetic resonance liver spectra from three volunteers showing progressive degrees of fatty infiltration. Spectrum (a) shows a liver with minimal fatty infiltration (1.0%), spectrum (b) shows a liver with moderate fatty infiltration (10.2%), and spectrum (c) shows a liver with severe fatty infiltration (74.9%). Resonances from water and IHCL-(CH<sub>2</sub>)<sub>n</sub> can be clearly identified. Values refer to the peak area of the IHCL peak with reference to the water peak after correcting for T<sub>1</sub> and T<sub>2</sub>. IHCL: intrahepatocellular lipids. Reproduced from Thomas *et al* Gut 2005; 54: 122-127, with permission from the BMJ Publishing Group.

cell membrane precursors, including phosphocholine (PC) and phosphoethanolamine (PE), adenosine monophosphate (AMP) and glycolytic intermediates, such as glucose-6-phosphate. Cell membrane degradation products, including glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE) and mobile phospholipids from the endoplasmic reticulum can also be identified. Depending on how the tissue is collected it is also possible to quantify contributions to the spectrum from inorganic phosphate and various nucleoside triphosphates (NTP). Such spectral resolution is difficult to achieve *in vivo*, and therefore a typical *in vivo* hepatic <sup>31</sup>P MR spectrum (Figure 4) consists of six dominant, composite resonances, arising from: (1) the phosphomonoester region (PME), which are mainly cell membrane precursors and sugar phosphates; (2) inorganic phosphate (Pi); (3) phosphodiester (PDE), which are mainly cell membrane degradation products, but also signal from mobile phospholipids contained in mitochondria and (4-6) three resonances from NTP, which are chiefly composed of adenosine triphosphate (ATP), but contain contributions from uridine triphosphate, guanosine triphosphate and cytosine triphosphate<sup>[12]</sup>.

Changes in the PME and PDE metabolites indicate



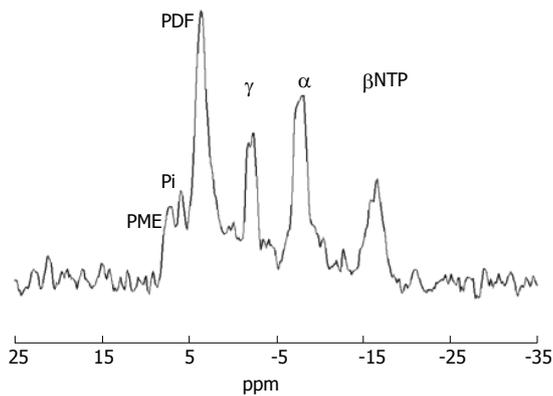
**Figure 3** Typical proton decoupled *in vitro* <sup>31</sup>P MR spectrum of perchloric acid extracted normal liver tissue. **A:** Full spectrum; **B:** PME and PDE regions. PME: phosphomonoesters; PDE: phosphodiester; NTP: nucleotide triphosphates; NDP: nucleotide diphosphate; PE: phosphoethanolamine; PC: phosphocholine; GPE: glycerophosphorylethanolamine; GPC: glycerophosphorylcholine; PCr: phosphocreatine; MDP: methylene diphosphonate. Reproduced from Taylor-Robinson *et al* Gut 1998; 42: 735-743, with permission from the BMJ Publishing Group.

modifications in rates of cell membrane synthesis, breakdown, cell death and regeneration, associated with increasingly rapid cell growth, turnover and development<sup>[13]</sup>. Thus, these important phosphorus-containing molecules are intricately involved in the cellular processes linked to cellular destruction, turnover and malignant transformation.

MRS also provides important insights into dynamic metabolic changes in the diseased liver. However, unlike most liver function tests, the information obtained is not dependent on blood flow. Furthermore, most standard liver function tests also depend on measurements of plasma components, rather than assessing markers at the site of disease. Recent studies on hepatitis C suggest that the MRS technique may be useful in monitoring response to treatment with interferon and ribavirin<sup>[8]</sup>.

#### Methodology for *in vitro* MR spectroscopy

Sample preparation of body fluids requires routine clinical documentation to ensure that the relevant patient details are adequately controlled or accounted for, including time of sample collection and drug use history. It may be necessary to dilute or buffer the sample, or alternatively to document and account for any spectral changes relating to



**Figure 4** Typical  $^{31}\text{P}$  magnetic resonance spectrum from the liver of a healthy volunteer (TR 10000 ms). PME: phosphomonoester; Pi: inorganic phosphate; PDE: phosphodiester; NTP: nucleoside triphosphate; ppm: parts per million. Reproduced from Mullenbach *et al* Gut 2004; 54: 829-834, with permission from the BMJ Publishing Group.

