Assessment of freezing effects and diagnostic potential of BioBank healthy and neoplastic breast tissues through HR-MAS NMR spectroscopy

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Abstract

HR-MAS NMR spectroscopy was employed to monitor the metabolic profiles of Modena BioBank breast samples over 1 year of freezing at

−80 °C. The study includes 22 adult female patients living in Modena and its hinterland, who underwent total mastectomy or quadrantectomy in 2011–2012. Variations occur, especially affecting phosphocholine (PC) and choline. This is not a trivial finding, since many studies base the distinction between neoplastic and healthy tissues or the assessment of tumor grade on the analysis of choline metabolites. Despite the changes observed, we established that the diagnostic power of the HR-MAS NMR spectra of frozen samples is preserved, at least as far as the distinction between neoplastic and healthy samples is concerned. Lactate (Lac), PC, phosphoethanolamine (PE), taurine (Tau), myo-inositol (Myo) and glucose (Glc) are biomarkers that can be used to distinguish healthy from neoplastic tissues, whereas some metabolite ratios, such as Lac + PE + Tau/Glc + Myo, seem to have even higher discrimination potential.

Keywords

Metabolomics
HR-MAS NMR
Breast cancer
Freezing effects on tissues

Abbreviations

Asc	Ascorbate
TG_{gl}	Bonded glycerol
CPMG	Carr–Purcell–Meiboom–Gill
Cho	Choline
ChoCC	Choline containing compounds
E	Ethanolamine
Glc	Glucose
GPC	Glycerophosphocholine
Gly	Glycine
HR-MAS	High-~~r~~Resolution ~~m~~Magic ~~a~~Angle ~~s~~Spinning
Lac	Lactate
Myo	Myo-inositol
NMR	Nuclear ~~m~~Magnetic ~~r~~Resonance

PLS-DA	Partial ŧLeast ŝSquares-ɗDiscriminant ɑAnalysis
PC	Phosphocholine
PE	Phosphoethanolamine
PCA	Principal ɕComponent ɑAnalysis
ROCCET	Receiver ɔOperating ɕCharacteristic ɕCurve ɕExplorer & ŧTester
Tau	Taurine

Electronic supplementary material

The online version of this article (doi:10.1007/s11306-014-0709-z) contains supplementary material, which is available to authorized users.

1. Introduction

The application of ɦHigh-ɾResolution ɱMagic ɑAngle ŝSpinning (HR-MAS) ɳNuclear ɱMagnetic ɾResonance (NMR) spectroscopy to the analysis of intact tissue biopsies and surgery samples dates back to 1997. It represents an actively investigated field, and it is used to obtain the metabolic fingerprints of tissues. It has been shown that HR-MAS NMR is able to differentiate between normal and neoplastic tissues in the case of breast, brain, head and neck squamous cell carcinoma, bladder, kidney, colon cancer and other malignancies in the gastrointestinal tract and to help in the characterization of metabolic abnormalities in cancer (Tripathi et al. 2012, 2013; Moestue et al. 2011; Bathen et al. 2010). The metabolic profile of healthy and neoplastic breast has already been studied by HR-MAS NMR spectroscopy. (Sitter et al. 2002; Cheng et al. 1998) The main metabolic differences between healthy and neoplastic breast tissues are in choline containing compounds (ChoCC), taurine (Tau), glycine (Gly) and glucose (Glc) (Moestue et al. 2011; Bathen et al. 2010).

Even though the feasibility of HR-MAS NMR has been proposed as a potential method for on-line analysis of resection margins during breast cancer surgery by different research groups (Bathen et al. 2013; Li et al. 2011; Kinross et al. 2011), as far as we know only one case of real-time metabolic profiling of biopsy specimens during a surgical operation has been reported (Piotto et al. 2012). At present, spatial localization of spectrometers and instrument time constraints prevent the direct application of HR-MAS NMR analysis on tissue samples immediately after resection in

most cases. Usually, samples are snap-frozen in liquid nitrogen and then stored to −80 °C or at lower temperatures. A critical issue to be addressed in these kind of approach is the interaction with the surgical teams, which can vary from time to time, with considerable risks of collecting samples with different thermal histories or contamination. In our experience, the best way to collect samples is that a unit of the spectroscopy staff go personally into the operatory theatre with a Dewar containing liquid nitrogen. Nevertheless, this is not always possible for difficulties associated with changes of surgical teams, last minute scheduling of operations, interference in operating theatre protocols and organization. These considerations prompted us to undertake a study on tissue samples collected by Modena BioBank, which represent an efficient supply channel of tissue samples. The Modena BioBank collects and stores tissue samples and associated data for medical-scientific research and diagnostic purposes.

