Supplementary Materials and Methods

Sample collection

OUS cohort: Immediately after radical prostatectomy, a pathologist identified and cut out the tumor area based on biopsy results, palpation and macroscopically visible morphology changes. The tissue sample was fresh frozen and one section was mounted onto slides for hematoxylin-eosin (HE) staining. The remaining part of the tissue sample was stored at -80 °C. Areas with high tumor content were marked on the HE-stained sections, and was used to guide excision of high-tumor samples (10.5 ± 2.1 mg) from the fresh frozen tissue specimen for HR-MAS MRS. Fractional distributions of cancer tissue, benign (glandular) epithelium, and stroma were evaluated on HE-stained section of tissue specimens analyzed by HR-MAS MRS. Where identification of cancerous areas was difficult, sections were stained with PIN-triple (P504S, 34βE12 and p63).

NTNU1 cohort: Based on tumor location from diagnostic needle biopsies, tissue samples (~10mg) were collected with a biopsy needle within ~2 minutes after radical prostatectomy and snap-frozen in liquid nitrogen (-196°C). The biopsies were stored at -196°C until HR-MAS MRS analysis. After HR-MAS MRS, the samples were formalin fixed and paraffin embedded for sectioning, and histopathological evaluation of HES stained sections was done according to the same protocol as previously described in (Hansen *et al*, 2016, Sandsmark *et al*, 2017).

NTNU2 cohort: A 2-mm tissue slice was snap frozen less than 10 minutes after RP and stored at -80°C (Bertilsson *et al*, 2011). A photo of the fresh tissue slice was fused with the two adjacent HE-stained slices, and samples (~10mg, range 1-6 (median 3) samples per

patient) were cored under frozen conditions according to the histopathologically confirmed cancer areas. A cryo-section from each frozen core was collected for HE staining before HR-MAS MRS analysis and evaluated by an experienced pathologist specialized in uropathology.

Supplementary tables

Supplementary Table S1: Metabolite concentrations and ratio levels in non-recurrent and recurrent prostate cancers. Median metabolite concentrations and ratios from all tumor samples included in the study are shown with corresponding interquartile ranges.

	No recurrence		Recurrence			
	Median	IQR	Median	IQR	*P*-value[a]	Q-value[b]
Metabolites (nmol/mg)						
Spermine	1.14	0.61 - 1.77	0.6	0.39 - 1.13	0.001	0.025
Citrate	7.09	4.08 - 10.9	4.18	2.89 - 7.82	0.007	0.063
Valine	0.4	0.25 - 0.53	0.49	0.39 - 0.61	0.08	0.382
Putrescine	0.1	0 - 0.36	0	0 - 0.18	0.082	0.382
Succinate	0.53	0.38 - 0.79	0.61	0.43 - 0.74	0.113	0.451
Glutamine	2.28	1.69 - 2.88	2.53	1.78 - 3.33	0.169	0.585
Creatine	2.4	1.75 - 3.14	2.24	1.66 - 2.68	0.188	0.585
Choline	1.02	0.67 - 1.42	1.29	0.93 - 1.73	0.231	0.641
Phosphocholine	0.61	0.36 - 0.95	0.71	0.51 - 1.06	0.252	0.641
Ascorbate[c]	0	0 - 0.53	0.23	0 - 0.75	0.375	0.75
Glutamate	4.22	3.12 - 5.63	5.21	3.61 - 6.88	0.364	0.75
Ethanolamine	0	0 - 0.46	0	0 - 0.60	0.426	0.756
Isoleucine	0.16	0.08 - 0.25	0.29	0.17 - 0.37	0.475	0.756
Glutathione[c]	0.8	0.4 - 1,0	0.6	0.40 - 1.00	0.492	0.756
Glucose[d]	0.03	0 - 0.41	0	0 - 0.33	0.524	0.756
Lactate	16.95	12.7 - 23.9	20.11	15.8 - 24.0	0.53	0.756
Glycine	2.44	1.69 - 3.24	2.86	2.03 - 3.64	0.557	0.756
GPC	0.86	0.53 - 1.27	0.74	0.41 - 1.07	0.591	0.756
Leucine	0.4	0.26 - 0.64	0.6	0.43 - 0.90	0.594	0.756
Alanine	2.21	1.64 - 2.94	2.32	1.65 - 3.14	0.65	0.791
PE	2.33	1.67 - 3.37	2.58	1.79 - 3.4	0.686	0.801
Taurine	4.57	3.56 - 6.33	4.86	3.50 - 6.54	0.826	0.919
Myo-inositol	8.31	6.54 - 10.3	9.04	6.53 - 10.4	0.864	0.919
GPE	0	0 - 0.46	0.05	0 - 0.46	0.896	0.919
Scyllo-inositol	0.44	0.32 - 0.63	0.43	0.31 - 0.59	0.919	0.919
Metabolite ratios						
tChoCre/Spm	4.38	2.73 - 9.1	9.65	4.81 - 12.4	0.002	0.025
tChoCre/Cit	0.72	0.44 - 1.26	1.17	0.65 - 1.88	0.011	0.079

[a]: Generalized linear mixed modeling of Box-Cox transformed variables.

