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RESEARCH ARTICLE

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Prediction of recurrence from metabolites and expression of TOP2A and EZH2 in prostate cancer patients treated with radiotherapy

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Background: The dual upregulation of TOP2A and EZH2 gene expression has been proposed as a biomarker for recurrence in prostate cancer patients to be treated with radical prostatectomy. A low tissue level of the metabolite citrate has additionally been connected to aggressive disease and recurrence in this patient group. However, for radiotherapy prostate cancer patients, few prognostic biomarkers have been suggested. The main aim of this study was to use an integrated tissue analysis to evaluate metabolites and expression of TOP2A and EZH2 as predictors for recurrence among radiotherapy patients.

Methods: From 90 prostate cancer patients (56 received neoadjuvant hormonal treatment), 172 transrectal ultrasound-guided (TRUS) biopsies were collected prior to radiotherapy. Metabolic profiles were acquired from fresh frozen TRUS biopsies using high resolution-magic angle spinning MRS. Histopathology and immunohistochemistry staining for TOP2A and EZH2 were performed on TRUS biopsies containing cancer cells ($n = 65$) from 46 patients, where 24 of these patients ($n = 31$ samples)

Abbreviations: BCR, biochemical recurrence; CI, confidence interval; CPMG, Carr-Purcell-Meiboom-Gill spin echo; EZH2, histone methyltransferase enhancer of zeste homolog 2; GEO, Gene Expression Omnibus; HDAC, histone deacetylase; HES, hematoxylin eosin saffron; HR, hazard ratio; HR-MAS, high resolution-magic angle spinning; IHC, immunohistochemistry; IQR, interquartile range; MALDI-TOF MSI, matrix-assisted laser desorption ionization time-of-flight imaging mass spectrometry; MRSI, magnetic resonance spectroscopy imaging; PA, polyamine; PCa, prostate cancer; PSA, prostate-specific antigen; RB, retinoblastoma; REC, Regional Committee of Medical and Health Research Ethics; RNAseq, RNA sequencing; RP, radical prostatectomy; SAT1, spermidine/spermine N1-acetyltransferase; SMOX, spermine oxidase; TCGA, The Cancer Genome Atlas; TOP2A, DNA topoisomerase IIα; TRUS, transrectal ultrasound guided.

received hormonal treatment. Eleven radical prostatectomy cohorts of a total of 2059 patients were used for validation in a meta-analysis.

Results: Among radiotherapy patients with up to 11 years of follow-up, a low level of citrate was found to predict recurrence, $p = 0.001$ (C-index = 0.74). Citrate had a higher predictive ability compared with individual clinical variables, highlighting its strength as a potential biomarker for recurrence. The dual upregulation of TOP2A and EZH2 was suggested as a biomarker for recurrence, particularly for patients not receiving neoadjuvant hormonal treatment, $p = 0.001$ (C-index = 0.84). While citrate was a statistically significant biomarker independent of hormonal treatment status, the current study indicated a potential of glutamine, glutamate and choline as biomarkers for recurrence among patients receiving neoadjuvant hormonal treatment, and glucose among patients not receiving neoadjuvant hormonal treatment.

Conclusion: Using an integrated approach, our study shows the potential of citrate and the dual upregulation of TOP2A and EZH2 as biomarkers for recurrence among radiotherapy patients.

KEYWORDS
citrate, EZH2, HR-MAS, prostate cancer, radiotherapy, recurrence, TOP2A

1 | INTRODUCTION

Radiotherapy, with or without androgen deprivation therapy (ADT), and radical prostatectomy (RP), are the most common and equivalent treatment modalities for prostate cancer (PCa).[1,2] Patients with non-metastatic PCa generally have a good prognosis, but 15-30% of men receiving radiotherapy experience recurrence.[2–4] Although recurrence has an impact on survival, it does not necessarily mean a patient will die due to PCa, and the association seems to be limited to patients with specific clinical risk factors.[5] Novel biomarkers capable of predicting aggressive, recurrent, or lethal PCa are highly needed to individualize treatment in both radiotherapy and RP patients.

Reprogramming of metabolism is recognized as a key hallmark of cancer,[6] and metabolite profiling of PCa tissue has previously revealed biomarkers for recurrence among RP patients. Specifically, decreased levels of the metabolites citrate and spermine (a polyamine) have been shown to identify aggressive PCa,[7] and further to be linked to PCa recurrence after RP.[8–10] Although citrate and spermine are promising biomarkers for recurrence in RP patients, their potential in radiotherapy patients has not been previously investigated. In addition, metabolites are proposed biomarkers for recurrence with a translational potential to in vivo magnetic resonance spectroscopy imaging (MRSI).

For PCa patients to be treated with radiotherapy, a few prognostic biomarkers have been suggested in the literature.[11] DNA topoisomerase IIα (TOP2A) and histone methyltransferase enhancer of zeste homolog 2 (EZH2) are two promising gene and protein expression biomarkers for recurrence in RP PCa patients. We will therefore investigate their potential as biomarkers for recurrence in radiotherapy patients. TOP2A is a key enzyme controlling the topologic state of DNA[12] and several studies have demonstrated TOP2A's importance in a range of cancers, including PCa.[13–15] TOP2A is vital for segregation of the replicated chromosomes at mitosis and has been found to be critical in carcinogenesis.[12,13,16,17] Abnormality of TOP2A plays a key role in chromosome instability and tumorigenesis.[12] Additionally, TOP2A interferes with anti-androgen therapy, increasing sensitivity to androgen receptor signaling,[18] and is linked to androgen resistance.[15] EZH2 is one of the most upregulated genes in aggressive PCa and is correlated to invasiveness, metastasis and poor patient prognosis.[19–24] EZH2, an epigenetic enzyme and the catalytic subunit of the polycomb repressive complex 2 (PRC2), works in concert with histone deacetylases (HDACs) as epigenetic modifiers.[25] Epigenetic regulators may mediate resistance to radiotherapy through several mechanisms,[26] and HDAC inhibitors can increase PCa radiosensitivity.[27] High expression of EZH2 in PCa tissue was recently associated with increased risk of metastasis after radiotherapy.[28] Further, a previous study found the dual upregulation of TOP2A and EZH2 ($TOP2A^+/EZH2^+$) to identify a subgroup of patients with a high rate of recurrence among RP patients.[29]

The overall aim of this study was to investigate whether an integrated analyses of tissue metabolites and expression of TOP2A and EZH2 could provide relevant biomarkers for recurrence among PCa patients scheduled for radiotherapy. Metabolite data, histopathology and immunohistochemistry (IHC) staining for TOP2A and EZH2 proteins were measured on the exact same samples using our previously published strategy for integration of multiplatform data, and patients were followed up to 11 years.[7,30–33] Additionally, we assessed the potential of TOP2A and EZH2 as biomarkers for recurrence through a meta-analysis combining gene expression data from more than 2000 RP patients in publicly available databases.