Nowadays breast cancer is still the most common malignant tumor diagnosed in adult female population worldwide. (Ferlay et al. 2013, http://globocan.iarc.fr) The areas of high risk are North America, Europe and Australia where 6 % of women develop invasive breast cancer before age 75. (Tavassoli and Devilee 2003). In Italy, although the breast cancer incidence and its mortality declined for younger women, its incidence rate is still high. (Rossi et al. 2013; Quaglia et al. 2013) In the Province of Modena the incidence of breast cancer amount to 29.8 % of the overall solid tumors in female gender with an increasing trend in the period 2001–2011. (Federico et al. 2013).

Here, we present a study on healthy and neoplastic breast samples collected by Modena BioBank in the period July 2011–April 2012 from 22 patients. It has two main goals: (i) to monitor the effect of freezing on the metabolic profile of tissues over time (12 months) and (ii) to verify if the metabolic information encoded in healthy and neoplastic tissues is preserved in the same period.

Healthy and neoplastic breast samples of Modena BioBank were analyzed through HR-MAS NMR 1, 6 and 12 months after collection.

2. Materials and methods

2.1. Samples collection

The study includes 22 adult female patients living in Modena and its hinterland, who underwent total mastectomy or quadrantectomy from July 2011 to April 2012 for previous cytological diagnosis of breast carcinoma. Tissue specimens were transported on ice from surgical room to the pathology department where they were immediately sampled. Three fresh samples of both neoplastic and macroscopically healthy appearing peritumoral breast tissue (2–3 cm from the tumor margins) were obtained from each patients and transferred immediately into a freezer at −80 °C for storage. The mean time interval from surgical specimen resection to freezing of samples was about 30 min.

2.2. Histopathological features

The main clinical and histopathological features of the cases are provided in Table 1. In particular the average age at diagnosis was 65 ± 16 years old (range 35–90). Tumors occurred in the right breast (15 cases) and in the left (7 cases) and included infiltrating ductal carcinoma (15 cases), infiltrating lobular carcinoma (5 cases), infiltrating mixed ductal–lobular carcinoma (1 case) and infiltrating ductal carcinoma with abundant extracellular mucin, (>50 % of the tumor component—mucinous carcinoma) (1 case). (Tavassoli and Devilee 2003). The average tumor dimension was 2.3 cm (range 1–5 cm), and the tumor grading (Elston and Ellis 1991) was G2 (moderately differentiated) in 16 cases and G3 (poorly differentiated) in 6. Twelve tumors were in stage I and 10 in stage II. (Sobin et al. 2009). Lymphovascular invasion was observed in five cases while perineural infiltration in two.

Table 1

Histological features of the 22 analyzed tumors

Age at diagnosis	Range	35–90
	Mean ± SD	65 ± 16
Gross morphology	Singular tumoral nodule	18
	Multifocal tumor	4
Surgical treatment	Quadrantectomy	5
	Quadrantectomy + axillary lumphectomy	6
	Mastectomy	8

	Mastectomy + axillary lumphectomy	3
Tumor site	Right breast	15
	Left brest	7
Tumoral histotypes	Infiltrating ductal carcinoma (IDC)	15
	Infiltrating lobular carcinoma (ILC)	5
	Mixed infiltrating ductal and lobular carcinoma (MLDC)	1
	Infiltrating ductal carcinoma with abundant extracellular mucin (>50 % of the tumor) (MUCINOUS)	1
Tumor grading	G2	6
	G3	16
Lymphovascular invasion	Presence	5
	Absence	12
Perineural infiltration	Presence	1
	Absence	11
Pathologic staging		
Tumor parameter (T)	pT1	14
	pT2	8
Nodal parameter (N)	N0	17
	N+	5

2.3. Ethical issue

All relevant ethical issues were identified and discussed with the local Ethical Committee who approved the Modena BioBank protocol for the collection of the specimens and the format of the written informed consent for the patients. All biological samples were anonymized prior to analysis. No further ethical approval was necessary to perform histological, immunohistochemical and other biochemical investigations.

