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Epithelial and Stromal Metabolite Changes in the Transition from Cervical Intraepithelial Neoplasia to Cervical Cancer: an in vivo $^1$H Magnetic Resonance Spectroscopic Imaging Study with ex vivo Correlation

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**ABSTRACT**

**Aim:** To investigate epithelial and stromal metabolite changes in cervical intraepithelial neoplasia [CIN] and cervical cancer *in vivo* and correlate findings with MR spectroscopy of tissue samples.

**Methods:** Forty-seven women (19 with CIN, 28 with cervical cancer) underwent endovaginal MR at 1.5T with T2-W and localized 2-D MR spectroscopic imaging (PRESS, TR=1500ms, TE=135ms). tCho, 2ppm and -CH$_2$ lipid peaks were measured in epithelial (>50%epithelium, no tumour), stromal (>50%stroma, no tumour) and tumour (>30%tumour) voxels. Unsuppressed water signal from the same voxel provided a concentration reference. $^1$H HR-MAS MR spectra were acquired from tissue in 37 patients (11.74T, pulse-acquire and cpmg sequences, with water pre-saturation).

**Results:** Analysable data from 17 CIN and 25 cancer patients showed significant increases in tCho (p=0.03) and 2ppm (p=0.007) in tumour compared with epithelial voxels from CIN patients but not with epithelial voxels from cancer patients. No significant differences were seen in stroma from cancer compared with CIN patients. Differences in -CH$_2$ lipids were not significant between groups. There was no significant correlation between *in vivo* and *ex vivo* tCho or –CH$_2$ lipids.

**Conclusion:** Estimated in vivo concentrations of tCho and 2ppm resonances increase in tumour and adjacent epithelium in progression from CIN to cervical cancer.
INTRODUCTION

In cancer tissues, a metabolic change with an increase in choline containing metabolites and mobile lipid resonances has been observed in a number of tumour types including cervix [1], breast [2] and prostate [3]. In the cervix, the recognition of a well-defined pre-invasive stage has enabled the documentation of these metabolites in tissue samples containing cervical intraepithelial neoplasia (CIN) as well as in cancer [4]. Magic angle spinning which permits the acquisition of spectra from tissue samples with a resolution close to that possible in tissue extracts, while retaining the intact sample for histopathology have shown increased lipid –CH₂ and –CH₃, and also choline in cancer relative to CIN or normal cervix [1, 4]. Studies have indicated that the metabolic abnormality is not confined to the tumour itself, but is likely to involve peritumoral tissue [5]. Acquiring such data in vivo would allow mapping of epithelial and stromal changes in patients with CIN and cancer thus identifying regional biological changes in the progression of preinvasive to invasive disease.

In vivo, the low signal-to-noise ratio of MR spectroscopic studies has limited its widespread use, with implementation of 2-D MRS methodology largely confined to brain [6-8], breast [9] and prostate [10-12] cancer. In studies of the uterine cervix, use of an endovaginal coil [13] improves the signal-to-noise ratio and enables ¹H MR spectroscopic data to be obtained, whilst also allowing identification of epithelial and stromal voxels from the morphological imaging data. For technical reasons, previous studies have primarily focussed on a single voxel technique with a 15X15X15mm resolution, but technical improvements have now made acquisition of 2-D data possible. The aim of this study therefore was to investigate the epithelial and stromal metabolite changes in CIN and cervical cancer in vivo and correlate findings with high-resolution magic
angle spinning (HR-MAS) MR spectroscopy of tissue samples in order to determine regional changes that occur with progression of preinvasive to invasive disease.
METHODS

Patients

Over 13 months (Apr 2007-May 2008), 47 women with CIN or invasive cervical cancer were studied with their written informed consent and approval of the local ethics committee. At diagnostic biopsy, 19 of these (aged 24-55 years, mean 32 years) had pre-invasive disease, and 28 (aged 26-75 years, mean 43 years) had cervical cancer. Women with dyskaryotic disease were identified from colposcopic clinic lists following an abnormal smear. Only 2 of these women had cone biopsies before the MR study, 2 and 5 years earlier. All patients with histological evidence of cervical tumour on diagnostic biopsy/smear were recruited. Ten of these patients had cone biopsies between 2 and 8 weeks (median 4 weeks) before MR imaging. Women with ferromagnetic implants were excluded. Patients were asked to abstain from caffeine for 4 hrs. before MRI.