2 | EXPERIMENTAL

2.1 | Radiotherapy cohort

To investigate the potential of metabolites, TOP2A and EZH2 in radiotherapy patients, a total of 172 transrectal ultrasound-guided (TRUS) needle biopsy samples (18 G, 1.2 mm) were collected from 90 patients scheduled for radiotherapy at St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway, between 2008 and 2011. Specifically, during implantation of gold seeds (gold fiducial markers used for fine-tuning the position of the treating x-ray beam), two additional TRUS-guided needle biopsy samples were obtained for this study (one or two samples per patient) (Figure 1). Gold seed implantation and sample collection were performed 1-2 weeks before radiotherapy, where the previous clinical TRUS-guided biopsies were used as a guide to target the cancer. In general, patients were scheduled for radiotherapy if they were not eligible for RP due to, eg, presence of comorbidities or locally advanced disease. This study was initiated before recommendations following the randomized SPCG Study 7 (radiotherapy with or without hormones)[34] were implemented. Therefore 62.9% ($N = 56$ patients) of the patients in the radiotherapy cohort received neoadjuvant hormonal treatment (median duration: 6 months). All patients received a total radiation dose of 78 Gy, except four patients receiving a total radiation dose of 39 Gy and one patient with no information regarding total radiation dose. Patients were followed up to 11 years, and clinical data were collected through inspection of hospital records. Gleason scores were categorized according to the ISUP (International Society of Urological Pathology) Grade Group System[35] after directly converting the reported Gleason scores: specifically, Gleason scores 6, 7a (3 + 4), 7b (4 + 3), 8 and 9-10 were converted to Grade Groups 1, 2, 3, 4 and 5, respectively. Collection and analysis of the samples from the radiotherapy cohort were approved by the Regional Committee of Medical and Health Research Ethics (REC) (approval no 2017/576), Central Norway. All patients gave written, informed consent.

2.1.1 | HR-MAS MRS and metabolite quantification

Proton high resolution-magic angle spinning (HR-MAS) MRS analysis was performed on the two TRUS biopsies collected during implantation of gold seeds from patients in the radiotherapy cohort. HR-MAS MRS spectra were acquired on a Bruker Avance III 600 MHz spectrometer (Bruker

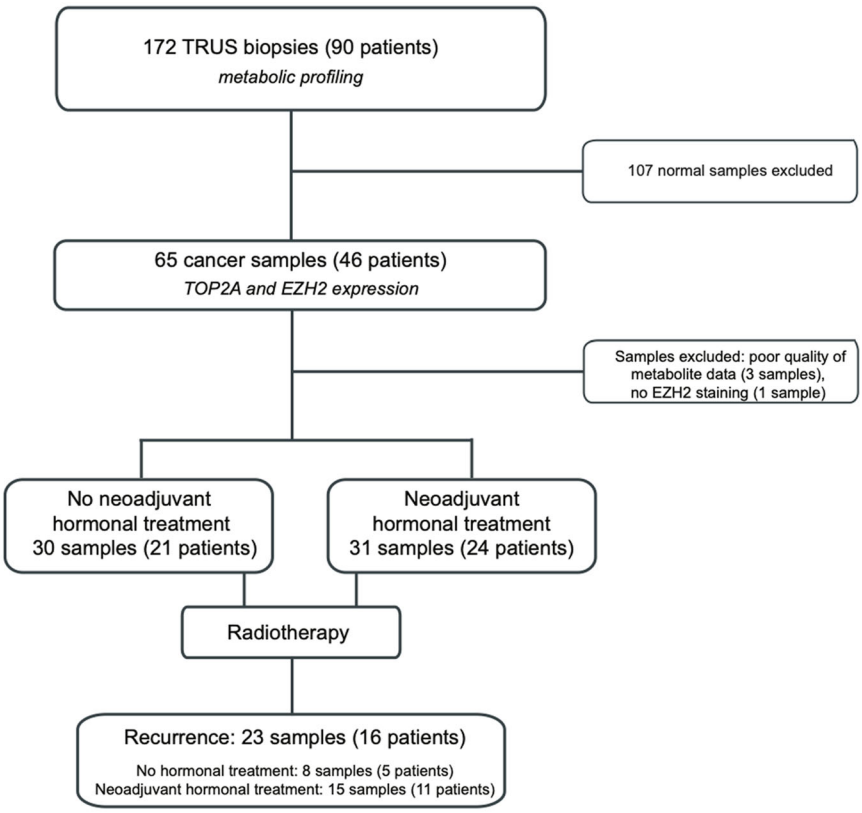

FIGURE 1 Flow chart of PCa patients included in the radiotherapy cohort. In total, 172 TRUS biopsies were sampled from 90 PCa patients scheduled for radiotherapy

BioSpin, Rheinstetten, Germany) equipped with a ^{1}H/13C MAS probe. A one-dimensional nuclear Overhauser effect spectroscopy pulse sequence ("noesygppr1d": spectral width 18 kHz, 96 k data points, relaxation delay 4 s with water suppression and 128 scans) and Carr-Purcell-Meiboom-Gill spin echo (CPMG) sequences ("cpmgpr1d": $[90^{\circ}\text{-}(\tau\text{-}180^{\circ}\text{-}\tau)_n]$, effective echo time 77 ms, spectral width 12 kHz, 72 k data points and 256 scans) were acquired at 5 °C with 5 kHz spin rate. Each biopsy was cut at both ends in order to avoid the inclusion of extra-prostatic tissue. The acquired spectral data were Fourier transformed with a line broadening of 0.30 Hz, and baseline and phase corrected using TopSpin (TopSpin 3.6, Bruker Biospin).

The relative amounts of 21 metabolites were estimated by integration of metabolite peaks of CPMG spectra (presat/noesy for polyamines) using MATLAB (R2019a, MathWorks, Natick, MA), and normalized by the weight of the sample. Metabolite peaks used for estimating the metabolite levels were guided by previous work in our group[7,9,32] (Supplementary Figure S1). Prior to integration, the spectra were further baseline adjusted (due to high levels of macromolecules/lipids) using a rolling ball algorithm from the baseline package in R (R Version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria), and aligned using the icoshift algorithm[36] in MATLAB.

Two metabolite ratios, transferable to metabolite ratios reported from in vivo prostate MRSI, were calculated based upon the integrated values using the HR-MAS MRS data: tChoCre/Cit = (total choline + creatine)/citrate and tChoCre/PA = (total choline + creatine)/polyamines. Three CPMG spectra from cancer samples were excluded prior to peak integration due to high levels of lipids (two samples) and high levels of exploration cream contaminants (one sample). In total, 62 cancer samples had CPMG spectra of sufficient quality, based on visual inspection of the spectra.