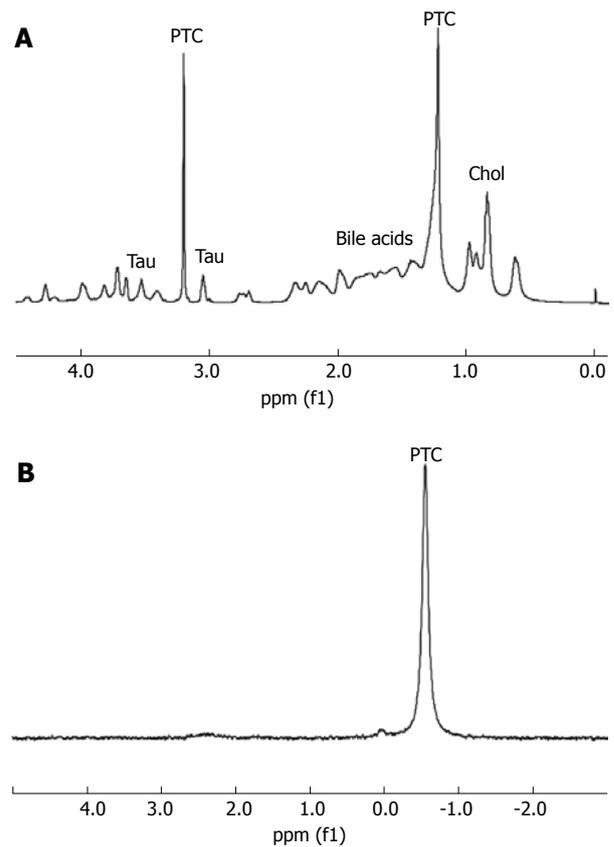
pH or concentration.

Sample storage should not cause a change in metabolite profile. Therefore storage temperature and conditions should be considered, and whether a change in sample temperature alters metabolite profile, for example during a freeze-thaw process<sup>[14,15]</sup>.

Immediately prior to data collection, a known amount of a NMR standard can be added for NMR referencing and quantification. For example, sodium trimethylsilyl- $[\text{}^2\text{H}_4]$  propionate (TSP) is routinely used as a reference compound for  $^1\text{H}$  MRS application. If the sample is required to be uncontaminated by any NMR reference compound for any further analysis, then either a reference compound can be placed in a capillary tube within the sample or an external standard is used.

The temperature of NMR data acquisition is under spectrometer control, so the sample temperature should be specified and constant throughout the study protocol. Many studies are performed at  $25^\circ\text{C}$ , but it may be necessary to study intact cells at lower temperatures to minimize the effects of ongoing cellular changes.

MR data acquisition includes adjusting the magnetic field homogeneity within the sample (a process known as 'shimming'). Data averaging is generally required, so as to improve the signal-to-noise ratio in the spectrum. Data can be acquired using pulse-collection methods or one- or two-dimensional NMR data collection protocols<sup>[16]</sup>. The water signal is generally suppressed using saturation techniques and/or spin-echo methods, in order to more readily observe the metabolite signals<sup>[17]</sup>. Intact tissue specimens may be analyzed using conventional solution MR spectroscopy techniques, by simply placing the biopsy sample within a NMR tube or by chemically extracting aqueous or lipid soluble fractions of the tissues. Studying intact tissue specimens directly within a conventional NMR tube has the disadvantage that magnetic field inhomogeneities within the sample do limit the achievable spectral resolution, but has the advantage that sample preparation is minimal and the sample remains intact and available for further analysis by other complementary techniques. Tissue extract studies have the advantage that



**Figure 5** A:  $^1\text{H}$  nuclear magnetic resonance spectrum of bile collected at laparoscopic cholecystectomy, after a history of recurrent cholecystitis. The spectrum has no contamination from contrast agents. The major peaks are assigned to phosphatidylcholine (PTC), bile acids, cholesterol (Chol), taurine (Tau) and the reference, sodium trimethylsilyl- $[\text{}^2\text{H}_4]$  propionate (TSP); B: Corresponding  $^{31}\text{P}$  nuclear magnetic resonance spectrum. The major peak is assigned to PTC. ppm, Parts per million. Reproduced from Khan SA, Cox IJ, Thillainayagam AV, Bansil DS, Thomas HC, Taylor-Robinson SD. Proton and phosphorus-31 nuclear magnetic resonance spectroscopy of human bile in hepatopancreaticobiliary cancer. *Eur J Gastroenterol Hepatol* 2005; 17: 733-738, with permission from Lippincott Williams & Wilkins, Inc.