MR imaging

A ring design receiver coil (37 mm diameter) of solenoid geometry, [13], was inserted endovaginally and positioned around the cervix. A feeding tube was passed into the posterior vaginal fornix along with the coil and air introduced during coil placement was aspirated to reduce B₀ field inhomogeneities at the air/tissue interface. The coil was immobilized by an external clamp. An antiperistaltic agent (20 mg hyoscine butyl bromide) was administered intramuscularly immediately before imaging in all cases to reduce peristalsis. In no case was the examination terminated because of patient discomfort.
MR imaging was performed on a 1.5T system (Intera, Philips Medical Systems, Best, The Netherlands). Conventional T2-W fast spin-echo (FSE) images were obtained in three orthogonal planes (TR 2000 ms, effective TE 90 ms; echo train length, 16) with a 256 x 256 matrix, 6 signal averages, 3 mm slice thickness and a 110 mm² field of view. The total imaging time was 12 minutes.

**In vivo MRS**

2-D magnetic resonance spectroscopic imaging (MRSI) was performed over a single slice coronal to the cervix, (thickness 15 mm, 16 x 16 grid and field of view 120 mm²). A midline slice was selected in CIN patients; a slice with abnormality on T2-W imaging was selected in cancer patients. Signal collection was limited to voxels over the cervix using a point-resolved spectroscopy (PRESS) localization technique (TR 888 ms, TE 135 ms) with 4 signal averages. All three orthogonal T2-W images were used to position the PRESS box (25 x 18 x 15 mm). An automated shimming method over the PRESS volume used the MR system’s software to adjust the x, y, and z shim currents in small increments and test for the best line shape. The line width at half-height of the total water signal within the PRESS box was measured following shimming, and if the value was greater than 15 Hz, the PRESS box was repositioned and re-shimmed or spectroscopy was abandoned. B₀ maps (dual gradient-echo; TR 50 ms TEs 7.6 and 17.6 ms, FOV 160 mm², slice thickness 5 mm, 1 signal average) obtained after shimming, ensured that there were no significant susceptibility artefacts or B₀ inhomogeneities. Water suppression was achieved during the PRESS sequence using band selective inversion with gradient dephasing (BASING, [14]). Unsuppressed water as a concentration reference also was measured with a TE of 135ms, resolution of 16 x 16 and 2 signal averages. Data acquisition took 17 minutes. The
spectroscopic data were voxel-shifted to align them with the PRESS box and were exported for analysis with LCMODEL software [15] using a basis set including metabolite spectra from choline, creatine, N-Acetyl Aspartate (NAA – as an approximation to a peak from acetate), glutamate, glutamine and lipids.

**Tissue collection**

A cervical punch biopsy additional to those taken for diagnostic purposes was taken at colposcopic examination following MR imaging, during local treatment for CIN or at examination under anaesthesia or hysterectomy in patients with cervical cancer. A gynaecologist determined the targeted biopsy site during colposcopy following acetowhite ning in the case of CIN and by visual inspection of the cervix when an area of tumour was clinically obvious. Tissue samples were frozen within 5 mins of excision and stored at –80ºC before MR analysis.

