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Ex vivo metabolic fingerprinting identifies biomarkers predictive of prostate cancer recurrence following radical prostatectomy

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Background: Robust biomarkers that identify prostate cancer patients with high risk of recurrence will improve personalised cancer care. In this study, we investigated whether tissue metabolites detectable by high-resolution magic angle spinning magnetic resonance spectroscopy (HR-MAS MRS) were associated with recurrence following radical prostatectomy.

Methods: We performed a retrospective *ex vivo* study using HR-MAS MRS on tissue samples from 110 radical prostatectomy specimens obtained from three different Norwegian cohorts collected between 2002 and 2010. At the time of analysis, 50 patients had experienced prostate cancer recurrence. Associations between metabolites, clinicopathological variables, and recurrence-free survival were evaluated using Cox proportional hazards regression modelling, Kaplan–Meier survival analyses and concordance index (C-index).

Results: High intratumoural spermine and citrate concentrations were associated with longer recurrence-free survival, whereas high (total-choline + creatine)/spermine (tChoCre/Spm) and higher (total-choline + creatine)/citrate (tChoCre/Cit) ratios were associated with shorter time to recurrence. Spermine concentration and tChoCre/Spm were independently associated with recurrence in multivariate Cox proportional hazards modelling after adjusting for clinically relevant risk factors (C-index: 0.769; HR: 0.72; $P = 0.016$ and C-index: 0.765; HR: 1.43; $P = 0.014$, respectively).

Conclusions: Spermine concentration and tChoCre/Spm ratio in prostatectomy specimens were independent prognostic markers of recurrence. These metabolites can be noninvasively measured *in vivo* and may thus offer predictive value to establish preoperative risk assessment nomograms.

Despite the curative intent of radical prostatectomy for localised prostate cancer, 15–30% of men who undergo resection develop biochemical recurrence (BCR) (Pound *et al*, 1999; Ward *et al*, 2003). Although BCR does not necessarily lead to lethal disease, 34% of men who experience BCR progress to develop metastases within 8 years following radical prostatectomy (Pound *et al*, 1999).

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Novel markers capable of predicting recurrence are thus needed to identify patients eligible for treatment and active surveillance.

Current decision-making nomograms are commonly based on clinical staging, serum prostate-specific antigen (PSA), and histological findings on tissue biopsies (Canfield *et al*, 2014). Unfortunately, limitations to these parameters used in clinical practice lead to overdiagnosis of men with indolent disease, and concurrently aggressive cancers are missed. Several promising molecular tests for predicting BCR following radical prostatectomy are emerging, such as the genetic signature-based Prolaris assay (Bishoff *et al*, 2014), OncotypeDX Genomic Prostate Score (Cullen *et al*, 2015), and the Decipher genomic classifier (Erho *et al*, 2013). These tests require, however, invasive sampling by needle biopsies of significant cancer foci, which is challenging owing to the multifocality and heterogeneity of prostate cancer (Canfield *et al*, 2014), and do not have the noninvasive potential of whole-prostate characterisation, such as magnetic resonance (MR) imaging modalities that are appealing alternatives as prognostic tools.

Magnetic resonance spectroscopy (MRS) enables concurrent identification and quantification of several metabolites, which collectively constitute the metabolic fingerprint of a tissue sample (Kumar *et al*, 2016). In 2010, a retrospective *ex vivo* study displayed the potential of distinguishing non-recurrent from recurrent prostate cancers in a small cohort of patients after RP using metabolite profiles obtained by *ex vivo* high-resolution magic angle spinning MRS (HR-MAS MRS) (Maxeiner *et al*, 2010). The paper received attention as the findings suggested a potential utility of MRS-based technology preoperatively (Sciarra, 2010). The potential clinical utility of this was further demonstrated with data from our group showing correlations between metabolites identified *ex vivo* and *in vivo* using HR-MAS MRS and MRS imaging (MRSI), respectively (Selnaes *et al*, 2013). We also showed that low concentrations of spermine and citrate as well as a high (choline + creatine + polyamines)/citrate ratio were associated with high Gleason score using HR-MAS MRS (Giskeodegard *et al*, 2013). Recently, the number of polyamine-free voxels from *in vivo* MRSI) was reported to be positively associated with BCR following radical prostatectomy (Zakian *et al*, 2016).

In vivo MRSI has lower sensitivity and resolution than *ex vivo* MRS, and metabolites visible in *in vivo* prostate MRSI are usually limited to citrate, total-choline, creatine, and polyamines. The low resolution causes overlapping peaks, which hampers quantification of individual metabolites, and the lack of robust internal standards has warranted calculations of metabolite ratios. However, new developments in MRSI allow for absolute metabolite quantification (Weis *et al*, 2016), and faster and more automated protocols. Furthermore, implementation of higher magnetic field strengths and new pulse sequences can increase sensitivity (Steinseifer *et al*, 2015; Lagemaat *et al*, 2016). Thus, MRSI has the potential to play a future role in clinical practice to improve precision medicine.

In the current study, we identified prognostic metabolites predicting recurrence using *ex vivo* HR-MAS MRS on tissue samples from 110 patients treated with radical prostatectomy. We show that metabolic profiling provides prognostic information independently of clinicopathological parameters, and discuss the possibility for *in vivo* use.