[b]: False-discovery rate-adjusted *P* values (Q-values, Benjamini-Hochberg).

[c]: Only quantified in OUS and NTNU1 cohorts.

[d]: Only quantified in NTNU1 and NTNU2 cohorts.

Abbreviations: GPC = Glycerophosphocholine; GPE = glycerophosphoethanolamine; IQR = interquartile range; PE = phosphoethanolamine; tChoCre/Cit = Total-choline+creatine/citrate; tChoCre/Spm = Total-choline+creatine/spermine.

Supplementary Table S2: Multivariate Cox proportional hazard ratios (HR) for candidate metabolites and ratios which were non-independent in multivariate analyses, and prostate cancer recurrence following radical prostatectomy.

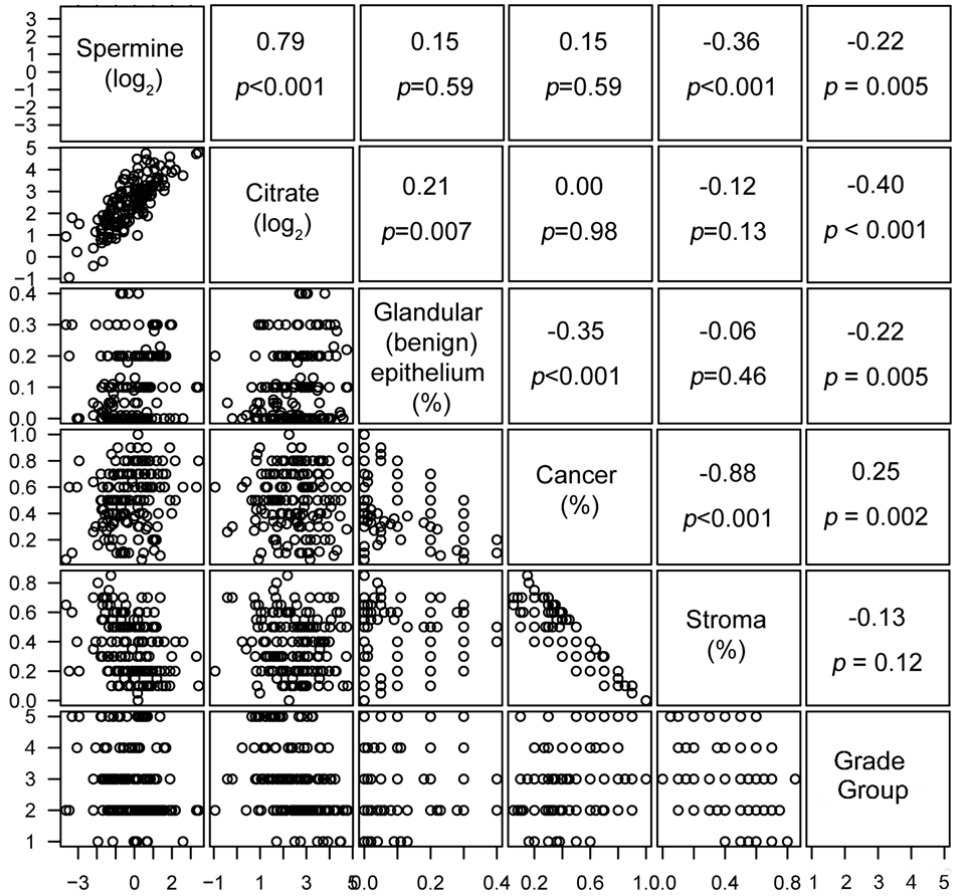
	Citrate (log_2)		Choline (log_2)		Creatine (log_2)		tChoCre/Cit	
Variable	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Metabolite (continuous)	0.82 (0.60-1.12)	0.21	1.22 (0.84-1.76)	0.30	0.80 (0.48-1.32)	0.37	1.14 (0.87-1.49)	0.33
Grade Group (RP)								
1	Reference		Reference		Reference		Reference	
2	0.60 (0.19-1.89)	0.38	0.76 (0.25-2.31)	0.63	0.71 (0.23-2.17)	0.55	0.63 (0.201.97)	0,43
3	0.85 (0.26-2.80)	0.79	1.16 (0.40-3.35)	0.78	1.15 (0.40-3.32)	0.79	0.93 (0.29-3.02)	0,90
4	2.65 (0.75-9.36)	0.13	3.23 (0.97-10.8)	0.057	3.18 (0.95-10.7)	0.061	2.84 (0.81-9.93)	0,10
5	2.34 (0.64-7.80)	0.21	3.22 (0.97-10.7)	0.056	2.93 (0.90-9.50)	0.074	2.39 (0.68-8.41)	0,17
EPE	3.25 (1.63-6.46)	<0.001	3.37 (1.71-6.66)	<0.001	3.54 (1.18-6.90)	<0.001	3.32 (1.66-6.61)	<0.001
SVI	1.11 (0.51-2.43)	0.79	1.28 (0.59-2.79)	0.54	1.22 (0.56-2.65)	0.62	1.16 (0.54-2.51)	0.71

Abbreviations: CI = confidence interval; EPE = extraprostatic extension; RP = radical prostatectomy; SVI = seminal vesicle invasion; tChoCre/Cit = (total-choline+creatine)/citrate.