2.4. HR-MAS NMR measurements

^{1}H HR-MAS NMR spectra were recorded with a Bruker Avance400 (Bruker

BioSpin, Karlsruhe, Germany) spectrometer operating at a frequency of 400.13 MHz. The instrument was equipped with a ^{1}H, ^{13}C HR-MAS probe. Before HR-MAS examination, 20 μL of D_2O were added to each sample to improve the homogeneity, the water suppression, and to add deuterium as a nucleus for the lock system. The sample was introduced in a MAS zirconia rotor (4 mm OD) maintained on ice, fitted with a 50 μL cylindrical insert to increase sample homogeneity, and then transferred into the probe cooled to 5 °C. Total time for sample preparation prior to NMR analysis was only a few minutes.

Experiments were performed at a temperature of 5 °C controlled by a Bruker cooling unit.

Samples were spun at ~~4,000 Hz~~4 kHz and three different types of one-dimensional (1D) proton spectra were acquired by using the sequences implemented in the Bruker software: (i) a composite pulse sequence (zgcppr) (Bax 1985) with 2.5 s water presaturation during the relaxation delay, 8 kHz spectral width, 32k data points, 32 scans, (ii) a water-suppressed spin-echo Carr–Purcell–Meiboom–Gill (CPMG) sequence (cpmgpr) (Meiboom and Gill 1958) with 1.5 s water presaturation during the relaxation delay, 1 ms echo time (τ) and 360 and 720 ms total spin–spin relaxation delay (2nτ), 8 kHz spectral width, 32k data points, 128–256 scans.

2.5. Data analysis

HR-MAS ^{1}H NMR CPMG data were processed by using MestReNova 8.1 (2012 Mestrelab Research S.L., Santiago de Compostela, Spain) software, by applying 1 Hz line broadening prior to Fourier transformation, manually phased and baseline corrected (third-order polynomial). The spectra are referenced to the chemical shift of choline at 3.206 ppm. Then they were binned (0.001 ppm), normalized (with respect to the total area in the range 4.20 and 2.95 ppm) and aligned in the region 4.70–2.95 ppm, obtaining 1,750 final data point sub-spectrum for each sample. The spectral intensities matrix was processed with MetaboAnalyst 2.0 (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp) (Xia et al. 2009, 2012a) and ~~p~~Principal ~~c~~Component ~~a~~Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) were run on the unscaled data. Data were filtered based on interquantile range and 1,049 variables were left for each sample.

One-way Analysis of Variance (ANOVA) was also run with MetaboAnalyst, using Fisher's Least Significant Difference method (Fisher's LSD), followed by post hoc analyses using Tukey's Honestly Significant Difference (Tukey's HSD).

Receiver Operating Characteristic Curve Explorer & Tester (ROCCET) (http://www.roccet.ca/ROCCET) (Xia et al. 2012b) software was applied on selected integrals in order to build a classification model.

The healthy CPMG (720 ms, 2nτ) dataset was formed by 56 spectra (15 spectra taken after 1 month, 21 after 6 months and 20 after 12 months). The neoplastic CPMG (720 ms, 2nτ) dataset was formed by 54 spectra (15 spectra taken after 1 month, 20 after 6 months and 19 after 12 months). Some spectra are lacking due to temporary instrumental unavailability at the scheduled times.