2.1.2 | Histopathology

After HR-MAS analysis, the exact same tissue biopsies as used in the HR-MAS analyses were formalin fixed, paraffin embedded and longitudinally sectioned both for hematoxylin eosin saffron (HES) and later IHC. One HES-stained tissue section from each biopsy sample was evaluated by an experienced uropathologist with regard to tissue composition (cancer, benign epithelia and stroma content) and Gleason score. Biopsy Gleason scores were directly converted to Grade Groups as previously described.

2.1.3 | Immunohistochemistry

IHC was performed on sections from the formalin-fixed and paraffin-embedded biopsy tissue containing cancer cells (hereafter termed cancer samples) using mouse monoclonal antibodies against TOP2A (Dako, Glostrup, Denmark, Clone SWT3D1, linker + 1:500) and EZH2 (Novocastra, Leica Biosystems, Newcastle, UK, 1:100), and counter-stained with hematoxylin (Figure 2). The IHC sections were evaluated using an H-score based scoring system (1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)) for TOP2A (nuclear) and EZH2 (nuclear). Scoring was guided by a pathologist experienced in IHC scoring and was performed blinded to patient characteristics and recurrence status. One sample detached from the glass slide during EZH2 staining and was excluded.

2.2 | Open access cohorts and NTNU/St. Olavs Hospital, Trondheim University Hospital RP cohort (NTNU2)

To validate the potential of TOP2A and EZH2 as potential biomarkers for PCa recurrence, we collected data from PCa cohorts with available gene expression or RNA sequencing (RNAseq) and recurrence data. As open access data from radiotherapy PCa patients could not be identified, data from 10 RP PCa cohorts from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) were included in the analyses. Eight of

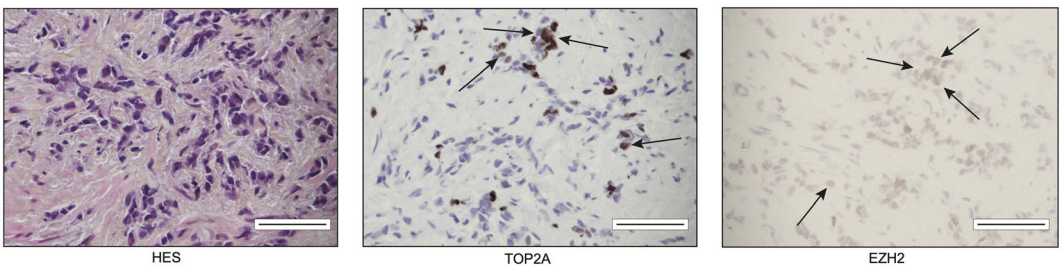

FIGURE 2 IHC staining of TOP2A and EZH2 in the radiotherapy cohort. Example showing sample classified as TOP2A^{+} and EZH2^{+}. Scale bar: 50 μm

these data sets have previously been collected as part of a previous study in our group.[37] Further, two additional cohorts were included. To summarize, the following cohorts were included in this study: Erho et al (GSE46691),[38,39] CAM (Cambridge) Ross-Adams et al (GSE70768),[40] STK (Stockholm) Ross-Adams et al (GSE70769),[40] Wang et al (GSE8218),[41–43] Sboner et al (GSE16560),[44] Taylor et al (GSE21035/32),[45] Mortensen et al (GSE46602),[46] Fraser et al (GSE84043),[47] Wu et al (GSE44353)[48] and TCGA PRAD.[49,50] Additionally, we included gene expression data from cancer samples from our NTNU/St. Olavs Hospital, Trondheim University Hospital RP cohort (hereafter and in previous publications named NTNU2).[9,30] Details regarding sample collection, processing and ethical approval of the NTNU2 cohort have previously been published by Bertilsson et al.[30] In total, 2137 cancer samples from 2059 patients were included in the meta-analyses.

2.3 | Statistical analysis

In the radiotherapy cohort, statistical analyses were restricted to cancer tissue samples. In total, 65 samples from 46 patients contained cancer cells. Four samples were excluded due to missing metabolite or IHC data, leaving 61 cancer samples from 45 patients to be included in the statistical analyses. Recurrence (with or without metastasis) was used as the end-point in the survival analysis, and defined as prostate-specific antigen (PSA) ≥ 2 ng/mL above the "nadir" level (lowest measurement after radiotherapy). One individual later developed bladder cancer and had a cystoprostatectomy, and in this case recurrence was defined as PSA ≥ 0.2 ng/mL. Time to event was calculated as the time in months between the date of the last radiotherapy (except for one individual with no information regarding the date for the last radiotherapy treatment, where the start-up date for radiotherapy was used instead) and the date of the PSA measurement indicating recurrence. Data regarding recurrence were obtained through inspection of hospital medical records, as individuals with potential recurrence of disease are referred to St. Olavs Hospital, Trondheim University Hospital, for follow-up and treatment. Individuals with no indication of recurrence were therefore assigned to the date of last data collection, or date of death (not due to PCa), as their last time-point in survival analyses.

Differences in rates of recurrence for dichotomized metabolite levels and IHC staining scores were estimated using Kaplan-Meier survival analysis, and survival curves were compared with the Mantel-Haenszel log-rank test. Metabolite levels and IHC staining scores were dichotomized into low versus high categories using the MaxStat package in R. This procedure identifies the optimal cut-off by finding the maximal log-rank statistics for cut-points between the 20% and 80% percentiles of metabolite levels or IHC staining scores. Samples were classified as $TOP2A^+$ and $EZH2^+$ if expression levels were higher than the identified cut-off values, and as $TOP2A^-$ and $EZH2^-$ if expression levels were lower than the identified cut-off values. A dual upregulation of TOP2A and EZH2 (ie upregulation of both TOP2A and EZH2 in the same tissue sample, hereafter termed $TOP2A^+/EZH2^+$) was present if the expression levels of both TOP2A and EZH2 were above their respective cut-offs in the same sample, ie classified as both $TOP2A^+$ and $EZH2^+$. For statistical analyses, $TOP2A^+/EZH2^+$ samples were compared with all other cancer samples without this dual upregulation, ie samples classified as $TOP2A^-/EZH2^-$, $TOP2A^-/EZH2^+$ and $TOP2A^+/EZH2^-$.

We assessed the univariate association between metabolite levels, metabolite ratios and dichotomous IHC staining score and recurrence using the Cox proportional hazards regression model and applied the multivariable Cox proportional hazards regression model to adjust for potential confounders. We investigated the association between clinical covariates, specifically tumor status (cT), PSA at diagnosis, neoadjuvant hormonal treatment, age at diagnosis, total radiation dose, histopathological classified Grade Groups of the tumor and the biopsies, and cancer, stroma and benign tissue content of the cancer samples, and recurrence. Clinical and histopathological variables found to be significantly associated with recurrence in univariate models (ie cT3a, cT3b and PSA at diagnosis) were included as covariates in multivariable models. The metabolite levels and metabolite ratios were mean centered and scaled (z-score) prior to Cox proportional hazards modelling. The predictive accuracies of models were tested using the concordance index (C-index), which is a generalization of the area under the ROC curve (AUC) used for survival analysis data. Statistical analyses were performed using all available cancer samples in the radiotherapy cohort, due to the moderate number. However, to validate our findings and check the robustness of the results, analyses were repeated selecting one random sample per patient, and the mean of 100 iterations was reported. Metabolite levels were $\log_{10}$ transformed prior to statistical analyses. Histograms and Q-Q plots were used to check the normality assumption and small deviations were accepted due to the robustness of the tests.