**Ex vivo MRS**

Rotor-synchronised high-resolution $^1$H magic angle spinning (MAS) spectra were recorded using an 11.74 T spectrometer (Bruker Avance, Germany) equipped with a $^{1}$H/$^{13}$C/$^{31}$P HR-MAS probe. Tissues (sample weight from CIN patients 16.7±12.2 mg, from tumour patients 31.9±9.3 mg) were thawed, washed in phosphate buffered saline (PBS) made with D$_2$O to remove any blood, loaded into a 35 $\mu$l Kel-F insert and placed in a cylindrical 4-mm ZrO$_2$ rotor. Samples were tuned, shimmed and spun at 3 kHz at a temperature of 4ºC to minimise metabolic activity. $^1$H HR-MAS spectra were acquired with water presaturation and a Carr-Purcell-Meiboom-Gill (CPMG) sequence (TE 135 ms, time between each 180-degree pulse 333.33µs). Each FID was the sum of 512 transients with 16 K data points, a repetition time of 4.8 s and a total acquisition
time of 41 minutes. In addition a pulse and acquire sequence with water pre-saturation (TR 4.3 s, 128 transients) was also obtained. Following MAS data acquisition, samples were fixed in formalin and routinely processed histologically. They were classified as predominantly epithelium, predominantly stroma or tumour by an expert histopathologist.

**Data processing**

**In vivo studies**

Tumour volume was calculated by drawing regions of interest (ROIs) around an intermediate signal intensity cervical mass on T2-W sagittal scans and multiplying total ROI area by slice thickness.

*Voxel assignment:* The five consecutive T2-W transverse slices that corresponded to that used for MR spectroscopic data acquisition were identified and an experienced radiologist assigned the voxels as epithelial (>50% epithelium, no tumour), stromal (>50% stroma, no tumour) or tumour (>30% tumour).

A total of 259 voxels were sited within the PRESS box in 47 patients (6 in 39 patients, 4 in 4 patients, 3 in 1 patient and 2 in 3 patients). A requirement that signal-to-noise (SNR) calculated by LCMODEL software was greater than 2 eliminated 21 voxels from the analysis. (SNR in LCMODEL is defined as maximum signal within the frequency range of interest in the baseline-corrected spectrum divided by twice the root-mean-square residuals [16]). In addition, voxels were included in the analysis only if there was a modest to good fit of the metabolite basis set and baseline to the *in vivo* spectra, enabling a calculation of the area under the curve. 40 further voxels were excluded on these criteria. Five voxels which contained fibroids in one patient, and
8 voxels which contained visible parametrial fat in 3 patients were also excluded. Thus, in total 185 voxels in 42 patients (17 CIN, 25 cancers) were included in the analysis.

The areas under the tCho (3.2ppm), 2ppm and -CH₂ lipid peaks were calculated for each voxel and normalized using the signal from tissue water in the same voxel (assumed tissue water content 79%). This yields a measure of the concentration of each metabolite, but uncorrected for differences in relaxation times. Values for each voxel type (epithelium, stroma, tumour) were averaged for each patient to eliminate statistical bias.

**Ex vivo studies**

Peak assignments were based on previously reported values [1,17] with creatine at 3.027ppm as the internal chemical shift reference. Choline-containing metabolites (3.20-3.23ppm, (using the CPMG spectra) and 1.3ppm -CH₂ lipids (using the pulse-acquire spectra and fitting lactate and –CH₂ resonances separately) were quantified by measuring peak areas (AMARES algorithm in jMRUI software package [18]). Concentrations were estimated by relating areas to those of a reference compound in a separate measurement (50 µl of 9.64 mM 3-(Trimethylsilyl) - Propionic acid-D4 sodium salt (TSP)), and correcting for sample weight. This used an identical CPMG sequence before the sample measurement. The probe was re-tuned and re-shimmed before every measurement.