3. Results and discussion

3.1. NMR spectra

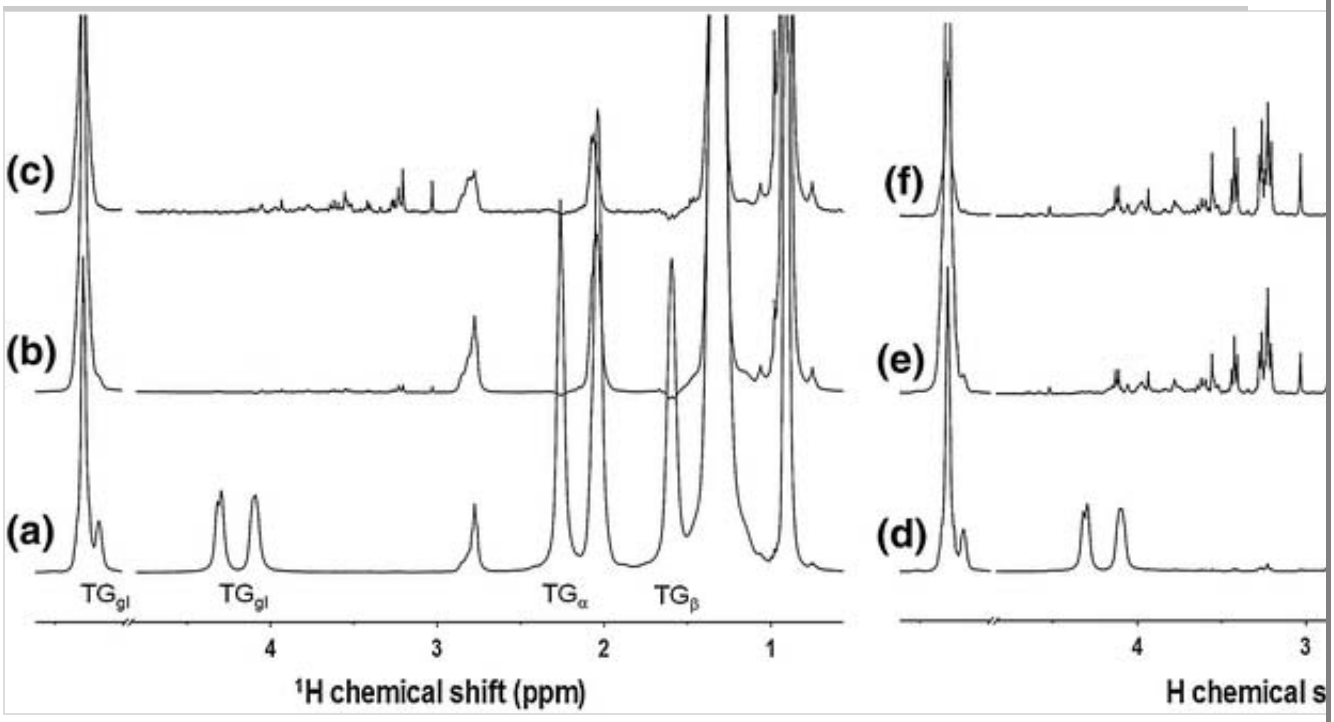
Breast tissue (especially the healthy one, see below) is rich in fat, that gives rise to very huge signals in presaturated 1D ^{1}H NMR spectrum. An experiment more suitable than the basic "pulse-and-acquire" one to detect signals from small metabolites is the CPMG sequence, that reduces triglyceride resonances. This attenuation is more efficient for signals coming from bonded glycerol (TG_{gl}) and from methylenes α and β to carbonyl in fatty acid chains (TG_α and TG_β) and it is a function of the total echo time employed in the experiment.

Figure 1 compares the standard presaturated 1D ^{1}H NMR spectra (traces a and d), obtained from a macroscopically healthy (a) and from neoplastic (d) sample, with the CPMG spectra acquired with 360 (traces b and e) and 720 ms (traces c and f) total echo times. It appears that, if the main aim is to observe changes in metabolites signal during frozen storage, only a total 720 ms allows to better detect signals between 4.7 and 2.9 ppm especially in the healthy samples. It is to be stressed that, having the protons different transverse relaxation times (T_2), this long total echo time alters the relative signal intensities for the observed metabolites with respect to those that could be found within a standard presaturated 1D ^{1}H NMR spectrum in the

absence of strong fat bands. Keeping this in mind, nothing prevents us to study how CPMG spectra of healthy or neoplastic samples, obtained at the same total echo time, change with time and to compare the spectra of healthy with those of neoplastic tissues.

Fig. 1

Healthy (*left*) and neoplastic (*right*) breast tissue: water presaturated (*a, d*) standard "pulse-and-acquire", (*b, e*) CPMG with 360 ms total echo time and (*c, f*) CPMG with 720 ms total echo time ^{1}H NMR spectra



3.2. Analysis of CPMG spectra 1, 6 and 12 months after surgery

The major changes clearly observed looking at the average CPMG spectra of frozen samples at −80 °C for 1, 6 and 12 months (reported in Fig. S1) is the enhancement of the free choline (Cho) signal at 3.21 ppm, both in healthy and neoplastic samples, and the lowering of the phosphocholine (PC) signal at 3.22 ppm, in the case of the healthy ones. These findings are in accordance with Jordan et al.'s paper (Jordan et al. 2007): they found minimal changes in metabolites during a study on prostate tissues in evaluating the metabolites concentration after 3 years storage at −80 °C. In our samples, the minimal alteration in ChoCC is consistent with their results. The increase of free Cho may be due to a small degradation of PC and glycerophosphocholine (GPC) during time, even if the mechanisms for the degradation of the phosphorylated Cho derivatives is not completely

understood. (Swanson et al. 2008).