Decision curve analyses were performed to visualize the net benefit of a model according to different threshold probabilities (ie chance of recurrence based on evaluated risk factors) at which clinicians may consider performing additional prognostic tests.[51] A basic (baseline) model containing PSA at diagnosis, cT3a and cT3b (associated with recurrence in univariate models) was compared with full models including the biomarkers identified as most promising in the radiotherapy cohort (A, citrate; B, TOP2A and EZH2; C, citrate, TOP2A and EZH2), using a "treat all" function calculated from the basic model and a "treat none" function. The net benefit was evaluated in the threshold probability range of 0-40% with bootstrapping ($n = 500$).

Independent sample t-test (unequal variance, two tailed) was used for comparisons of differences in metabolite levels, metabolite ratios and IHC staining scores between samples from patients receiving and not receiving neoadjuvant hormonal treatment. Selected p-values were corrected for multiple testing using the Benjamini-Hochberg procedure[52] and corrected p-values (q-values) are presented in respective tables.

Open access cohorts with gene expression data and end-point data were included in a random-effects model meta-analysis, to account for potential heterogeneity between studies. Gene expression levels were $\log_2$ transformed, while RNAseq data were transcripts per million (TPM) normalized (ie normalized for total intensity per sample) prior to analyses and dichotomized into high versus low groups using the median expression value among cancer samples in the respective cohort as a cut-off. When more than one microarray probe for TOP2A or EZH2 existed in a cohort, the probe with the highest variance was chosen for statistical analyses. For the two cohorts with multiple samples per patients (the NTNU2 and Wang et al cohorts), the meta-analyses were performed selecting one sample per patient. Specifically, one random sample was selected and the mean of 100 iterations was calculated and applied in the meta-analyses. Due to no data on time to event in the Erho et al and Wu et al cohorts, logistic regression was used for analyses of these, and results were only included for comparisons in forest plots. Further, one cohort (Sboner et al) had PCa death as the end-point, and was not included in the calculations, but presented in forest plots for comparisons.

Statistical analyses were performed using Stata (StataCorp, College Station, TX, Stata 16), and R (R Version 3.5.1, R Foundation for Statistical Computing). *p*-values less than 0.05 were considered statistically significant, unless otherwise stated.

3 | RESULTS

In the radiotherapy cohort, 172 TRUS biopsies were obtained from 90 patients (Figure 1). In total, 65 samples from 46 patients contained cancer cells (cancer samples). Four samples were excluded due to missing metabolite or IHC data, leaving 61 cancer samples from 45 patients to be included in the statistical analyses. Cancer samples had a median sample weight of 5.1 mg (inter-quartile range (IQR): 4.1-6.1 mg) and a median cancer content of 15% (IQR: 10-20%). Patients with recurrence had a median follow-up time of 7.0 years (range: 1.1-10.5 years), while patients not experiencing recurrence had a median follow-up time of 9.9 years (range: 4.6-11.4 years). Among 45 patients with cancer samples and metabolite and IHC data, 16 individuals experienced recurrence (12 patients developed metastases during the follow-up time of the study). Characteristics of the radiotherapy cohort are presented in Table 1 and as a flow chart in Figure 1.

3.1 | The predictive value of tissue biomarkers in cancer biopsies from radiotherapy patients

In cancer-containing biopsies, including samples from patients both with and without hormonal treatment, differences in time until recurrence between dichotomous high and low levels of metabolites were found for seven of 21 metabolites (citrate, polyamines, glutamine, glutamate, isoleucine, choline and creatine) after correction for multiple testing, $q < 0.05$ (Supplementary Table S1). No difference in dichotomous high versus low expression of the IHC biomarkers TOP2A and EZH2, individually, were found, $p > 0.05$, while the combined biomarker TOP2A$^+$/EZH2$^+$ was associated with a shorter recurrence-free time, $q < 0.05$. Kaplan-Meier plots of citrate, polyamines and TOP2A$^+$/EZH2$^+$ are presented as Figure 3. Box plots of citrate, polyamines, TOP2A and EZH2 are presented in Supplementary Figure S2.

In univariate Cox proportional hazard models (Table 2 and Supplementary Table S2), decreased levels of the metabolites citrate (HR 0.52, 95% confidence interval (CI) 0.35-0.78, $p = 0.001$), polyamines (HR 0.61, 95% CI 0.40-0.92, $p = 0.019$), choline (HR 0.58, 95% CI 0.37-0.90, $p = 0.016$) and creatine (HR 0.55, 95% CI 0.35-0.87, $p = 0.011$) were associated with shorter time until recurrence in radiotherapy PCa patients. Additionally, increased levels of the metabolite ratio tChoCre/Cit (HR 1.63, 95% CI 1.13-2.35, $p = 0.009$) and TOP2A$^+$/EZH2$^+$ (HR 2.82, 95% CI 1.15-6.87, $p = 0.023$) were associated with a shorter time until recurrence. The metabolite ratio tChoCre/PA was borderline significantly associated with recurrence (HR 1.49, 95% CI 0.99-2.24, $p = 0.057$). When comparing the predictive ability of metabolites and IHC biomarkers, we found the level of citrate to have the highest predictive ability (C-index = 0.74). Results for the other measured metabolites, TOP2A and EZH2 are presented in Supplementary Table S2.

3.2 | Combining metabolites and IHC biomarkers as predictors for recurrence

We further investigated if combining expression levels of TOP2A and EZH2 with metabolites could yield a better prediction for recurrence in cancer samples. We focused on metabolites significantly associated with recurrence (citrate, polyamines, choline and creatine) and combined them with TOP2A$^+$/EZH2$^+$. Low citrate/TOP2A$^+$/EZH2$^+$ and low polyamines/TOP2A$^+$/EZH2$^+$ were associated with a shorter time until recurrence, both $p = 0.007$. Although these two combined biomarkers were significantly associated with recurrence, they had a lower predictive ability than citrate alone, and a comparable predictive ability to TOP2A$^+$/EZH2$^+$, with a C-index of 0.60. Combining TOP2A$^+$/EZH2$^+$ with creatine was borderline significantly associated with recurrence, $p = 0.054$, while no association was found when combined with choline, $p > 0.05$ (Supplementary Table S2).

