# Metabolite analysis of preinvasive and invasive cervical cancer tissues using 1H and 31P HR-MAS spectroscopy

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**Introduction:** Cervical cancer is preceded by a well-defined pre-invasive stage called cervical intraepithelial neoplasia (CIN) and therefore is a useful model for studying early events in the biology of epithelial malignancies. Elevated levels of choline have previously been identified in a number of tumours including cervical cancer [1] but whether these metabolites are elevated in precancerous tissue is debated. The observed choline resonances include phosphocholine (PC), glycerophosphocholine (GPC) and choline (Cho), with PC relatively elevated in cancer tissue (e.g breast cancer [2] and prostate cancer cell lines [3]). The aim of this is to determine the metabolite differences between low grade and high grade CIN and cancer tissues using $^{1}$H HR MAS MRS of intact tissue and to establish which components of choline were selectively alerted using $^{31}$P HR MAS MRS.

**Methods:** ***Tissue Collection:*** All women were studied with their written informed consent and with the approval of the local ethics committee. Criteria for inclusion- Abnormal smears (low/high grade CIN or cancer). Regions were biopsied at colposcopy examination or by visual inspection of the cervix by the gynecologist when invasive disease was clinically obvious. Average tumour biopsy sample size ~30 mg, Average CIN tissue biopsy sample size ~10 mg.

***MAS:*** Tissue samples were thawed, and washed with phosphate buffered saline solution (PBS) to remove excess blood, loaded into 40µl sample inserts, topped up with D2O and then placed inside 4mm ZrO rotors. NMR measurements were performed on a Bruker Avance 11.74T spectrometer. All spectra were acquired with spin rate of 3kHz, number of scans 512 and temp 4°C. $^{1}$H MAS data were acquired using a CPMG sequence (TE=134 ms, TR=4.8s), expt time 41 mins, internal chemical shift ref-creatine 3.03 ppm. $^{31}$P MAS spectra for high grade CIN and cancer tissue were acquired using a hydrogen decoupled pulse acquire sequence (TR= 3.35 s) and 1H decoupling with 1024 scans, expt time 1 hr 1 min. Peak assignments were based on chemical shift. Peak areas were measured using the AMARES algorithm included in the jMRUI software package. Concentrations from $^{1}$H spectra were obtained using the peak area of a reference compound in a separate measurement (50 µ l of 9.64 mM 3-(Trimethylsilyl)- Propionic acid-D4, sodium salt (TSP)), and corrected for the weight of each sample. Concentrations from $^{31}$P spectra were obtained using the peak area of a reference compound in a separate measurement (40 µ l of 20 mM methylene diphosphonic acid (MDPA)), and corrected for the weight of each sample. Calculations are based on the assumption that the total reference volume is detected by the coil results in the area under the peak. Statistical analysis was performed using ANOVA and a *p*- value of <0.05 was considered significant.

**Results:**

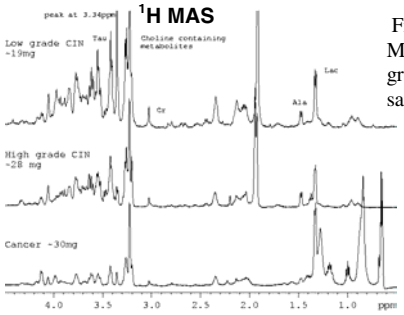


Figure 1 Example of 1H MAS of low grade, high grade CIN + tumour sample with NS = 512

| | Aln | Cr | Cho | PCh | Tau |
|---|---|---|---|---|---|
| **Low grade CIN (n=5)** | 0.73± 0.31 | 1.52± 0.75 | 0.47± 0.49 | 1.09± 0.54 | 1.56± 0.19 |
| **High grade CIN (N=31)** | 0.53± 0.31 | 0.91± 0.69 | 0.41± 0.24 | 0.94± 0.49 | 1.60± 1.85 |
| **Cancer (N=19)** | 0.73± 0.53 | 0.69± 0.47 | 0.94± 0.79 | 1.76± 1.31 | 1.55± 0.89 |