Also Gly singlet (overlapped to a Myo signal) seems to slightly enhance in healthy samples, whereas a singlet at 3.34 ppm (labelled X, which could be methanol or trimethylamine *N*-oxide, but could not be unambiguously assigned in this study, for the signals of metabolites are not detected in HSQC spectra, due to the huge H, C correlations of TGs that causes dynamic range problems) seem to increase and then decrease with time in healthy tissue and to enhance in the neoplastic ones. Finally, a decrease of Tau is observed in tumoral samples.

3.3. Chemometric analysis of healthy and neoplastic tissues

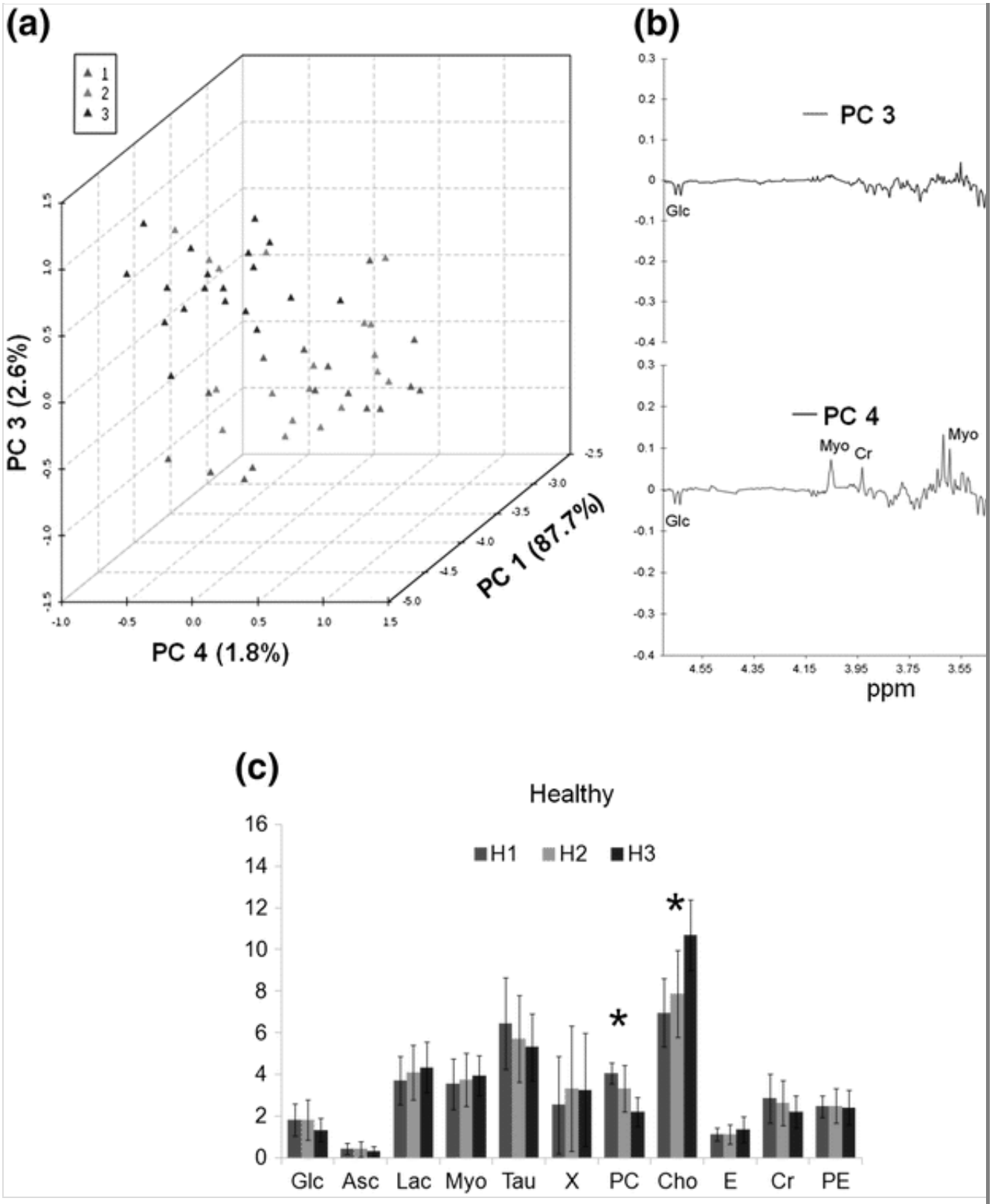
3.3.1. Healthy breast

PCA was applied to the spectra set of healthy breast samples in order to see if some time-dependent clustering occurred and to better highlight metabolite variations. The first four PC account for 95.7 % of the total variance and allow us to infer information on the metabolites that can be responsible for the observed clustering. PC1, PC3, PC4 score plot, reported in Fig. 2a, shows that some trends with time should be present in our sets, at least as far as spectra acquired after 1 and 12 months are concerned, whereas the scores of the spectra acquired after 6 months appear to be more scattered.

Fig. 2