the sample which is finally available for MRS study that has taken place after the extraction process, has become homogeneous so that the spectral peaks are well defined. However, the obvious disadvantages include the total destruction of the sample, the additional influence of metabolite solubilities in the extract medium and the relatively large amount of tissue required (upwards of 100 mg). The recently introduced method of magic angle spinning (MAS) MRS can overcome the limitations of both of the above techniques<sup>[18]</sup>.

### Magic angle spinning (MAS) MRS

The line-broadening effects in an intact semi-solid are caused by constraints on molecular motion from cellular architecture, chemical shift anisotropy and dipolar couplings, and these can be reduced by spinning the sample at an angle of  $54.7^\circ$  to the main magnetic field, known as the 'magic angle'. While such MAS MRS techniques have been used for many years in the NMR spectroscopy study of true solids, MAS MRS has only recently been applied to the study of intact tissue<sup>[18]</sup>. A distinct advantage is that a MAS MR spectrum can be obtained from as little as 2

mg tissue and the tissue remains sufficiently intact to allow subsequent histological or genetic analysis<sup>[19]</sup>.

As in conventional MRS studies, accurate determination of metabolite concentrations is pivotal to the success of tissue MAS MRS studies. A number of factors need to be considered for metabolite quantitation in MAS MRS, including possible leakage of metabolites during washing of biopsies prior to analysis<sup>[15]</sup>, temperature at which the spectrum is acquired and appropriate choice of reference standard. The question of how to define a standard for quantitation is particularly important. For example the tissue water signal measured from a proton spectrum without water presaturation, may be used as an internal standard. Silicone rubber, for example, provides a useful external standard. Alternatively peak area ratios can be used to compare different spectra, but this has limitations as with *in vivo* MRS studies. For example, not only can changes in absolute concentrations be missed by the use of metabolite ratios, but an increase in the area of one peak can have the same result on the area ratio as a decrease in the other. Therefore, relying on the ratio of signal areas may mask metabolite differences inherent in the spectra.

## APPLICATIONS OF *IN VITRO* MR SPECTROSCOPY IN LIVER DISEASE

### Cellular bioenergetics

Owing to the ubiquity of phosphorus-containing moieties in energy metabolism, <sup>31</sup>P MRS has been used to assess energy states in living systems<sup>[20]</sup>. However, the rapid degradation of phosphorylated nucleotide intermediates *ex vivo*, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), has made snap-shot *in vitro* assessment difficult, although efforts have been made to minimize the ischemic time. Oxidation of these metabolites can occur at harvesting, snap freezing, thawing and during tissue extraction. In order to obtain metabolically useful information, the samples should be snap-frozen as soon as possible. In order to eliminate the need for the extraction of aqueous and lipid fractions, MAS MRS techniques could be used but data collection may need to be done at 4°C to minimise further metabolic reactions during data collection.

An alternative methodology to assess bioenergetics is to use an *in vitro* perfusion system. Applications have included the effects of normoxia and anoxia in rat livers<sup>[21]</sup>, the response to cyanide intoxication<sup>[22]</sup> and the response of damaged liver to fructose loading<sup>[23]</sup>.

### Tumours

<sup>31</sup>P and <sup>1</sup>H MR spectra from aqueous soluble metabolites in extract of liver tumours of different types (hepatocellular carcinoma, colorectal metastases and secondary lung carcinoma) have been compared with samples from normal liver tissue and from histologically normal liver of the tumour host<sup>[24]</sup>. A significant increase in phosphoethanolamine (PER), phosphocholine (PC), taurine and lactate has been reported, with a reduction in GPE glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC) in tumours, compared to normal liver controls<sup>[24]</sup>. In addition, the spectral changes identified by <sup>31</sup>P MRS in

tumour tissue have been seen at a lesser magnitude in the tumour host tissues<sup>[24]</sup>.