MATERIALS AND METHODS

Patient selection. Patients ($n = 136$) radically operated for prostate cancer between 2002 and 2010 at Oslo University Hospital (Oslo, Norway) (OUS cohort, $n = 55$) or at St Olavs Hospital (Trondheim, Norway) (NTNU1 cohort, $n = 39$ and NTNU2 cohort, $n = 42$) were retrospectively included in this study. Patients were excluded owing to the lack of follow-up data ($n = 5$), lack of available tumour tissue ($n = 5$), unsatisfactory HR-MAS MRS spectral quality ($n = 4$), and due to administration of adjuvant and/or neoadjuvant therapy ($n = 12$), leaving a total of 110 patients eligible for analysis. Recurrent prostate cancer was defined as BCR (PSA $\geqslant 0.2$ ng ml^{-1} with a subsequent rise). If PSA measurements were missing ($n = 2$ patients), time of recurrence was set to onset of salvage radiation therapy or salvage androgen deprivation therapy.

Ethical approval of studies and informed consent. The studies were approved by the Regional Committee for Medical and Health Research Ethics (REC) (2009/1028 and 2013/1713 REC South-East and 2009/1161 and 010-04 REC Central), and informed written consent was obtained from all included patients.

Sample collection. Samples were kindly provided by the 'Registry and Biobank for Urological Diseases' (OUS cohort, $n = 55$), MR Biobank (NTNU1 cohort, $n = 39$), and Biobank1, St Olavs Hospital (NTNU2 cohort, $n = 104$). The tissue samples from the OUS cohort were collected at the Oslo University Hospital immediately after prostatectomy by excising and fresh-freezing tissue samples from areas identified as tumour by a pathologist. The NTNU1 cohort (MR Biobank) consists of tissue biopsies from St Olavs University Hospital taken within ~1–2 min after surgical removal of the prostate and immediately stored in liquid nitrogen (Hansen *et al*, 2016; Sandsmark *et al*, 2017). The biopsies (~10 mg) were aimed at cancer areas previously detected by transrectal ultrasound guided biopsies with more than 1 mm tumour. The NTNU2 cohort (Biobank 1) was collected as 2-mm fresh-frozen prostate slices from the middle of the prostate, and tissue cores (~10 mg) were extracted based on clinical histopathology on the two adjacent tissue slices (Bertilsson *et al*, 2011). Further protocols for sample collection following radical prostatectomy are described in Supplementary Materials and methods.

***Ex vivo* HR-MAS MRS.** *Ex vivo* HR-MAS MRS metabolic fingerprinting was performed as described previously (Giskeodegard *et al*, 2013) using frozen, intact tissue samples (mean weight 12.5 mg, range 3.00–21.9 mg) on a Bruker Avance DRX600 (14.1T) Spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a $^{1}H/^{13}C$ HR-MAS probe. Absolute quantification of the MR spectra was performed using LCModel (Provencher, 1993; Giskeodegard *et al*, 2013; Hansen *et al*, 2016) and reported in nmol mg^{-1} wet weight. Total choline was calculated as the sum of choline, phosphocholine, and glycerophosphocholine (GPC).

Pathology. Experimental procedures for pathological examination of the OUS cohort (AS and BK) following HR-MAS MRS spectral acquisition are described in Supplementary Materials and methods. The pathological procedures on paraffin sections (NTNU1 cohort) and cryosections (NTNU2 cohort) are described in Hansen *et al* (2016), Sandsmark *et al* (2017), and Giskeodegard *et al* (2013), respectively. In all three cohorts, percentages of cancer, benign (glandular) epithelium, and stromal tissue were reported for each sample.

Statistics. Linear mixed modelling (LMM) was applied to test each metabolite concentration or ratio as a function of recurrence status at 5 years while correcting for multiple samples per patient (R version 3.0.3, NLME package). Metabolite concentrations and ratios were Box–Cox-transformed before analysis, and the Benjamini–Hochberg method (Benjamini and Hochberg, 1995) was used to adjust for multiple testing, giving false discovery rate-corrected *P*-values (*Q*-values, Q).

Recurrence was used as the end point in survival analyses, with time to event calculated from date of radical prostatectomy to onset of recurrence. Cox proportional hazards modelling (R version 3.0.3, survival package) was performed on univariate

and multivariate models to evaluate the association of metabolites with recurrence. Clinicopathological variables were found to be significantly associated with recurrence in univariate models, and were included as covariates in multivariate models. In all analyses, Gleason scores were categorised according to the ISUP (International Society of Urological Pathology) Grade Group System (Gordetsky and Epstein, 2016) after directly converting the reported Gleason scores as follows: Gleason score 6, 7a, 7b, 8, and 9 corresponds to Grade Group 1, 2, 3, 4, and 5, respectively. The predictive accuracies of the models were tested with the Harrell's concordance index (C-index) (Harrell *et al*, 1982). To compare models with different number of covariates and investigate overfitting, leave-one-out cross-validation of C-indexes (LOOCV C-index) was performed. Leave-one-out cross-validation of C-index was performed in R v.3.03 as follows: For each individual i, we first fit a Cox model to the survival data, leaving i out in the estimation. The resulting coefficient estimates, β_{-i}, were then used together with individual i's covariate values x_i, to obtain the linear predictor $\eta_i = \beta_{-i} x_i$ for each individual i. This was done successively for each individual, thus obtaining a vector of linear predictors $\eta = \eta_1, \eta_2, \ldots, \eta_n$, where n is the number of individuals. This vector η was then used as a single covariate in the Cox proportional hazard model to calculate the C-index, which we refer to as the LOOCV C-index.