a Scores plots (*left*) of PCA on healthy samples after 1 month (*red*), after 6 months (*green*) and after 12 months (*blue*). **b** Loadings profiles (*right*) of PC3 and PC4. **c** *Bar plots* showing the relative amounts (mean ± SD) of metabolites obtained from integrals of NMR signals in spectra of healthy samples acquired after 1 month (*red*, H1), 6 months (*green*, H2) and 12 months (*blue*, H3). *$p < 0.000001$

AQ1



The loadings profile of PC1 (Fig. S2) follows very close the global spectrum and does not help in highlighting trends over time, as does PC2 (Fig. S2) that captures mainly the variance due to X. This signal is not reported by other authors (Bathen et al. 2013; Li et al. 2011) and it is probably due to the thermal history of the samples. If we cut out X and repeat PCA analysis, we observe a very similar clustering only in the PC1, PC2, PC3 scores plot (Fig. S3).

Inspection of the loadings of PC3 and PC4 (Fig. 2a), seems to indicate that Cho, Cr, Myo, PC, X and Glc are the metabolites that vary with time in healthy samples.

In order to check which of these metabolites varies significantly among the three sets of spectra, one-way Analysis of Variance (ANOVA) and post hoc test was employed. Statistically significant differences ($p < 0.0001$) were found only for Cho at 3.21 ppm and PC at 3.22 ppm, when 1 versus 12 and 6 versus 12 months classes are compared. In the case of PC, also 1 and 6 months classes were significantly different.