**Statistical methods**

Statistical analysis was performed using SPSS, version 11 for Windows. tCho values were normally distributed but the 2ppm and 1.3ppm values were not; the natural log of values was
therefore used to normalize these data. For \textit{in vivo} MR data, the tCho, signal at 2ppm and –CH$_2$ lipid were compared between patients with CIN and cancer. For the \textit{ex vivo} MR data, tCho and 1.3ppm lipid were compared between groups, as the 2ppm peak was not identified. One-way analysis of variance (ANOVA) with Bonferroni correction investigated differences between groups [19]. Independent two-sample $t$-tests identified significant differences between epithelium and tumour and between stroma from both patient groups. All significance tests were two-sided, with a $p$-value of less than 0.05 denoting statistical significance. In patients from whom tissue was obtained, \textit{ex vivo} metabolite concentrations (classified as epithelium, stroma or tumour) were correlated with \textit{in vivo} relative concentrations obtained from voxels of the same tissue type.
RESULTS

In vivo studies

Patients were classified as CIN or cancer according to the result of their diagnostic biopsy. In 23 of 25 patients with cancer, tumour was identified on the conventional T2-W scans. In 2 patients with large cone biopsies, residual tumour was not identified. Tumour volumes ranged from 0-62 cm³ (median 2.7 cm³, quartiles 1.4 and 10.8 cm³). Voxel types were 24 epithelial and 50 stromal in CIN patients, 51 tumour, 14 epithelial and 46 stromal in cancer patients. Choline-containing metabolites (3.2ppm) were detected in all epithelial, 94/96 stromal and all tumour voxels. A peak at 2ppm was observed in 36/38 epithelial, 93/96 stromal and all tumour voxels. A lipid peak at 1.3ppm was detected in 37/38 epithelial, 91/96 stromal and 47/51 tumour voxels (Fig. 1). Relative metabolite concentrations for each patient category are summarized in Tables 1 and 2.

\(t_{Cho}\): \(t_{Cho}\) was significantly different between groups (ANOVA, \(p=0.038\)) with significant increases in tumour compared with epithelial (\(p=0.033\)) voxels from women with CIN, but not compared with epithelial voxels from women with cancer (Fig. 2). Increases in stromal values of \(t_{Cho}\) in cancer compared with CIN patients were not significant.

2ppm peak and \(-CH_2\) lipids: The peak at 2ppm was significantly different between groups (ANOVA \(p=0.028\)) (Table 2) but there were no significant differences in the \(-CH_2\) lipid peak between groups. The 2 ppm peak, which was fitted as a combination of acetate (using the available basis spectra of NAA, NAAG), glutamine, glutamate, lipids and macromolecules visible at this resonant frequency was significantly greater in tumour than in epithelial voxels from CIN patients (\(p=0.007\)), but not than in epithelial voxels from cancer patients. This pattern
of increase was the same as for tCho. Although stromal values were higher in stroma of cancer compared to CIN patients, differences were not significant (Table 2, Fig. 3a).

For the lipids at 1.3ppm, there was a large variability with high values for lipid in the stroma of both groups, likely due to contribution from parametrial fat not detected on the T2-W images. There was no significant difference in the 1.3ppm lipid between stroma of CIN patients and cancer patients. The epithelial voxels, being centrally placed within the cervix, were not subject to contamination by parametrial fat. However, there was no significant increase of -CH₂ lipids in tumour compared to epithelium from CIN or cancer patients (Fig. 3b).

**Ex vivo studies**

Biopsy samples were obtained from 37 of these patients (14 CIN, 23 cancers). Estimated concentration of tCho were significantly greater (p=0.034) in biopsies from patients with cancer compared with CIN. Estimated –CH₂ lipid levels at 1.3ppm were also significantly greater (p<0.005) in patients with cancer compared with CIN (Table 3).

There was a no significant correlation between the levels of *in vivo* and *ex vivo* tCho (r=-0.03, p=0.87) possibly because the spectral resolution of the *ex vivo* data ensures that this peak is much more rigorously defined. Similarly, no correlation was seen for the –CH₂ lipid concentrations (r=0.07, p=0.74) *in vivo* and *ex vivo*.