Hepatic <sup>31</sup>P MRS has been translated to the *in vivo* setting by Cox and colleagues and others<sup>[25]</sup>. The observed hepatic *in vivo* MR spectrum demonstrated a significantly raised PME/PDE ratio in tumour compared to controls. These peaks are thought to comprise increased PE and PC, and decreased GPE and GPC respectively, as elicited by *in vitro* analysis of tissue extract<sup>[24]</sup>.

*In vitro* <sup>1</sup>H MRS in conjunction with a statistical classification strategy has been used by Soper and colleagues to differentiate histologically normal liver, cirrhotic nodules and hepatocellular carcinoma<sup>[26]</sup>. Multivariate and pattern recognition techniques enable all data points to be incorporated into the analysis, no matter whether the metabolites comprising the spectral peaks have been identified. In this study, cirrhotic nodules were distinguished from hepatocellular carcinoma in 98% of cases<sup>[26]</sup>.

### Chronic liver disease

Initial hepatic MRS studies of chronic liver disease focused on cirrhosis of varying aetiologies. Human livers with histologically proven cirrhosis have been assessed using *in vitro* <sup>31</sup>P NMR spectroscopy<sup>[7]</sup>. Spectra from these patients with end-stage liver disease, all of whom had tissue obtained at the time of liver transplantation, showed significant elevations in phosphoethanolamine (PE) and phosphocholine (PC) and significant reductions in glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC), when spectra were compared with those from histologically normal livers. Whether the patient had compensated or decompensated disease did not significantly alter the spectra obtained. Further work<sup>[27]</sup> by the same authors correlated such *in vitro* findings with <sup>31</sup>P MRS *in vivo* and these studies suggest a potential clinical utility for *in vivo* MRS as an addendum to a standard MRI protocol in staging of the end-stage liver disease in patients that are being imaged for surveillance of hepatocellular carcinomas.

More recently, *in vivo* hepatic <sup>31</sup>P MRS has been used to stratify inflammation and fibrosis caused by hepatitis C virus, compared to histological staging from standard liver biopsy<sup>[8]</sup>. The PME/PDE ratio was found to be elevated in mild, moderate/severe fibrosis and cirrhosis compared to normal, healthy volunteers. These changes have been thought to represent increased cell membrane turnover, although differences between means for each group were statistically significant, there was some overlap between the patient groups<sup>[8]</sup>. While histology remains the gold-standard, sampling error is inherent in the technique and it has been postulated that hepatic MRS could potentially provide a more accurate representation of the disease process, owing to the fact that it provides metabolite information from most of the liver<sup>[8]</sup>. Further studies directly correlating *in vivo* and *in vitro* MRS findings are awaited in this context.

While *in vivo* hepatic <sup>31</sup>P MRS has been shown to correlate closely with disease severity in hepatitis C, its availability as a technique is not widespread. *In vivo* hepatic <sup>1</sup>H MR spectroscopy would be more generally applicable on standard MRI scanners. Cho and colleagues have used this

technique to stratify chronic hepatitis compared to histology<sup>[28]</sup>. They were able to detect differences between groups, but the signal-to-noise ratio was low and assignment of the spectral peaks is open to debate<sup>[29]</sup>. A rational approach would be to identify the change in spectral peaks *in vitro*, using <sup>1</sup>H MAS MRS, prior to translating the technique to the *in vivo* setting. With this in mind, Martínez-Granados and colleagues performed <sup>1</sup>H MAS MRS on needle biopsies of liver tissue from 16 patients with chronic hepatitis or cirrhosis and one specimen from autopsy, nominated a “normal” control. High quality spectra were obtained and a number of metabolites assigned to previously unidentified peaks. Data collection was carried out at 4°C to minimize tissue degradation. Differences in signal intensities between disease states and “normal” liver were noted, particularly increases in mobile fatty acids and glycogen in the former. However, the “normal” liver was harvested 24 h post mortem, so assumptions of metabolic normality are questionable<sup>[30]</sup>.

### Hepatic steatosis

The intrahepatocellular lipid (IHCL) component of lipid extracts from steatotic liver specimens as assessed by <sup>1</sup>H MR spectroscopy has been calibrated with *in vivo* MR spectroscopy measurements to estimate the lipid volume fraction in fatty liver disease<sup>[31]</sup>. There is an agreement with CT estimations but histomorphometry appears to underestimate the fat volume in samples. Szczepaniak and colleagues used localised *in vivo* <sup>1</sup>H MRS in 2349 patients to assess the hepatic triglyceride content and estimated the prevalence of hepatic steatosis in the study group as 33.6%<sup>[32]</sup>. Further MRI studies *in vivo* have demonstrated a close relation between hepatic steatosis and body adiposity, and a close correlation between MRI estimation of adiposity and histological assessment in two of these patients<sup>[11]</sup>. Although studies have shown a close association between *in vivo* estimates and biopsies, *in vitro* MRS assessment of lipid content in liver biopsies by MAS MRS would allow direct comparison with histology, reducing the effect of sampling error<sup>[33]</sup>.