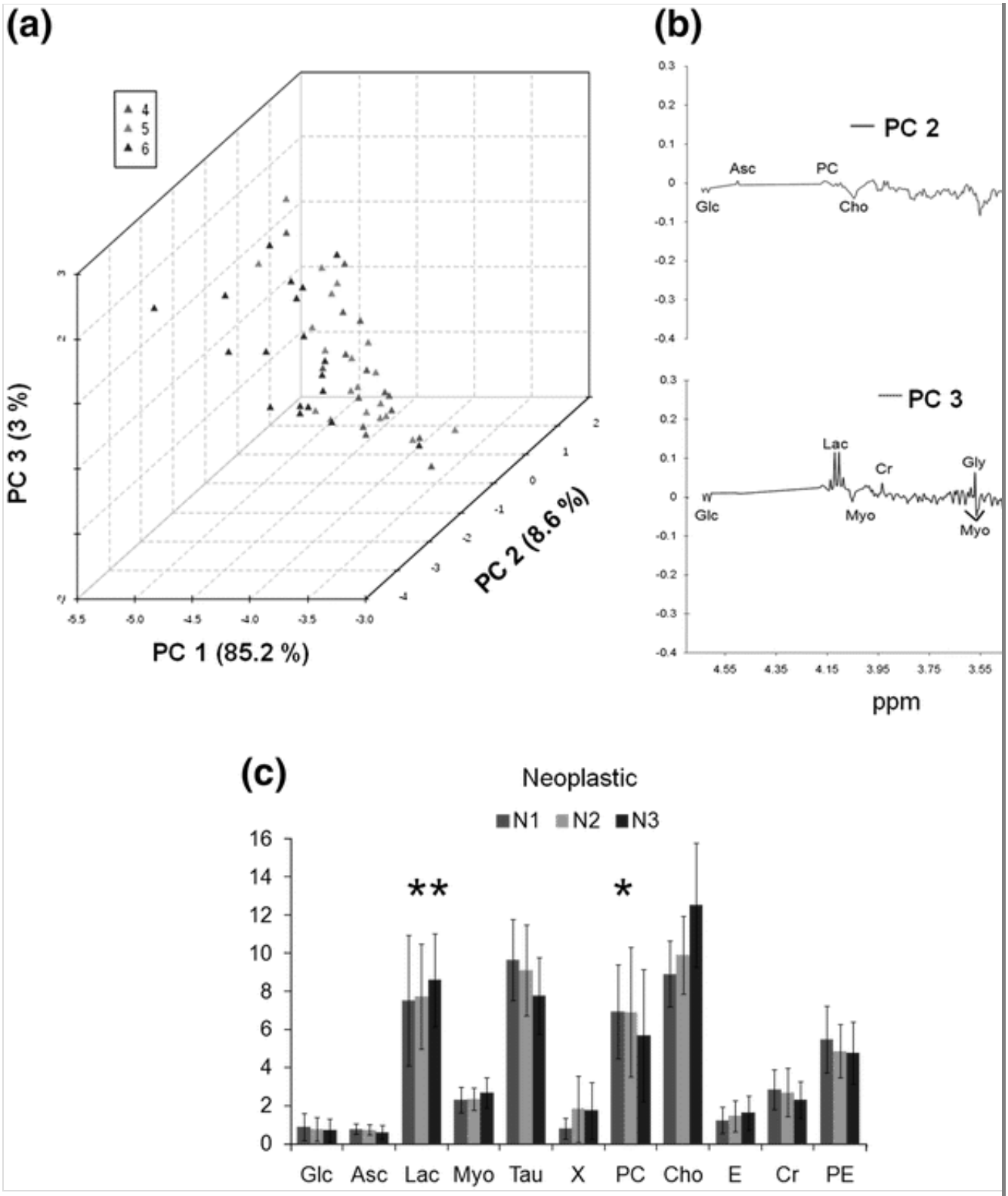
To confirm the trends observed on spectra, the signals of metabolites less overlapped, i.e. β-Glc (4.65 ppm), ascorbate (Asc 4.51 ppm), Lac (4.12 ppm), Myo (4.05 ppm), phosphoethanolamine (PE, 3.98 ppm), Tau (3.40 ppm), X (3.34 ppm), PC (3.22 ppm), Cho (3.21 ppm), ethanolamine (E, 3.13 ppm) and Cr (3.03 ppm) were integrated (Fig. 2b). Gly was not integrated for it strongly overlaps to a signal of Myo at the magnetic field employed. One-way ANOVA and post hoc test of the integrated areas showed that PC is significantly different ($-\mathrm{Log}(p) > 6$) in the three classes, whereas Cho changes are significant ($-\mathrm{Log}(p) > 6$) only when 1 versus 12 months and 6 versus 12 months classes are compared.

3.3.2. Neoplastic breast

The same approach employed for healthy samples was applied to spectra of the neoplastic ones. In this case PCA shows a less defined trend with time (Figs. 3a, S4). The first three PCs account for 95.3 % of the total variance and the principal component that seems to better describe some time-trend is PC2, the loadings profile of which is reported, together with that of PC3, in Fig. 3a (the loading of PC1 follows very close the global spectrum and it is not shown).

Fig. 3

a Scores plots (*left*) of PCA on neoplastic samples after 1 month (*red*), after 6 months (*green*) and after 12 months (*blue*). **b** Loadings profiles (*right*) of PC2 and PC3. **c** *Bar plots* showing the relative amounts (mean ± SD) of Cho obtained from integrals of NMR signals in spectra of neoplastic samples acquired after 1 month (*red*, N1), 6 months (*green*, N2) and 12 months (*blue*, N3). $*p < 0.0002$, $**p < 0.05$



The PC2 loadings profile seems to indicate that Cho, PC and Tau can be the metabolites varying more with time in neoplastic samples, whereas one-way ANOVA and post hoc test on spectra point to Cho as the only metabolite changing significantly in time, at least when comparing spectra acquired after 1 and 12 months and 6 and 12 months ($p < 0.0001$).

Integrals of selected metabolites (Fig. 3b) were computed also for normalized spectra of neoplastic samples. One-way ANOVA and post hoc