Selection of metabolites to be modelled in survival analyses was based on Q-values from LMM and the possibility of measuring by *in vivo* MRSI in its current technological state. The metabolite concentrations and ratios were $\log_2$-transformed before Cox proportional hazards modelling to obtain scale-independent hazard ratios. For all survival analyses, one sample was randomly selected for inclusion where more than one tumour sample was present for a patient (applicable only for the NTNU2 cohort). To validate the Cox proportional hazard models, 1000 computations with random selections of samples from patients with multiple available samples were run.

Decision curve analyses were performed in R (version 3.0.3, DecisionCurve package) to evaluate the multivariate models at different threshold probabilities for recurrence within the 5-year mark. Recurrence-free patients lacking 5 years of follow-up were excluded ($n = 4$). One patient lacked only 15 days to reach 5 years of follow-up, but was included nonetheless, leaving a total of 106 patients for the analyses. A basic model containing pathological variables included in the Cox proportional hazards multivariate models, a full model with spermine or (total-choline + creatine)/spermine (tChoCre/Spm), a 'treat all' function (calculated from the basic model), and a 'treat none' function were tested. Briefly, net benefit was evaluated in the threshold probability range of 0–40%, with bootstrapping ($n = 500$).

The Kaplan–Meier method was used to depict differences in recurrence-free survival among patients harbouring tumours with above or below median values of the metabolites of interest, and the Mantel–Haenszel log-rank test was used to evaluate the differences in the distributions (R v.3.03, survival package). To test the validity of the results from the OUS cohort in the two other cohorts, the cutoff points yielding the lowest log rank P-values were used (Budczies *et al*, 2012).

Student's t-tests and exact Mantel–Haenszel linear-by-linear association tests were used to test for differences in distributions of clinicopathological demographics according to recurrence, and Mann–Whitney U-tests were performed to test for metabolite levels in the OUS and NTNU cohorts individually (all three tests were performed in SPSS v.21; IBM, Chicago, IL, USA). Spearman's rank correlation analyses were performed using R (R v.3.03, Hmisc package).

Partial least-squares discriminant analysis (PLS-DA) was performed in MATLAB (Mathworks, Natick, MA, USA) and PLS-toolbox (Eigenvector Research, Manson, WA, USA) to search for systematic differences in metabolite concentrations between the cohorts. PLS-DA was performed with leave-one-out cross-validation using $n = 10\%$ of the patients ($n = 11$). Permutation testing ($n = 1000$) was performed to examine the significance of the resulting model.

RESULTS

Patient characteristics. Of the 110 patients eligible for analysis, recurrence was observed for 50 patients at the time of follow-up. The median follow-up time was 2366 days for patients without recurrence and 900 days for patients with recurrence. The recurrent group had a significantly higher proportion of patients with extraprostatic extension (pT3a), seminal vesicle invasion (⩾pT3b), and had higher Gleason grades, whereas no significant differences in age, preoperative PSA, and surgical margin status were detected. All clinicopathological characteristics of the pooled cohort are presented in Table 1.

Metabolite concentrations in non-recurrent and recurrent prostate cancers. A total of 25 metabolites were quantified from the HR-MAS MRS spectra (Figure 1 and Supplementary Table S1). The spermine concentration (the most predominant polyamine in the polyamine region; Giskeodegard *et al*, 2013) was significantly lower in the recurrent compared with the non-recurrent group ($P = 0.001$, $Q = 0.025$), whereas citrate approached statistical significance towards a decrease after correcting for multiple testing ($P = 0.007$, $Q = 0.063$). Neither choline, total choline (not shown), nor creatine were significantly different between the groups.

The tChoCre/Spm ratio was significantly higher in samples from patients who experienced recurrence within 5-years of follow-up ($P = 0.002$, $Q = 0.025$). The (total choline + creatine)/citrate (tChoCre/Cit) ratio approached statistical significance towards separating the groups ($P = 0.011$, $Q = 0.079$) (Figure 1B and Supplementary Table S1). Additionally, spermine and citrate concentrations were found to be highly correlated (Spearman's $\rho = 0.79$, $P < 0.001$) (Supplementary Figure S1).

Table 1. Clinical and pathological characteristics of included patients from the pooled cohort

	No recurrence (%)	Recurrence (%)	*P*-value
N	60	50	
Follow-up Median (IQR), d	2366 (455)	900 (1148)	
Age Mean ± s.d. (years)	61.7 ± 6	62.5 ± 5	0.42[a]
Preoperative PSA Mean ± s.d. (ng ml^{-1})	9.4 ± 5.8	10.3 ± 5.1	0.44[a]
Grade group (RP) 1 2 3 4 5	 4 (7) 39 (65) 15 (25) 2 (3) 0 (0)	 5 (10) 14 (28) 17 (34) 7 (14) 7 (14)	<0.001[b]
EPE	9 (15)	31 (62)	<0.001[b]
SVI	4 (7)	12 (24)	0.014[b]
PSM	15 (25)	21 (42)	0.12[b]
PCa-specific death	0	3 (6)	

Abbreviations: EPE = extraprostatic extension; IQR = interquartile range; PCa = prostate cancer; PSA = prostate-specific antigen; PSM = positive surgical margins; RP = radical prostatectomy; SVI = seminal vesicle invasion.
[a]Student's *t*-test.
[b]Exact Mantel–Haenszel linear-by-linear association χ^2 test.