### Orthotopic liver transplantation

Liver transplantation is used as a definitive therapy for acute hepatic failure, severe chronic liver disease and some cases of hepatocellular carcinoma. Demand for donor livers is high and suboptimal specimens may be used. At present there is no reliable non-invasive method to assess the viability of livers between organ harvesting and implantation. A program of research to address these problems demonstrates translational techniques from animal models *in vitro* to the clinical environment.

*In vitro* and *ex vivo* MRS studies of animals have established a model, which follows human organ harvesting and storage protocols<sup>[34,35]</sup>. Rapid reductions in ATP levels, readily measured by *ex vivo* <sup>31</sup>P MRS have been seen in a pig model compared to rodent models and levels could be replenished by hypothermic reperfusion<sup>[34]</sup>. Now that MR probes have been designed to fit within organ retrieval boxes, clinical studies of organs within a transplant program are required<sup>[36]</sup>.

In a preliminary study, Duarte and colleagues used *in vitro* <sup>1</sup>H MAS MRS to assess biopsies taken at three time-points from six livers, before removal from donors, during cold perfusion and following implantation into the recipient<sup>[33]</sup>. The biopsies with the highest concentration of peaks reflecting fatty acyl chain (triglyceride) resonances were also identified as those also estimated to have the highest fat content on histological analysis. Other metabolites were identified, including glycerophosphocholine (GPC), which were reported to decrease from pre- to post-transplant<sup>[33]</sup>. However, in further studies such spectral changes need to be correlated with a range of clinical endpoints, including the pre-morbid clinical history of the liver donor, the pre-transplantation clinical history and nutritional status of the recipient, the subsequent post-transplantation liver function tests, pre-and post-transplantation indices of nutrition in the recipient and ultimately, the final clinical outcome.

Moving towards using MRS as a non-invasive methods for assessing graft dysfunction following transplantation, Taylor-Robinson and colleagues used correlative *in vitro* MRS of liver biopsy material and *in vivo* whole-body clinical hepatic <sup>31</sup>P MRS to examine chronic ductopenic rejection of human liver allografts and noted an increased PME/NTP metabolite ratio reflecting associated altered phospholipid metabolism<sup>[37]</sup>. The study also included electron microscopy of liver tissue and the alteration in the phospholipid component was judged to be related to a change in biliary phospholipid excretion in these cholestatic patients<sup>[37]</sup>. Such an increase in the *in vivo* PDE resonance, seen in the *in vivo* hepatic MR spectrum is not a specific finding in patients with chronic allograft rejection, because a similar, albeit less marked change has been found in patients with primary biliary cirrhosis and obstetric cholestasis<sup>[38,39]</sup>.

### Iron studies

Iron overload may be a result of iatrogenic causes such as multiple blood transfusions in beta-thalassaemia, or of metabolic causes such as hereditary haemochromatosis, and is commonly associated with liver dysfunction. At the current time, hepatic iron stores are still usually estimated using liver biopsy with the associated risks that this procedure carries, but are readily studied by MR, since paramagnetic iron compounds cause magnetic inhomogeneities, which shorten the nuclear relaxation time<sup>[40,41]</sup>. However, imaging is difficult on account of rapid relaxation caused by these iron moieties. In addition, use of different MR sequences is required and equipment may require recalibration. Relaxometry allows measurement of relaxation times and provides information as to iron-proton interactions. An *in vitro* approach allows direct quantification of iron following MR analysis<sup>[42]</sup>. *In vivo*, Wang and colleagues demonstrated single voxel MRS measurement of T<sub>2</sub> in liver iron overload that correlates strongly with iron quantification from biopsy and overcomes the difficulty of lack of detectable signals in conventional MRI<sup>[43]</sup>. Furthermore, Gandon and colleagues have proposed the use of a liver to muscle intensity ratio, which is transferable between equipment and sequences<sup>[44]</sup>.