**Table 1** Choline metabolites and creatine concentrations (µmol/g wet wt) for CIN and tumour tissue samples (mean ± sd) obtained from $^{1}$H MAS spectra

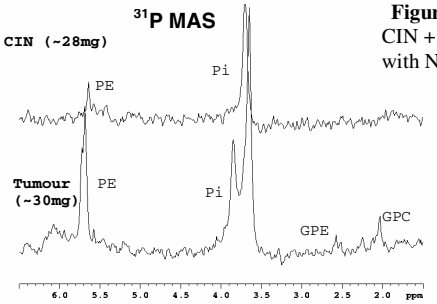


**Figure 2** $^{31}$P MAS of a CIN + tumour sample with NS = 1024

| | Pi | PE | PC | GPC | GPE |
|---|---|---|---|---|---|
| **CIN (N=31)** | 7.29± 5.26 | 2.5± 1.7 | 1± 0.6[#] | ND | ND |
| **Cancer (N=19)** | 9.96± 4.26 | 5.27± 2.59 | 2.30± 1.51 | 1.07± 1.11* | 1.43±0.95* |

**Table 2** Calculated concentrations (µmol/g wet wt) in tumour tissue samples (mean ± sd). [#] PC is only observed in 10 CIN samples.* GPE and GPC only detected in 4 samples. N.D. = Not detected

**$^{1}$H HRMAS** - PC (Anova one way test, *p*=0.004) and Cho (Anova one way test, *p*=0.001) were significantly increased in cancer compared to low or high grade CIN tissue. Triglycerides were also observed to be present in greater concentration in cancer biopsy tissue. The signal observed between 1.9-2 ppm in the CIN spectrum corresponds to the frequency of the acetyl moiety. This is found in high concentration in CIN samples due to 'Acetowhitening' applied during colposcopy. Cr concentration in biopsy samples is seen to reduce with increasing abnormality. The singlet peak observed at 3.35 ppm in high concentration in low grade tissue has been tentatively assigned as "scyllo-inositol" until further clarification can made. This peak is substantially decreased in high grade CIN and cancer.

**$^{31}$P HRMAS -** Inorganic phosphate (Pi) (Anova one way test, *p*=0.047), PE (Anova one way test, *p*=0.0002) and PC (Anova one way test, *p*=0.017) concentrations are significantly increased in tumour samples on 31P spectra.

**Discussion:** Increased PC observed in invasive cancer compared to CIN may be caused by elevated levels of choline transport and/or phosphorylation [5] and/or breakdown of phosphatidiylcholine [2] common in many tumours. Triglycerides found in much higher concentrations in invasive carcinoma tissue samples compared with preinvasive CIN biopsy tissue could be due to several factors such as enhanced triglyceride synthesis, hindered secretion of cellular triglycerides or excess release of fatty acids from intracellular membrane phospholipid stores [6]. Pi was observed in all and PE and PC only in some CIN samples with $^{31}$P MAS. However, PC was detected in all CIN samples using $^{1}$H MAS. This illustrates the advantage of the greater sensitivity and presence of equivalent 1H nuclei for detecting the peak using 1H MAS.

**Conclusion:** Spectral differences between preinvasive and invasive disease can be monitored with $^{1}$H and $^{31}$P HR MAS and offer potential for monitoring the underlying metabolic changes in the progression from dysplastic to tumour phenotype.

**References:** [1] deSouza, NM *et al*. NMR in Biomedicine, 2004;17:144-153.. [2] Zaver, B. M *et al*, Cancer Research, 1999, *59*, 80-84. **[3]** Zaver, B. M *et al*, Cancer Research, 2001, *61*, 3599-3603. [4] Govindaraju, V et al. NMR in Biomedicine, 2000;13:129-153. [5] Degani, H et al, Cancer Research, 2002, 62, 1996-1970. [6] Hakumaki, JM et al. TIBS, 2000, 25, 357-362.

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