**Correlation of peritumoral metabolites with tumour volume:**

In the cancer patients, no correlation was seen between *in vivo* tCho or –CH₂ lipids within peritumoral voxels (stromal or epithelial) and tumour volume indicating that the metabolic changes in the peritumoral tissues are not related to tumour size. In fact, the 2 patients with no
visible tumour on T2-W MR imaging both had relatively high levels of tCho and 1.3ppm lipids in peritumoral voxels.
DISCUSSION

This study extends the previous work done with single voxel methods [4] and shows the feasibility of using 2-D MRSI to study tumoral and peritumoral metabolic changes: measured tCho within tumour voxels was increased compared with epithelial voxels from women with CIN but not compared with epithelial voxels in cancer patients. This was confirmed on the ex vivo samples of non-tumour containing peritumoral tissue in cancer patients where complete sectioning affirmed no contamination with tumour cells. Previous ex vivo data also showed that Cho and phosphocholine (PC) were significantly increased in histologically confirmed cancer biopsies compared with high grade CIN [5] and that PC was not significantly different between tumour, epithelium and stromal voxels from patients with cancer. These observations may be explained by rapid cell membrane turnover resulting in accumulation of choline metabolites not only in the cancer cells but also in the peritumoral tissue. In the current study, although the resolution of the technique resulted in imperfect divisions between epithelial and stromal voxels in vivo, there was a definite increase in tCho in normal appearing epithelium from cancer patients compared with CIN patients suggesting increased tissue metabolic activity adjacent to a malignancy.

Signals at 2ppm were observed in women with CIN and invasive cancer. Previous studies on gynaecological lesions [4,20], brain tumour cystic fluid [21] and sinus mucocele [22] also observed a peak at 2ppm. Lee et al observed a peak at 2ppm in patients with adenocarcinoma but not squamous cell carcinoma and suggested that this peak was a biomarker for adenocarcinoma [23]. This is not consistent with our data where this peak was visible in all patients, with and without tumours. In vivo the 2ppm peak is a broad resonance potentially encompassing
contributions from acetate, glutamine, glutamate, macromolecules and lipids [24]. As separation between these is not possible at the resolution available in vivo, we considered them collectively. It is possible that the increase in lipid components of this peak in stroma from cancer patients is offset by depletion of metabolites such as glutamine and glutamate leading to no overall change in stroma adjacent to cancer. Depletion in metabolites such as alanine has previously been reported in peritumoral tissue [5], but metabolites such as glutamine and glutamate have not specifically been measured. In future improved resolution using an endovaginal coil at 3T together with 2-D techniques such as J-PRESS and L-correlation spectroscopy (COSY) may make differentiation of the components of the 2ppm peak possible in vivo.

The assignment of the 2ppm peak itself remains debatable. It previously has been assigned as monosaturated fatty acyl protons [25], to the N-CH$_3$ group of sialic acid [26] or to the N-acetyl compounds present in mucus glycoproteins (mucins) [22,27,28] Mucus glycoproteins have complex carbohydrate chains covalently attached to their peptide backbones [29], primarily in the form of oxygen linkages to oligosaccharides. The monosaccharides that make these oligosaccharides include N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), sialic acid, galactose, glucose mannose, xylose and fucose [30]. Therefore, the most likely metabolites contributing to the 2ppm peak would be N-acetylated sugars GalNAc, GlcNAc and sialic acid [27]. In a review of GlcNAc-Serine (O-glycans) biosynthesis, Brockhausen suggested that mucins from patients with intestinal cancer had less total carbohydrate and that the reduction may be due to both shorter chain length and fewer O-glycan chains [31]. Therefore, structural and antigenic properties of glycoproteins may be altered in cancer. It is possible that glycoproteins,
present in cervical mucus, are altered in cancer. It may be that we were unable to identify this peak \textit{ex vivo}, because adherent mucus was washed off before measurement.

All \textit{in vivo} MRS studies on cervical cancer have noted the presence of lipid resonances. Comparison of proton spectra pre and post radiation therapy showed differences between triglyceride peaks (0.9 and 1.3 ppm) and choline (3.2 ppm) in normal and cancerous tissue [24]. In-phase \textit{CH}_2 lipid peaks 1.3 ppm have been described as a biomarker of cervical cancer [4]. In our study, stromal voxels at the periphery of the cervix potentially had a high contamination with parametrial fat so that reliable measurements for \textit{CH}_2 lipids were difficult to achieve. In future, effective use of presaturation bands may improve this.