### Gene therapy

Gene therapy offers the opportunity to replace defective genes in phenotypically abnormal tissue with recombinant genes. Integration into the host cell genome allows expression of the desired protein. However, methods of conveying the gene to the required location and monitoring delivery and expression are required. Viral vectors and non-viral means such as naked DNA, liposomes and molecular conjugates have all been used. Expression varies with time and between tissues. MR techniques using MR spectroscopy and/or MR imaging, offer non-invasive methods of monitoring expression<sup>[45,46]</sup>. As in drug monitoring techniques where a MR detectable moiety or metabolite is linked to the active drug, genes expressing markers may be combined with the therapeutic gene. Phosphoarginine produced by the enzyme arginine kinase in *Drosophila*, but not present in mammalian skeletal muscle, is expressed in mouse skeletal muscle and detected by <sup>31</sup>P MRS following injection of an adenovirus vector<sup>[47]</sup>. The expression in neonatal mice could continue for up to eight months. This demonstrates elegantly the principle of 'marker metabolite' using xenogenetic material. This principle has been demonstrated *in vitro* using a hepatocyte cell line<sup>[48]</sup>. Hepatocytes do not express creatinine kinase so phosphocreatine has not been seen on the hepatic MR spectrum. Integration of the cytoplasmic creatine kinase into hepatocytes *in vitro* could lead to detection of phosphocreatine by <sup>31</sup>P MRS, raising the possibility that combined with a hepatotropic gene delivery system, gene expression may be monitored *in vivo* through a MR-visible marker.

## APPLICATIONS OF *IN VITRO* NMR SPECTROSCOPY IN BILIARY DISEASE

### Introduction

Bile is predominantly an aqueous solution containing numerous constituents. Bile acids (BA), phosphatidylcholine (PTC) and cholesterol are the predominant lipid components of bile. Other components include electrolytes, organic anions (bilirubin), plasma proteins, hepatocyte proteins, peptides and amino acids, nucleotides, heavy metals and vitamins, xenobiotics and toxins<sup>[49]</sup>. In health the concentration of these biliary constituents is tightly controlled.

Primary bile acids such as chenodeoxycholic acid (CDCA) and cholic acid (CA), and secondary bile acids such as lithocholic acid (LCA) and deoxycholic acid (DOCA) are conjugated with the amino acids taurine and glycine and secreted as sodium or potassium salts into the biliary canaliculi via the ABC biliary transporter proteins<sup>[50]</sup>. Phosphatidylcholine, the predominant biliary phospholipid, is synthesized in hepatocytes and transported into the biliary canaliculi by the flippase multidrug resistant protein 3 (MDR3)<sup>[51]</sup>. Its main function is to form mixed micelles with primary and secondary bile acids and cholesterol essential for the emulsification of fats. More recently it has been shown to be cytoprotective to the biliary epithelium<sup>[52]</sup>. Cholesterol is solubilized by the formation of vesicles with PTC or mixed micelles with bile salts and PTC.

Sampling of bile for diagnostic purposes has become a common clinical practice since the introduction of endoscopic retrograde cholangiopancreatography (ERCP). Bile research has advanced in the last decade mainly as a consequence of the alarming rise in incidence of biliary tract cancer (cholangiocarcinoma)<sup>[53-55]</sup>.

Advanced cytological techniques such as digital image analysis and fluorescence *in situ* hybridization (FISH) of bile are being used to improve the diagnostic accuracy of cholangiocarcinoma in certain experimental units<sup>[56]</sup>. More recently proteomic analysis of cholangiocarcinoma bile has identified 87 unique proteins including several novel proteins whose functions are unknown and a large number of proteins not previously described in bile<sup>[57]</sup>. Advances in molecular research and imaging technologies have vastly improved the diagnostic sensitivity and specificity in this area but there is still a clear need to identify novel, highly sensitive and specific biomarkers for fluid-based detection of biliary tract cancer, as well as other diseases of the biliary tree. Metabolic profiling of bile using *in vitro* MRS is a valuable experimental tool for the identification of such early biomarkers of the disease.