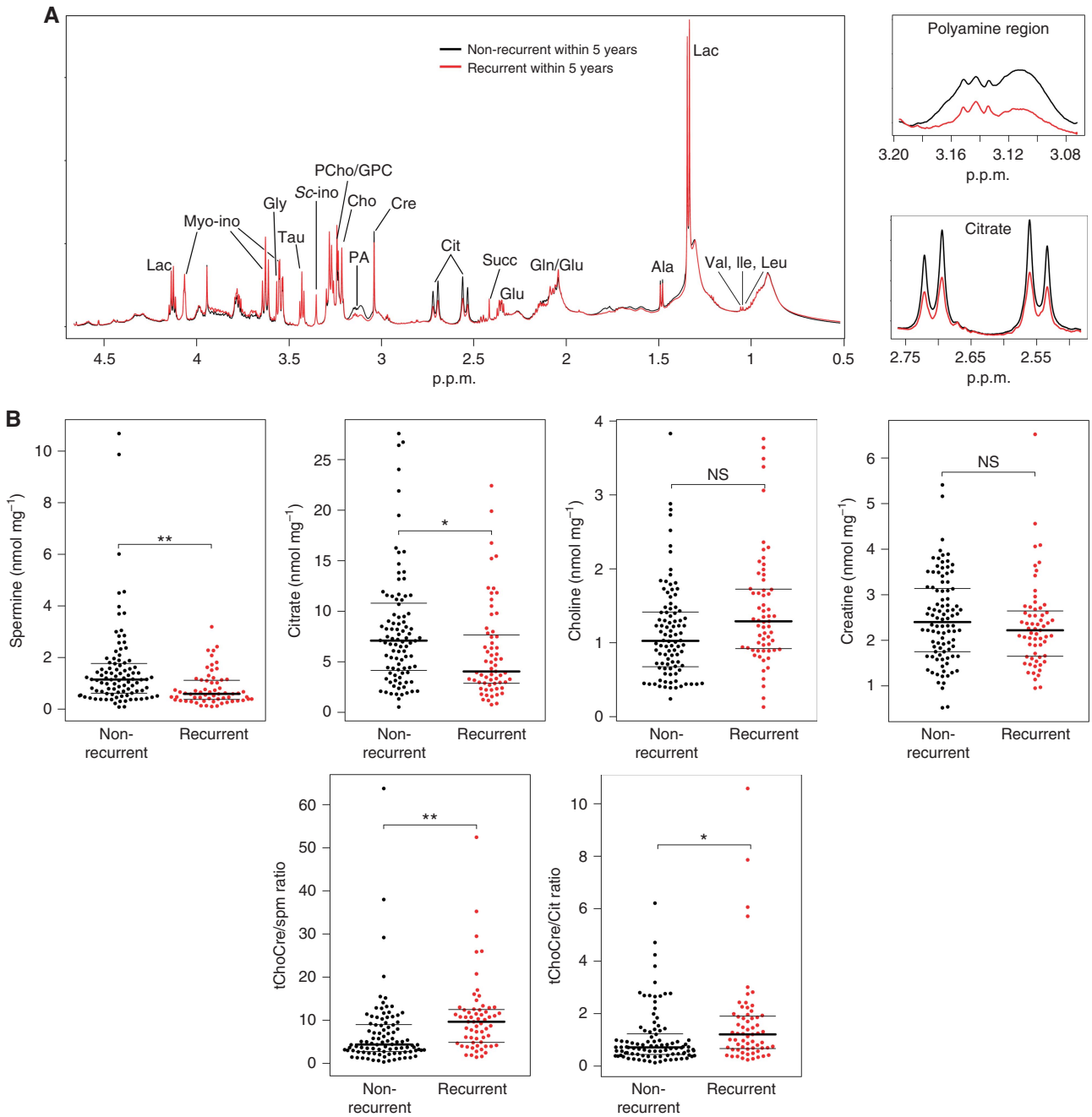

Figure 1. Metabolite levels in non-recurrent and recurrent prostate cancers at the 5-year mark. (**A**) Average HR-MAS MRS spectra from tumours from non-recurrent (black) and recurrent (red) prostate cancer groups in the NTNU1 cohort. Magnifications of the polyamine (containing spermine and putrescine) and citrate regions are shown. (**B**) Quantified peak integrals of spermine, citrate, choline and creatine (nmol mg^{-1}), and tChoCre/Spm and tChoCre/Cit ratios in the groups from all samples in all included cases from the three cohorts are shown as beeswarm plots ($n = 158$). The thin horizontal lines make out the 25% and 75% quartiles, and the median value is shown as thick black horizontal lines. Statistical significance was calculated by LMM to account for multiple samples per patient (*$P<0.05$, **$Q<0.05$). Ala, alanine; Cho, choline; Cit, citrate; Cre, creatine; Gln, glutamine; Glu, glutamate; Gly, glycine; Lac, lactate; Myo-ino, myo-inositol; NS, nonsignificant; PA, polyamines; PCho/GPC, phosphocholine and glycerophosphocholine peaks; *Sc*-ino, *scyllo*-inositol; Spm, spermine; Succ, succinate; Tau, taurine; tCho, total choline.