Shimming can be a major limitation in data acquisition and required care. In our study difficulties in shimming due to local inhomogeneities arose from the presence of air, distance of the PRESS box from the center of the coil, haemorrhage following recent biopsies and by the presence of different tissue types including fat and blood vessels within the shimmed volume. Care was also required to ensure that the position of the cervix did not alter with time due to bladder filling or bowel peristalsis. The former was limited by fluid restriction of patients before imaging and the latter was reduced with administration of intramuscular antiperistaltic agents.

Metabolite peak fitting was another limitation in our study. The three peaks consistently visible in our \textit{in vivo} spectra (tCho, 2 ppm peak and \textit{CH}_2 lipid) were analysed using a general basis set and not a set specific for cervical metabolites. Based on MAS data [5] the tCho peak will contain contributions from taurine whilst at 2 ppm there is a contribution from glutamate. In our study
LCMODEL was used to fit known peaks to the *in vivo* data which were then summed. *Ex vivo* data did not use the sum of closely resonating multiple metabolites, which could have accounted for the poor correlations between the *in vivo* and *ex vivo* data. Development of a specific basis set of cervical metabolites for *in vivo* analysis would be useful for future studies.

In conclusion, 2-D MRSI has enabled interrogation of metabolic activity in tumour and peritumoral tissue and shown that relative tCho and 2ppm concentrations were increased in tumour and peritumoral epithelium in patients with cancer compared with epithelium in women with CIN indicating altered metabolic activity in tissue surrounding tumour.

**ACKNOWLEDGEMENTS**
REFERENCES


5. [blinded]


FIG. LEGENDS

Fig. 1 In vivo MR imaging and spectroscopy of uterine cervix (\(^1\)H PRESS, TR 888ms TE 135ms) in a patient with CIN (a) and a patient with cancer (b). The central panel shows the coronal T2-W image on which the PRESS box was planned. Example spectra from epithelial voxels in the right hand panel show increased tCho, 2ppm and –CH\(_2\) lipids in epithelium from cancer patients. The left hand panel shows these peaks in a stromal voxel in (a) and a tumour voxel in (b) (not to scale).

Fig. 2 Comparison of relative tCho concentration in epithelial, stromal and tumour voxels from women with CIN and cervical cancer showing increased levels in tumour compared with epithelium in CIN patients, but not compared with epithelium in cancer patients. Lower quartile, bottom line of the box; median, middle line of the box; upper quartile, top line of the box; lower whisker, lower value; upper whisker, upper value.

Fig. 3 Comparison of relative 2ppm concentration (consisting of a combination of NAA, NAAG, glutamine, glutamate and macromolecules and lipids) (a) and -CH\(_2\) lipid concentration at 1.3ppm (b) in epithelial, stromal and tumour voxels from women with CIN and cervical cancer. In a, there is an increased level of the 2ppm peak in tumour compared with epithelial voxels from CIN patients but not from cancer patients. Lower quartile, bottom line of the box; median, middle line of the box; upper quartile, top line of the box; lower whisker, lower value; upper whisker, upper value.)
Fig. 4 High-resolution magic angle spinning spectra acquired using a CPMG sequence (left panel, a, b, c) and a pulse-acquire sequence (right panel, d, e, f) in epithelium from a CIN patient (top row), peritumoural epithelium from a cancer patient (middle row) and tumour tissue (bottom row). Choline containing metabolites (3.2ppm) are increased in CPMG spectra and –CH₂ lipids are increased (1.3ppm) in pulse-acquire spectra in tumour tissue (c, f) as well as in peritumoural epithelium (b, e). The acetate impurity at 2ppm in the CIN patient (a, d) is from acetowhitening during colposcopy.

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