### Bile physiology by MRS

The majority of MRS studies on bile have been carried out using <sup>1</sup>H MR spectroscopy. The hydrophobic association of conjugated bile acids and biliary lipids in micelles has been confirmed in early MR studies<sup>[58]</sup>. Bile acids have also been quantified in both animal and human bile using *in vitro* <sup>1</sup>H MRS. Rat bile studies with <sup>1</sup>H MRS have derived peak assignments for C-18 methyl proton of bile acids at 0.7 ppm and the C-19 at 0.9 ppm, while the taurine moiety of taurine-conjugated bile acids resonated at 3.1 ppm and 3.5 ppm, respectively<sup>[59]</sup>. Conjugated bile acids in human gallbladder bile have been quantified using two dimensional MR<sup>[60]</sup>. Amide (-NH) proton resonances in glycine- and taurine-conjugated bile salts are in the region of 7.8-8.1 ppm<sup>[60]</sup>.

More recently, <sup>1</sup>H MRS has demonstrated cholesterol in human bile that can be differentiated from other lipid components in bile<sup>[61]</sup>. It has also been utilized for studying the effects of cholesterol on the fluidity of human gallbladder bile as well as for quantifying micellar PTC concentrations<sup>[61,62]</sup>.

<sup>31</sup>P NMR spectroscopy has been used to quantify phospholipids in red cell membranes and in model bile salts<sup>[63]</sup>. Such <sup>31</sup>P MRS studies have shown higher concentrations of PTC and Pi in gallbladder bile compared to canalicular bile<sup>[64]</sup>.

### MR spectroscopy of bile in disease

The biliary epithelium is exposed to numerous constituents of bile. Bile acids, PTC and cholesterol are of particular importance in disease if the cytoprotective mechanism of cholangiocytes is disrupted<sup>[65]</sup>. Biliary disease has a major effect on biliary composition as the level of cellular function determines the production and biochemical constituents of bile. Biliary composition of these lipids also varies in cholestatic diseases of the liver and malignancy of the biliary tree. Current applications of

MRS in biliary disease include assessment of bile content in cholesterol gallstones, rejection in liver transplantation, primary biliary cirrhosis and biliary tract malignancy, as well in biliary excretion of xenobiotics<sup>[64,66-69]</sup>.

MRS has been used to assess the distribution of biliary lipids between vesicles and micelles, which is believed to have a role in gallstone disease, and there is evidence that cholesterol from vesicular aggregates may be responsible for the deposition of cholesterol stones in the gallbladder<sup>[70]</sup>. Changes in the pattern of fatty acids of PTC with an increase of arachidonic acid have also been observed in bile from patients with gallstones<sup>[71]</sup>. Fusion and aggregation of phospholipid-cholesterol vesicles which form liquid crystalline droplets leading to nucleation of cholesterol monohydrate crystals are thought to be responsible for the formation of cholesterol gallstones<sup>[70]</sup>. A selective reduction in biliary phospholipids has been suggested to be responsible for cholesterol gallstones in certain populations<sup>[72]</sup>.

Initial <sup>31</sup>P MRS studies on bile from patients with primary biliary cirrhosis have shown reduced levels of PTC and Pi when compared to bile from healthy volunteers<sup>[64]</sup>, but the significance of these findings still remains to be determined in more extensive studies.

*In vitro* MRS has also been demonstrated to effectively identify and quantify xenobiotic metabolites in human and animal bile. Dioxins have been identified in biliary-cannulated rodents<sup>[67]</sup>.

*In vitro* <sup>1</sup>H MRS analysis of bile has also been applied in human liver transplantation in an attempt to assess donor liver integrity. Melendez and colleagues studied twenty-four hepatic bile samples from eight liver donors<sup>[69]</sup>. The livers from two donors were steatotic on histological analysis, while the rest were normal. <sup>1</sup>H MRS analysis could show more intense PTC resonances in bile from steatotic donor livers, compared to bile from histologically normal donor livers. Seventeen hepatic bile samples from four recipients collected immediately after donor liver reperfusion were also analyzed by <sup>1</sup>H MRS and showed bile from donor livers with good early graft function had a progressive increase in the bile acid peaks which represents restoration of bile flow. When compared to grafts with early graft dysfunction, the relatively reduced bile acid peaks in these spectra suggested slow recovery of bile secretion. In this study a total quantification of bile acids was not feasible due overlapping signals from other biliary lipids, notably cholesterol and PTC<sup>[69]</sup>.

Our preliminary studies have revealed differences in phospholipid metabolites that may help distinguish between malignant and non-malignant causes of pancreaticobiliary obstruction. A reduced PTC resonance was seen in the bile from the majority of patients with hepatobiliary cancer compared to bile from patients with non-malignant indications for ERCP. This preliminary observation was confirmed by significant differences in the peak area ratios of PTC, referenced to the TSP standard in the <sup>1</sup>H MR spectra ( $P = 0.007$ )<sup>[68]</sup>.