TABLE 1 Clinical characteristics of the radiotherapy cohort ($n = 45$ patients, 61 cancer samples, included in the statistical analyses)

	No recurrence	Recurrence
Patients	29	16
Follow-up time (median (range), months)	119 (55-137)	85 (13-126)
Age (median (range), years)	69.0 (57-77)	66.0 (45-76)
PSA at diagnosis (median (range), ng/mL)	11.3 (5.1-29)	24.0 (6.2-51)
Tumor status (cT)*		
cT1	3	2
cT2	7	1
cT3	19	12
cT3a	*15*	*2*
cT3b	*1*	*8*
Gleason group**		
1	1	1
2	12	3
3	2	2
4	10	4
5	4	4
Received neoadjuvant hormonal treatment	13	11
Cancer samples	38	23
Grade Groups (biopsies)		
1	6	4
2	21	12
3	5	1
4	4	4
5	2	2
Tissue composition		
Cancer content (median (range), %)	15.0 (2-50)	12.0 (3-40)
Stromal content (median (range), %)	75.0 (40-88)	80 (60-95)
Benign epithelia (median (range), %)	7.5 (0-35)	5 (0-20)

*One patient had missing data on tumor status (cT).
**Two patients had missing data on Gleason.

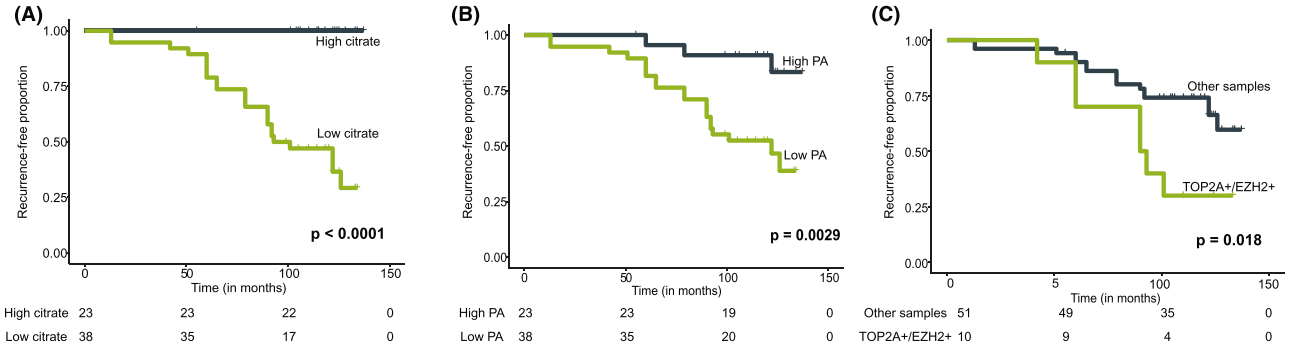

FIGURE 3 Kaplan-Meier plots of high and low levels of citrate (A) and polyamines (PA) (B) and of $TOP2A^{+}/EZH2^{+}$ versus other cancer samples (ie $TOP2A^{-}/EZH2^{-}$, $TOP2A^{+}/EZH2^{-}$, $TOP2A^{-}/EZH2^{+}$) (C). *p*-values from log-rank test

TABLE 2 Univariable and multivariable Cox proportional hazard analysis of selected metabolite levels (continuous) and IHC biomarkers (dichotomous) in cancer samples in the radiotherapy cohort. Multivariable model adjusted for PSA at diagnosis and tumor status cT3a and cT3b. A complete table of all results is presented as Supplementary Tables S2 and S5. *$p < 0.05$

	Univariable model ($n = 61$ samples)			Multivariable model ($n = 59$ samples)		
Biomarker	**HR (95% CI)**	**C-index**	***p*-value**	**HR (95% CI)**	**C-index**	***p*-value**
Metabolites						
Citrate	0.52 (0.35-0.78)	0.74	0.001*	0.77 (0.47-1.30)	0.83	0.296
PA	0.61 (0.40-0.92)	0.67	0.019*	0.81 (0.49-1.30)	0.82	0.396
IHC biomarkers						
TOP2A	1.79 (0.76-4.24)	0.56	0.183	0.98 (0.34-2.84)	0.82	0.970
EZH2	2.23 (0.94-5.27)	0.57	0.068	3.26 (1.20-9.11)	0.82	0.024*
$TOP2A^+/EZH2^+$	2.82 (1.15-6.87)	0.59	0.023*	2.78 (0.98-7.89)	0.82	0.054
Combinations						
Low citrate/$TOP2A^+/EZH2^+$	3.47 (1.42-8.51)	0.60	0.007*	3.03 (1.05-8.79)	0.83	0.041*
Low PA/$TOP2A^+/EZH2^+$	3.47 (1.42-8.51)	0.60	0.007*	3.03 (1.05-8.79)	0.83	0.041*

3.3 | The influence of multiple samples per patient

We performed repeated analyses randomly selecting one sample per patient, to account for the potential bias due to multiple samples per patient. In these analyses, citrate, tChoCre/Cit, tChoCre/PA and $TOP2A^+/EZH2^+$ were significant predictors of recurrence, $p < 0.05$ (Supplementary Table S3). These results indicate citrate, tChoCre/Cit, tChoCre/PA and $TOP2A^+/EZH2^+$ to be robust biomarkers for recurrence.

3.4 | The influence of clinical covariates

Among the clinical and histopathological covariates investigated in this study, PSA at diagnosis and cT3 status (specifically cT3a and cT3b) were associated with recurrence, $p < 0.05$ (Supplementary Table S4) and were included as covariates in the multivariable models. Although the hazard ratio (HR) estimates were only moderately attenuated, the individual metabolites were not associated with recurrence in the multivariable models, eg citrate HR 0.77, 95% CI 0.47-1.30, $p = 0.30$, potentially due to lack of statistical power. However, EZH2 and the combined biomarkers low citrate/$TOP2A^+/EZH2^+$, low polyamines/$TOP2A^+/EZH2^+$ and low creatine/$TOP2A^+/EZH2^+$ were independent biomarkers for recurrence in these multivariable models, $p < 0.05$ (Supplementary Table S5). $TOP2A^+/EZH2^+$ was associated with recurrence in a multivariable model, although only borderline statistically significant (HR 2.78, 95% CI 0.98-7.89, $p = 0.054$) (Table 2 and Supplementary Table S5). Despite not being statistically significant in multivariable models, the predictive ability of citrate was higher than that of the clinical variables individually (Supplementary Tables S2 and S4). As PCa tissue is heterogeneous, repeated analyses were performed to adjust for tissue composition (benign, stroma and cancer content). However, this did not change the obtained results substantially (Supplementary Table S6).

In decision curve analyses a positive net benefit was found on adding citrate to a basic model (including PSA at diagnosis, cT3a and cT3b) at decision threshold probabilities from about 13 to 28%, while on adding TOP2A and EZH2 the net benefit was positive in the decision threshold range of approximately 13 to 19% (Supplementary Figure S3).