After stratifying patients based on median metabolite or ratio levels, patients with high levels of spermine and citrate had a longer recurrence-free survival than patients with low levels of these metabolites as shown in the Kaplan–Meier plots (Figure 2). For metabolite ratios, low ratios were associated with longer recurrence-free survival.

Predictive accuracy of metabolites for prostate cancer recurrence. Candidate metabolites with translational potential for *in vivo* MRSI were evaluated in Cox proportional hazards univariate and multivariate modelling to investigate their association with recurrence-free survival. In univariate Cox analyses, higher spermine and citrate concentrations were associated with a decreased risk of recurrence, whereas higher choline concentration, as well as the tChoCre/Spm and tChoCre/Cit ratios, were associated with an increased risk of recurrence (Table 2). The spermine concentration and the tChoCre/Spm ratio were independently associated with recurrence-free survival in multivariate

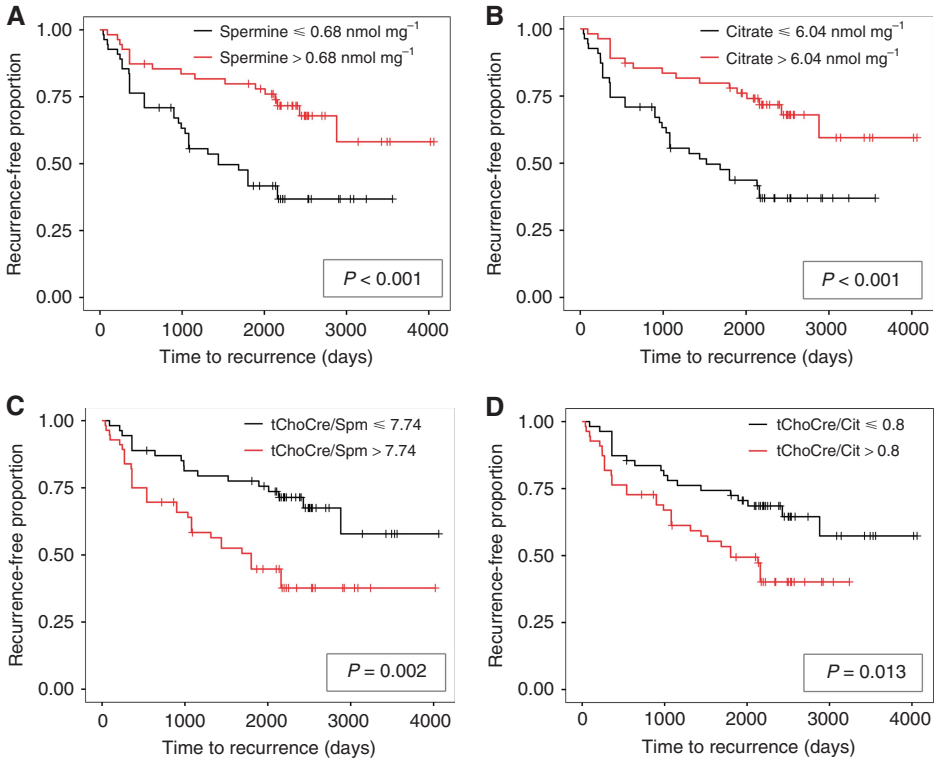

Figure 2. Kaplan-Meier curves for patients with high and low levels of metabolites and ratios. Recurrence-free proportions plotted against time to first report of recurrence for spermine (**A**), citrate (**B**), tChoCre/Spm (**C**), and tChoCre/Cit (**D**) dichotomised to above and below median concentrations. Mantel–Haenszel log-rank test was used to test for the null hypothesis of equal survival distributions. tChoCre/Cit, (total-choline + creatine)/citrate; tChoCre/Spm, (total-choline + creatine)/spermine.

models after adjusting for variables found to be predictive of recurrence in univariate models (Grade Group, EPE, SVI) (spermine model and tChoCre/Spm model; Table 2). The calculated hazard ratios were in good compliance with the output from random sampling where more than one sample was available for a patient (Supplementary Figure S2). The hazard ratio of citrate was statistically significant in the univariate Cox analysis, suggesting high citrate levels to be associated with lower risk of recurrence. However, citrate was not statistically significant in the multivariate Cox model (Supplementary Table S2). Similarly, although not statistically significant, creatine was associated with a lower risk of recurrence, whereas choline and tChoCre/Cit were associated with an increased risk of recurrence in multivariate models.

The spermine concentration and the tChoCre/Spm ratio reached univariate C-statistics (predictive accuracy) of 0.667 and 0.660, respectively, which were higher than that of seminal vesicle invasion, but not extraprostatic extension and Grade Group (Table 3). A basic clinicopathological model containing Grade Group (1–5), extraprostatic extension and seminal vesicle invasion reached a LOOCV C-index of 0.749. Adding spermine or tChoCre/Spm to the basic model increased the LOOCV C-index to 0.769 and 0.765, respectively, thus both provided additional predictive power over clinicopathological parameters alone.