Nishijima and colleagues have observed a lactate peak in bile spectra from patients with hepatic and biliary malignancy but not in bile from patients with non-malignant disease or bile from healthy controls<sup>[73]</sup>. The

significance of this finding has yet to be determined in larger studies, where the collection and storage of bile for analysis are performed according to uniform protocols without potential contamination from ERCP contrast agents or the uncertainty of a long storage time that may lead to lactate accumulation.

## FUTURE STUDIES

Although MRS is primarily a research tool and its use to study hepatobiliary disease is a relatively new area, the ability of both *in vivo* and *in vitro* MRS to provide quick, repetitive, and non-invasive assessments of organ function raises several possible future development areas, including non-invasive diagnosis and staging of disease.

*In vitro* MRS holds a particular promise in the metabolic profiling of body fluids, such as urine, plasma and bile to pinpoint the potential disease mechanisms and to assess the response of the body to treatment regimens. This is of particular relevance when patients are having *in vivo* MRS studies, since an *in vivo/in vitro* correlation of the metabolite profile obtained may highlight disease processes and inform further genetic profiling studies<sup>[39]</sup>.

Several clinical areas may be of potential interest. In the oncology field, phospholipid profiling to assess changes by *in vivo* <sup>31</sup>P MRS may be useful in monitoring responses to the treatment of hepatic tumors, with co-temporaneous *in vitro* MRS analysis of plasma and urine. For example, chemoembolization of hepatic tumors has been found to correlate with a decrease in PME and increase in ATP concentrations using *in vivo* hepatic MRS<sup>[74]</sup>.

Correlative *in vitro* MRS of body fluids and *in vivo* hepatic MRS techniques can also be applied to chronic liver disease to monitor liver fibrosis and progression towards cirrhosis. With increasing prevalence of alcohol-related liver disease, obesity-related liver disease (non-alcoholic hepatitis or NASH) and viral hepatitis, such as hepatitis C in particular, a reliable and repeatable monitoring system is required that obviates the morbidity and mortality associated with liver biopsy. *In vivo* and *in vitro* MRS may hold some of the answers to this, but further, larger scale studies are required to assess the true utility of these techniques with respect to other methodologies, such as microbubble contrast-enhanced ultrasound, ultrasound elastography and use of serological markers of fibrosis<sup>[75,76]</sup>.

Future technical advances will boost the clinical potential of *in vivo* MRS, for example improving the delineation of multi-component signals, such as PME and PDE, with proton decoupling<sup>[77]</sup>. Increasing magnetic field strengths for *in vivo* studies may also provide better signal-to-noise and increased resolution, and 3T magnets are becoming steadily more available. Furthermore, *in vivo* <sup>1</sup>H MRS may find a role in monitoring hepatic lipid content in interventional treatment studies of patients with non-alcoholic fatty liver disease in the future. *In vivo* <sup>13</sup>C MRS for measurement of lipid metabolism, glycogen storage and gluconeogenesis looks promising for the future, but current sensitivity issues mean that this capability is currently confined only to a few centers<sup>[78-80]</sup>. *In vitro* MRS will find a correlative role in this context for screening plasma and urinary metabolites.

With respect to *in vitro* applications in bile, the ratio of taurine to glycine conjugates of bile acids and conjugated to unconjugated bile acids varies in hepatobiliary disease, and it has been widely accepted that elevated levels of bile acids in hepatocytes are toxic, leading to cholestasis<sup>[65]</sup>. More recently, the role of bile acids in cholangiocytes has been highlighted. *In vitro* studies on bile acids has demonstrated that they can act as ligands for the epidermal growth factor receptor on cholangiocytes and *via* the mitogen-activated protein kinase cell signalling pathway, leading to disordered cell cycling and cholangiocyte proliferation<sup>[81]</sup>. The measurement of bile acids is therefore necessary for an understanding of the pathophysiology of these diseases. *In vitro* MRS may find a useful role in screening bile in this context.

It is true that the significance of *in vivo* MRS needs to be assessed in larger studies with greater numbers of patients with various hepatobiliary diseases. Correlative *in vitro* NMR spectroscopy will help to elucidate some of the conundra. Trials in large populations in well-defined clinical settings are needed to determine if both *in vivo* and *in vitro* MRS can provide independent diagnostic and prognostic indices in management.

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