3.5 | The influence of hormonal treatment on tissue biomarkers for recurrence

We further explored the potential of tissue metabolites and IHC biomarkers in cancer samples as predictors for recurrence in patients receiving and not receiving neoadjuvant hormonal treatment. In patients receiving neoadjuvant hormonal treatment (total androgen blockade, $N = 23$ patients; anti-androgens, $N = 1$ patient; $n = 31$ samples), low levels of citrate (HR 0.54, 95% CI 0.31-0.93, $p = 0.027$), glutamine (HR 0.54, 95% CI 0.31-0.95, $p = 0.033$), glutamate (HR 0.50, 95% CI 0.27-0.92, $p = 0.027$) and choline (HR 0.48, 95% CI 0.25-0.90, $p = 0.023$) were associated with a shorter time until recurrence (Supplementary Table S2).

Among patients not receiving neoadjuvant hormonal treatment ($N = 21$ patients, $n = 30$ samples), a low level of glucose in cancer biopsies was a significant predictor of recurrence (HR 0.41, 95% CI 0.18-0.90, $p = 0.027$), while a decreased level of citrate approached significance (HR 0.56, 95% CI 0.30-1.00, $p = 0.061$). Additionally, $TOP2A^+/EZH2^+$, TOP2A and EZH2 were significantly associated with recurrence among patients not receiving neoadjuvant hormonal treatment (HR 14.3, 95% CI 2.83-72, $p = 0.001$; HR 7.09, 95% CI 1.40-35.3, $p = 0.017$, and HR

19.5, 95% CI 2.40-160, $p = 0.006$, respectively). In comparison, among patients receiving neoadjuvant hormonal treatment, TOP2A$^+$/EZH2$^+$, TOP2A and EZH2 were not significantly associated with recurrence (HR:1.61, 95% CI 0.51-5.07, $p = 0.42$; HR 1.47, 95% CI 0.50-4.32, $p = 0.48$, and HR 1.54, 95% CI 0.56-4.28, $p = 0.40$, respectively). Among patients not receiving neoadjuvant hormonal treatment, TOP2A$^+$/EZH2$^+$, EZH2 (both with a C-index = 0.84) and the combined biomarkers low citrate/TOP2A$^+$/EZH2$^+$ and low polyamines/TOP2A$^+$/EZH2$^+$ (both with a C-index = 0.86) had the highest predictive ability among all biomarkers in the present study (Supplementary Table S2).

This suggests a promising potential of tissue expression of TOP2A$^+$/EZH2$^+$, EZH2 and either low levels of citrate or polyamines as biomarkers, particularly among PCa patients not receiving neoadjuvant hormonal treatment. To summarize, citrate was the only individual biomarker that was predictive or borderline predictive of recurrence in patients both treated and not treated with neoadjuvant hormonal treatment.

When comparing metabolite levels and IHC tissue expression of cancer samples from patients receiving neoadjuvant hormonal treatment with cancer samples from patients not receiving this therapy, decreased levels of nine metabolites were found in samples from patients receiving hormonal treatment. After correction for multiple testing, only levels of choline ($q = 0.017$) and creatine ($q = 0.006$) were decreased. Further, lower expression levels of TOP2A and EZH2 were detected in cancer samples from patients receiving neoadjuvant hormonal treatment compared with cancer samples from individuals not receiving this therapy ($q = 0.009$ and $q = 0.006$, respectively) (Supplementary Table S7).

3.6 | Open access data confirm the potential of TOP2A and EZH2 in RP patients

The potential of TOP2A$^+$/EZH2$^+$ as a biomarker for recurrence was validated through meta-analysis. As no open access data for radiotherapy PCa patients were identified, analyses were performed among RP PCa patients. In total, 1101 samples from 1023 RP PCa patients were included in the calculations, while the remaining samples and patients were included for comparisons in forest plots (due to lack of time to event (two cohorts) or different end-point (one cohort)). An overview of key clinical and histopathological characteristics of the open access and the NTNU2 cohort is presented as Table 3. The meta-analyses supported TOP2A$^+$/EZH2$^+$ as a potential biomarker for recurrence in RP PCa patients, $p < 0.0001$ (HR 2.29, 95% CI 1.79-2.93) (Figure 4). Additionally, dichotomous levels of TOP2A and EZH2, individually, were significant predictors of recurrence in meta-analyses, $p < 0.0001$ and $p = 0.0005$, respectively (Supplementary Figure S4A and S4B).

4 | DISCUSSION

In the present study, we investigated whether tissue metabolites and/or tissue expression of TOP2A and EZH2 in cancer biopsies could be novel biomarkers for PCa recurrence in radiotherapy patients. Our study suggests low level of citrate to be particularly valuable as a biomarker for recurrence. The combined biomarker TOP2A$^+$/EZH2$^+$ and the increased tissue expression of EZH2 were suggested as predictive biomarkers in patients not receiving neoadjuvant hormonal treatment. These biomarkers are predictive of recurrence, prior to radiotherapy, and accessible in clinically relevant samples obtained through standard protocols.

4.1 | The predictive value of tissue biomarkers in cancer biopsies from radiotherapy patients

In radiotherapy patients, low level of citrate was associated with shorter time until recurrence and was the biomarker with the highest predictive ability in our study. Specifically, citrate had a higher or comparable predictive ability compared with the clinical variables individually, highlighting its strength as a potential biomarker for PCa aggressiveness and recurrence. Despite citrate being well acknowledged as a potential biomarker in PCa, it has to our knowledge not been investigated as a biomarker for recurrence among radiotherapy PCa patients. However, our finding of citrate being a predictor of recurrence is in agreement with a previous study from our group, where citrate was found as a biomarker for recurrence among RP patients.[9] Abnormal citrate metabolism is a unique trait of the prostate.[7,31] While the normal prostate accumulates high concentrations of citrate and secretes it into the seminal fluid,[53] this metabolic trait is lost in PCa, with decreased level of citrate in cancer compared with benign tissue.[7] The reduction in citrate is proposed to be due to lower import of zinc in PCa tissue, regaining the activity of aconitase, leading to a subsequent utilization of citrate in the TCA cycle.[54,55]

Decision curve analyses revealed a net benefit of adding citrate to a basic model containing PSA at diagnosis, cT3a and cT3b. Thus, citrate may serve as an adjunct predictive parameter to these factors. Although citrate seems to be a promising biomarker for recurrence, we recognize that our radiotherapy cohort, with a limited number of cancer samples, may not have the sufficient statistical power to determine its dependence on clinical parameters. Larger radiotherapy cohorts are therefore needed for validating the potential of citrate as a biomarker for recurrence, and to further explore the additional predictability by including citrate to existing clinical and histopathological variables.