Decision curve analyses visualise the net benefit of a model according to different threshold probabilities (i.e. chance of recurrence based on evaluated risk factors) at which patients or clinicians may consider performing additional prognostic tests (Vickers and Elkin, 2006). In this study, decision curve analyses revealed a positive net benefit of adding spermine to the basic clinicopathological model at decision threshold probabilities from 13 to 28% (Supplementary Figure S3). For tChoCre/Spm, the net benefit was positive in the decision threshold range 14–20%.

Variability between the three pooled cohorts. As the three cohorts were pooled, we investigated the reproducibility across the cohorts. The clinicopathological characteristics of each cohort is presented in Supplementary Table S3. The histopathological composition of the tissue samples analysed is shown in Supplementary Figure S4. The distribution of cancer tissue was highest in the NTNU2 cohort (mean 62%), which had the highest distribution of Grade Group $\geqslant 4$ samples, whereas tissue samples from the NTNU1 and OUS cohorts had less cancerous tissue (mean 38% and 35%, respectively), but higher stromal content (mean 40% and 57%, respectively). The amount of benign tissue was nonsignificantly lower in recurrent than in non-recurrent samples from the NTNU1 cohort.

Bar charts and a PLS-DA score plot describing the metabolic differences between the three cohorts are shown in Supplementary Figure S5. The median metabolite concentrations across the cohorts showed similar trends, although the median concentrations and size of interquartile ranges displayed intercohort variance. A PLS-DA model with three latent variables showed significant differences in metabolite concentrations between the cohorts ($P<0.001$) with an average classification accuracy of 92% (sensitivity = 91.7%, specificity = 92.3%). The loadings revealed that the OUS cohort was systematically different from the other two cohorts in its levels of isoleucine, leucine, glycerophosphoethanolamine and citrate. The separation of the NTNU1 cohort was characterised by its taurine, valine, glutamate and myo-inositol levels, whereas NTNU2 had differential GPC, glutamine and ethanolamine levels. The Spermine concentration did not contribute to the variance observed between the cohorts.

We tested the validity of results obtained in the OUS cohort, which was most balanced in terms of clinicopathology, in the NTNU1 and NTNU2 cohorts separately. The cutoff points for metabolite concentrations and ratios that best separated the recurrent and non-recurrent group in the OUS cohort were

Table 2. Univariate and multivariate Cox proportional hazard ratios for metabolites and metabolite ratios and prostate cancer recurrence following radical prostatectomy

	Univariate analysis		Multivariate analysis		Multivariate analysis	
			Spermine model		tChoCre/Spm model	
Variable	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Spermine (log_2)	0.63 (0.50–0.80)	<0.001	0.72 (0.55–0.94)	0.016		
tChoCre/Spm (log_2)	1.55 (1.23–1.96)	<0.001			1.43 (1.08–1.91)	0.014
Citrate (log_2)	0.67 (0.51–0.86)	0.002				
tChoCre/Cit (log_2)	1.38 (1.11–1.71)	0.004				
Choline (log_2)	1.59 (1.05–2.39)	0.028				
Creatine (log_2)	0.75 (0.48–1.19)	0.22				
Age (continuous)	1.02 (0.97–1.07)	0.42				
Preoperative PSA	1.02 (0.98–1.07)	0.329				
Grade group (RP)						
1	Reference		Reference		Reference	
2	0.38 (0.14–1.05)	0.062	0.55 (0.18–1.70)	0.30	0.53 (0.17–1.65)	0.27
3	0.92 (0.34–2.51)	0.87	0.83 (0.28–2.44)	0.74	0.73 (0.24–2.23)	0.59
4	2.12 (0.67–6.71)	0.20	2.73 (0.81–9.18)	0.11	2.58 (0.76–8.72)	0.13
5	3.01 (0.95–9.56)	0.061	2.04 (0.61–6.85)	0.25	2.00 (0.60–6.71)	0.26
EPE	4.65 (2.61–8.27)	<0.001	3.03 (1.54–5.96)	0.0013	2.98 (1.51–5.89)	0.0017
SVI	3.39 (1.76–6.54)	<0.001	1.02 (0.46–2.72)	0.95	1.06 (0.49–2.31)	0.87
PSM	1.67 (0.95–2.94)	0.074				

Abbreviations: CI = confidence interval; EPE = extraprostatic extension; HR = hazard ratio; PSA = prostate-specific antigen; PSM = positive surgical margins; RP = radical prostatectomy; SVI = seminal vesicle invasion; tChoCre/Cit = (total-choline + creatine)/citrate; tChoCre/Spm = (total-choline + creatine)/spermine.