We have previously suggested a high level of tChoCre/Spm as a better predictor of recurrence than tChoCre/Cit in RP PCa patients.[9] In the current study, low levels of polyamines were significant in predicting recurrence, but had a lower C-index compared with citrate. Neoadjuvant

TABLE 3 Overview of clinical and histopathological characteristics of the 11 RP PCa cohorts included in the meta-analyses. Abbreviations: BCR—biochemical recurrence, PCa-death—PCa-specific death

	Cohorts included in the meta-analysis calculations								Other cohorts (only included in forest plots)		
Clinical variables	**NTNU2**[9,30]	**Mortensen et al (GSE46602)**[46]	**Wang et al (GSE8218)**[41–43]	**Taylor et al (GSE21035/32)**[45]	**CAM Ross-Adams et al (GSE70768)**[40]	**STK Ross-Adams et al (GSE70769)**[40]	**TCGA-PRAD**[49,50]	**Fraser et al (GSE84043)**[47]	**Sboner et al (GSE16560)**[44]	**Erho et al (GSE4669)**[38,39]	**Wu et al (GSE44353)**[48]
Cancer samples (patients)	103 (34)	36 (36)	65 (56)	131 (131)	111 (111)	92 (92)	490 (490)	73 (73)	281 (281)	545 (545)	210 (210)
Age at diagnosis (median (range), years)	64 (48-69)	63 (46-71)	64 (43-77)	58 (37-73)	62 (41-73)		61 (41-78)	61 (44-72)	74 (51-91)	65.3 ± 6.4	–
PSA before surgery (median (range), ng/mL)	9 (4.0-45.8)	16 (5.0-43)	6.8 (1-75)	5.9 (1.0-46)	7.8 (3.2-23.7)	7.95 (1.5-117)	7.4 (0.7-107)	6.6 (1.7-39.5)	6.6 (1.0-75)	–	–
Grade Groups											
Low (1/2)	51 (50%)	32 (89%)[a]	50 (77%)	107 (82%)	81 (73%)	56 (61%)	207 (42%)	56 (77%)	162 (58%)	334 (61%)[a]	183 (87%)[a]
High (3-5)	52 (50%)	4 (11%)[a]	15 (23%)	24 (18%)	30 (27%)	34 (37%)	289 (58%)	17 (23%)	119 (42%)	211 (39%)[a]	27 (13%)[a]
Missing data						2 (2%)					
Pathological T stage											
pT1			1 (2%)			1[d] (1%)			281[c] (100%)		
pT2	24 (71%)	19 (53%)	32 (57%)	85 (65%)	34 (31%)	47 (52%)	187 (38%)	40 (55%)		219 (40%)	155 (74%)
pT3	9 (26%)	17 (47%)	20 (35%)	40 (30%)	76 (68%)	42 (45%)	293 (59%)	33 (45%)		253 (47%)	55 (26%)
pT4			1 (2%)	6 (5%)	1 (1%)		9 (2%)				
Missing data	1 (3%)		2 (2%)			2 (2%)	8 (1%)			73 (13%)	
Follow-up											
End-point	BCR	BCR	BCR	BCR	BCR	BCR	BCR	BCR	PCa-death	Metastasis	BCR
Occurred	13 (38%)	22 (61%)	29 (52%)	27 (21%)	19 (17%)	45 (49%)	91 (18%)	16 (22%)	165 (59%)	212 (39%)[b]	83 (40%)
Not occurred	21 (62%)	14 (39%)	27 (48%)	104 (79%)	92 (83%)	47 (51%)	399 (80%)	57 (78%)	116 (41%)	333 (69%)[b]	127 (60%)
Missing data	–						7 (2%)				
Follow up time (median (range), months)	72 (2-97)	32 (1-81)	14 (0.5-79)	46 (1-149)	30 (1-65)	58 (0-103)	35.7 (1-119)	71 (2-154)	100 (6-274)	–	–

[a]In Erho et al, Wu et al and Mortensen et al Low Grade Group was defined as Grade Groups 1-3.
[b]In Erho et al, metastatic progression at 10-year patient follow-up.
[c]Clinical T-stage.
[d]pT0.

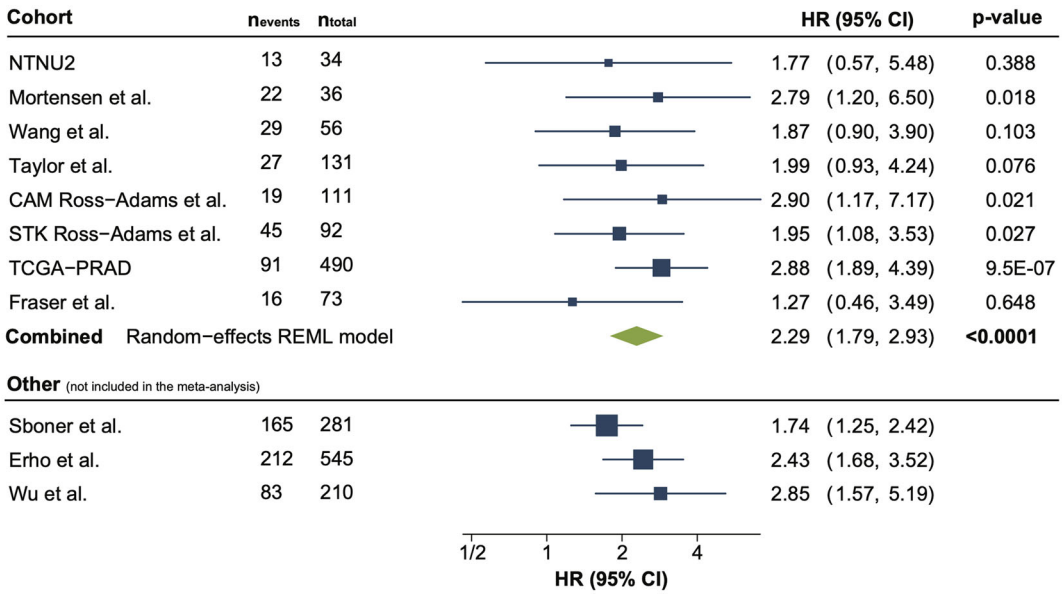

FIGURE 4 Forest plot from meta-analysis of TOP2A$^+$/EZH2$^+$ using open access gene expression data including data from 2059 individuals in 11 cohorts of RP PCa patients. REML, restricted maximum likelihood

hormonal treatment may affect polyamine levels, in line with findings of the current study (borderline significant after multiple testing), which may explain these partly conflicting results. In contrast to other cancer types,[56] PCa is in general characterized by decreased levels of polyamines.[7,57,58] Enhanced expression of spermine oxidase (SMOX) and spermidine/spermine N1-acetyltransferase (SAT1), two key enzymes important in the polyamine metabolism, have been reported in PCa tissue and in PCa tissue from patients who later progress to metastatic disease.[59,60] In a previous study from our group, the upregulation of SAT1, SMOX and spermidine synthetase (SRM) were associated with decreased spermine level.[61] These observations fit with our findings of low levels of polyamines being associated with recurrence. Additionally, although the polyamines have structural similarities, they have been suggested to have distinct functions.[62] This might explain why the individual polyamines might have different predictive abilities than polyamines as a group, as exemplified by Tsoi et al.[63]

A key finding in our radiotherapy cohort was TOP2A$^+$/EZH2$^+$ as a predictor of recurrence. This result is in accordance with previously reported findings in RP patients, where TOP2A$^+$/EZH2$^+$ was shown to identify a subgroup of patients with a high risk of recurrence.[29] In decision curve analyses, we found a positive net benefit of adding TOP2A and EZH2 to a basic model (containing PSA at diagnosis, cT3a and cT3b), although in a slightly smaller range of decision threshold probabilities compared with citrate. Further, our results indicate that TOP2A$^+$/EZH2$^+$ may be an independent predictor of recurrence, borderline significant after adjustment for clinical and histopathological variables.