Table 3. C-index for univariate and multivariate models including spermine and the tChoCre/Spm metabolite ratio

	Univariate analysis		Multivariate analysis		
Variable	C-index		C-index	LOOCV C-index[a]	ΔC-index
Spermine (log_2)	0.667	Full models	0.769	0.769	0.020
tChoCre/Spm (log_2)	0.660		0.765	0.765	0.016
Grade group (RP)	0.694	Basic model	0.749	0.749	
EPE	0.697				
SVI	0.595				

Abbreviations: C-index = concordance index; EPE = extraprostatic extension; LOOCV = leave-one-out cross-validated; RP = radical prostatectomy; SVI = seminal vesicle invasion; tChoCre/Spm = (total-choline + creatine)/spermine. Grade group was categorised from 1 to 5. Full model refers to models containing metabolites on top of the basic model, whereas basic model refers to the pathological model without added metabolites.
[a]LOOCV C-indexes were used to calculate the change in C-index upon adding either spermine or tChoCre/Spm to the basic model (ΔC-index).

applied to dichotomise the patient populations in the two other cohorts. Kaplan–Meier analyses showed good reproducibility for spermine and citrate, and fair reproducibility for tChoCre/Cit (Supplementary Figure S6). The cutoff value for tChoCre/Spm could not significantly separate the groups in the NTNU1 and NTNU2 cohorts.

Associations of metabolites with clinicopathology. The Spermine and the citrate concentrations were negatively correlated with Grade Group and pT-stage, whereas tChoCre/Spm and tChoCre/Cit were positively correlated with Grade Group and pT-stage (Figure 3 and Supplementary Figure S7). Using the Grade Groups from the HR-MAS-analysed tissue samples gave similar correlation coefficients as using diagnostic Grade Groups from the RP specimens (results not shown). Citrate and tChoCre/Cit had higher correlations to Grade Group than spermine and tChoCre/Spm. Extraprostatic extension was negatively correlated with spermine and citrate concentrations, whereas a positive correlation was observed for tChoCre/Spm and tChoCre/Cit. Seminal vesicle invasion was more modestly associated with the four metabolites/metabolite ratios, and none of the metabolites correlated with patient age at operation. Of note, patient age, RP Grade Group, and extraprostatic extension were all positively correlated.

DISCUSSION

In this study, we demonstrate by *ex vivo* HR-MAS MRS that both the concentration of spermine and the tChoCre/Spm ratio in radical prostatectomy specimens are independent biomarkers of recurrence. A similar trend was found for citrate concentration. A combination of metabolite concentrations and standard clinicopathological parameters gave better accuracies towards predicting recurrence than clinicopathological parameters alone, although the relative increases in predictive accuracies were modest. Furthermore, decision curve analyses revealed a net benefit of adding metabolic fingerprinting to already established risk factors, with spermine granting the greatest overall improvement in net benefit. Thus, metabolic fingerprinting may serve as an adjunct predictive parameter to established decision-making nomograms.

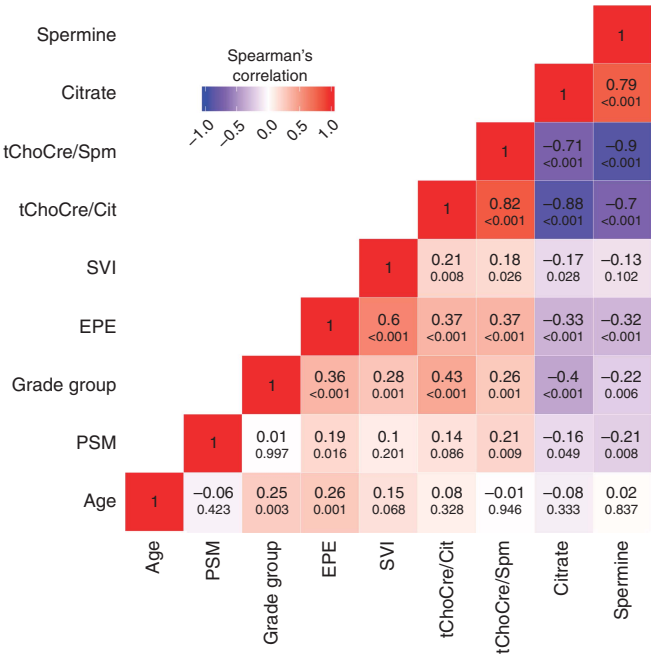

Figure 3. Correlation heatmap. Correlations between metabolites and clinicopathological factors. The Spearman's correlation coefficients are shown inside the boxes (large font), with corresponding *P*-values below. The colour scale indicates the sign of the correlation coefficients and the degree of correlation, where blue indicates negative correlation, white no correlation, and red positive correlation. EPE, extraprostatic extension; PSM, positive surgical margins; SVI, seminal vesicle invasion; tChoCre/Cit, (total-choline+creatine)/citrate; tChoCre/Spm, (total-choline+creatine)/spermine.

Spermine and citrate concentrations were the metabolites that best correlated with recurrence within 5 years following prostatectomy. The relative levels, but not concentrations, of polyamines, therein spermine, have previously been shown to predict BCR in prostate cancer (Maxeiner *et al*, 2010). In support of this, a recent *in vivo* MRSI study demonstrated that the number of voxels with undetectable levels of polyamines was associated with recurrence (Zakian *et al*, 2016). Furthermore, polyamine concentrations have been reported to be reduced in malignant compared with benign prostate tissue (Van Der Graaf *et al*, 2000), and further decreased from low to high Gleason score (Giskeodegard *et al*, 2013).