PCa tissues are inherently heterogeneous. In a recent study from our group, we show by using matrix-assisted laser desorption ionization time-of-flight imaging mass spectrometry (MALDI-TOF MSI) that specific tissue compartments within PCa samples have distinct metabolic profiles.[64] As tissue heterogeneity may be of concern, we performed repeated analyses adjusting for tissue composition in this study. Due to a low cancer content in the current biopsy cohort, the stroma content was relatively high (no recurrence 75% and recurrence 80%). Since the compared groups have more or less equal amounts of stroma, we expect minimal influence on the comparison of tumor tissue in recurrent samples and non-recurrent samples. Although citrate remained significant in these models, MALDI-TOF MSI, spatial transcriptomics and single cell sequencing may reveal important information regarding the biomarkers included in this study, which may be hidden in traditional bulk analyses.

4.2 | The influence of hormonal treatment status on tissue biomarkers

Despite being associated with recurrence, TOP2A$^+$/EZH2$^+$ had a relatively low predictive ability in our study. However, among patients not receiving neoadjuvant hormonal treatment, TOP2A$^+$/EZH2$^+$ had a high predictive ability, better reflecting its potential as a biomarker for recurrence. This may suggest an influence of neoadjuvant hormonal treatment on TOP2A and EZH2 tissue expression levels, as detected in the current study. Based on these findings, tissue for evaluation of TOP2A and EZH2 should be collected prior to administration of neoadjuvant hormonal treatment, to serve as useful biomarkers for PCa recurrence. Neoadjuvant hormonal treatment may influence EZH2 expression via the retinoblastoma (RB) and p130-dependent pathways,[65] while a close link between TOP2A and the androgen receptor signaling pathway has been reported,[18] suggesting potential connections between neoadjuvant hormonal, TOP2A and EZH2 expression levels. Further, among patients not

receiving neoadjuvant hormonal treatment, increased expression of TOP2A and EZH2 individually were predictive for recurrence, while no significant associations were found among individuals receiving neoadjuvant hormonal treatment for these two biomarkers. The potential of TOP2A[13,14,18] and EZH2[19,28] as individual predictors of recurrence have been suggested in previous studies, in line with our study. A previous study reported an association between cytoplasmic and total (nuclear + cytoplasmic) EZH2 expression and metastasis after radiotherapy.[28] In our radiotherapy cohort, cytoplasmic staining of EZH2 was not observed (potentially due to weaker staining) and therefore not evaluated. The potential of EZH2 should be investigated in future studies, to reveal potential differences in cytoplasmic versus nuclear EZH2 staining. In addition, patients scheduled for radiotherapy may include patients with occult micro-metastases or with local disease recurrence at time of treatment. Recent diagnostic advancements may more accurately evaluate local versus metastatic disease, and further clarify the role of cytoplasmic versus nuclear EZH2 in PCa recurrence. Further, systematic biopsies were the standard diagnostic technique at the time of this study. Future studies using MR-guided biopsies may give a better representation of the index tumor.

While citrate was a significant biomarker independent of hormonal treatment status, the current study indicated a potential of glutamine, glutamate and choline as biomarkers for recurrence among patients receiving neoadjuvant hormonal treatment, and glucose among patients not receiving neoadjuvant hormonal treatment. Neoadjuvant hormonal treatment has previously been shown to influence metabolite levels,[66,67] in line with our current findings. However, results from the stratified analyses should be carefully interpreted, due to the moderate number of patients included in these analyses.

4.3 | Open access data confirms the potential of TOP2A$^+$/EZH2$^+$ in RP PCa patients

The potentials of TOP2A$^+$/EZH2$^+$, TOP2A and EZH2 were validated as biomarkers for recurrence, by meta-analyses including data from 11 independent PCa cohorts and 2059 patients. The meta-analysis supported the potential of the combined biomarker TOP2A$^+$/EZH2$^+$ as a biomarker for recurrence among RP patients, as well as TOP2A and EZH2 individually. No open access data from radiotherapy PCa patients were found by search, and meta-analyses were therefore performed using only RP patient data. In summary, our results suggest that TOPA and EZH2 may be useful biomarkers for recurrence among both radiotherapy and RT patients. Additional studies among radiotherapy patients are thus needed to strengthen and clarify TOP2A$^+$/EZH2$^+$, TOP2A and EZH2 as potential biomarkers for recurrence among this patient group.

4.4 | Conclusions

In conclusion, a low level of citrate was associated with shorter time until PCa recurrence and may serve as a prognostic biomarker for patients scheduled for radiotherapy. Citrate is detectable in patient in vivo MRSI examinations, which may be a 10 minute sequence of a regular clinical prostate MRI, offering a relatively easy translational potential of our findings. Although TOP2A$^+$/EZH2$^+$ and EZH2 were strong predictive biomarkers among patients not receiving neoadjuvant hormonal treatment, citrate may serve as a more robust biomarker for recurrence in patients both receiving and not receiving neoadjuvant hormonal treatment. These biomarkers need further validation in larger cohorts specific for PCa radiation patients due to the limited number of biopsies obtained in this study.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Collection and analysis of the samples from the radiotherapy were approved by the Regional Committee of Medical and Health Research Ethics (REC) (approval no 2017/576), Central Norway.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Data included in the meta-analyses were downloaded from public databases: GEO (https://www.ncbi.nlm.nih.gov/geo/) and TCGA (http://cancergenome.nih.gov/).

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. A.F.H., T.S.H., K.M.S., M.B.R. and M.-B.T. gathered data and performed experiments. Ø.S. performed the pathology evaluation. A.F.H. and T.S.H. performed IHC scoring with guidance from A.M.B. Data analyses were performed by A.F.H. and T.S.H. with consultation from G.F.G., M.B.R. and M.-B.T. All authors contributed to the interpretation of the results. The paper was drafted by A.F.H., T.S.H. and M.-B.T., and all authors edited and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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