Like spermine, citrate concentrations are significantly lower in tumours with high compared with low Gleason score (Giskeodegard *et al*, 2013), likely due to loss of capacity to accumulate zinc during neoplasia and progression leading to increased inhibition of citrate accumulation (Costello and Franklin, 1997; Bertilsson *et al*, 2012). Hence, both citrate and spermine are markers for benign, glandular structures, and their levels are expected to drop during dedifferentiation (Zakian *et al*, 2016). In support of previous reports (Swanson *et al*, 2003; Giskeodegard *et al*, 2013), we observed that spermine and citrate concentrations were significantly negatively correlated with Grade Group in radical prostatectomy specimens, although the correlation for spermine was modest. Both metabolites are constituents of the seminal fluid, and are most likely produced by luminal cells due to their positive association with luminal space (Lynch *et al*, 1994; Sandsmark *et al*, 2017). The finding that spermine, but not citrate, remains independently associated with recurrence in multivariate models, may relate to spermine's lower adherence to Gleason grade. This indicates a tissue architecture-independent association of spermine with prostate cancer aggressiveness.

Both Spermine oxidase (SMOX) and Spermidine/Spermine *N*1-acetyltransferase (SAT1) are enzymes that catalyse reactions in the polyamine pathway, with reactive oxygen species as byproducts (Goodwin *et al*, 2008; Huang *et al*, 2015). Interestingly, both enzymes are believed to play a role in prostate cancer progression (Goodwin *et al*, 2008; Huang *et al*, 2015). We have previously shown that when the tissue composition was balanced between prostate cancer and normal tissue samples, a significant upregulation of SAT1 and SMOX accompanied by decreased spermine levels, but without a significant upregulation of spermine synthase was measured in cancer samples (Tessem *et al*, 2016). This fits with a significant reduction in spermine concentration in prostate cancer samples. These observations may explain the association of spermine with prostate cancer recurrence. However, the polyamine pathway is complex and involved in multiple signalling pathways in human cells (Pegg, 2009) and whether lower spermine levels relates to dedifferentiation and loss of luminal characteristics of prostate tissue should be further investigated.

Aside from the proposed utility of postoperative *ex vivo* HR-MAS MRS to predict recurrence, *in vivo* MRSI sequences may easily be added to multiparametric MR imaging (mpMRI) protocols before treatment (Shukla-Dave *et al*, 2009, 2012). Of the available mpMRI sequences, MRSI has primarily been applied in research settings because of its dependence on radiologist expertise on the field, prolonged overall scan time, and the associated costs (Loffroy *et al*, 2015). Implementation of standardised and faster MRSI protocols at higher magnetic field strengths, and computer-assisted spectral interpretation, may circumvent issues related to reproducibility of MRSI across institutions due to varied degree of radiologist training. This could, in turn, lower the overall costs of implementation of MRSI in the clinic. Importantly, studies have shown that the molecular information provided by MRSI may improve sensitivity towards detection and localisation of clinically significant prostate cancers compared with MRI alone (Wefer *et al*, 2000; Barentsz *et al*, 2012; Weinreb *et al*, 2016).

As the current study was performed on radical prostatectomy specimens, the interpretation of the results is limited to a postsurgery setting where treatment decision-making is limited to adjuvant or salvage treatment modalities. Ultimately, due to the large clinical challenge of improving the sensitivity towards identifying high-risk patients, as well as the specificity towards identifying truly indolent disease, the incremental value of adding MRSI protocols in the diagnostic setting should be investigated. Our study indicates that intratumoural metabolites may add value to clinically applied nomograms, and the translational potential from *ex vivo* HR-MAS MRS to *in vivo* MRSI has previously been demonstrated through a positive correlation between preoperative MRSI and HR-MAS MRS data from spatially matched tissue samples (Selnaes *et al*, 2013). Furthermore, the prognostic value of spermine presented in this study is in line with a previous MRSI study performed preoperatively where the authors looked at the number of polyamine-free image voxels (Zakian *et al*, 2016), indicating that spermine and possibly other polyamines may indeed predict risk for recurrence.

We hypothesise that MRSI may have a presurgical clinical applicability in detecting recurrent and aggressive characteristics in patients diagnosed with low- and intermediate-risk cancers. These patients may be offered radical treatment or adjuvant approaches such as radiation therapy and/or hormonal therapy (Zapatero *et al*, 2015), as well as extended lymph node dissection (Gordetsky and Epstein, 2016). Furthermore, mpMRI-based approaches may detect tumours not discovered by biopsies (Hambrock *et al*, 2010), and confirmation of low- or very-low-risk diagnoses may aid urologists in identifying patients eligible for active surveillance. Thus, future studies should be performed to establish the clinical utility of MRSI in prostate cancer precision medicine.

We recognise some limitations of this study. First, the cohorts collectively contain a modest number of patients, and despite the fair reproducibility observed between the cohorts, validation in larger cohorts balanced in terms of clinicopathological parameters should be conducted. Second, the retrospective design may have introduced potential confounding that we have not been able to control for.

In summary, high concentration of spermine and low tChoCre/Spm ratio, determined by HR-MAS MRS of prostate cancer tissue, were associated with shorter time to recurrence following radical prostatectomy. Both spermine and tChoCre/Spm are visible in *in vivo* MRSI, which opens for clinical translation of these candidate metabolic biomarkers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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