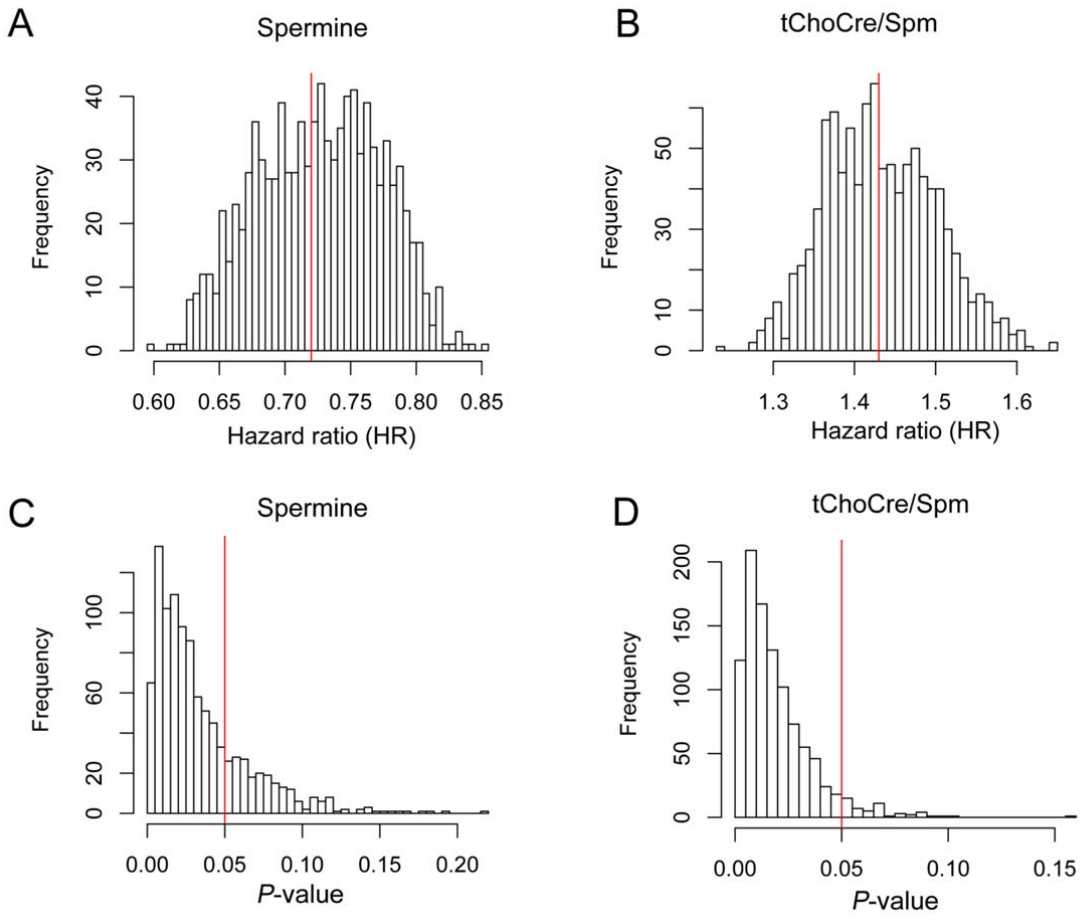
Supplementary Table S3: Clinicopathological characteristics of included patients from each cohort separately.						
	OUS		**NTNU1**		**NTNU2**	
Recurrence	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**
N	13	30	21	9	26	11
Time-period of surgeries	2002 - 2010		2008 - 2010		2007 - 2009	
Follow-up time Median (IQR), d	3240 (482)	720 (1350)	2196 (396)	986 (1215)	2342 (358)	639 (1096)
Age Mean ± SD, yr	62 ± 6	62 ± 5	62 ± 6	64 ± 6	61 ± 6	64 ± 5
Preoperative PSA Mean ± SD, ng/ml	7.7 ± 4.5	9.9 ± 5.7	9.2 ± 2.8	10.6 ± 3.9	10.3 ± 7.8	11.1 ± 4.3
Grade Group (RP)						
1	3	5	0	0	1	0
2	6	10	16	3	17	1
3	3	12	5	3	7	2
4	1	3	0	2	1	2
5	0	0	0	1	0	6
EPE	5	18	3	6	1	7
SVI	2	7	2	0	0	5
PSM	7	15	2	2	6	4
PCa-specific death	0	2	0	0	0	1

Abbreviations: NTNU1 = Norwegian University of Science and Technology cohort 1; NTNU2 = Norwegian University of Science and Technology cohort 2; OUS = Oslo University Hospital cohort; EPE = extraprostatic extension; PSA = prostate-specific antigen; PSM = Positive surgical margins; RP = Radical prostatectomy; SVI = seminal vesicle invasion, PCa = prostate cancer.

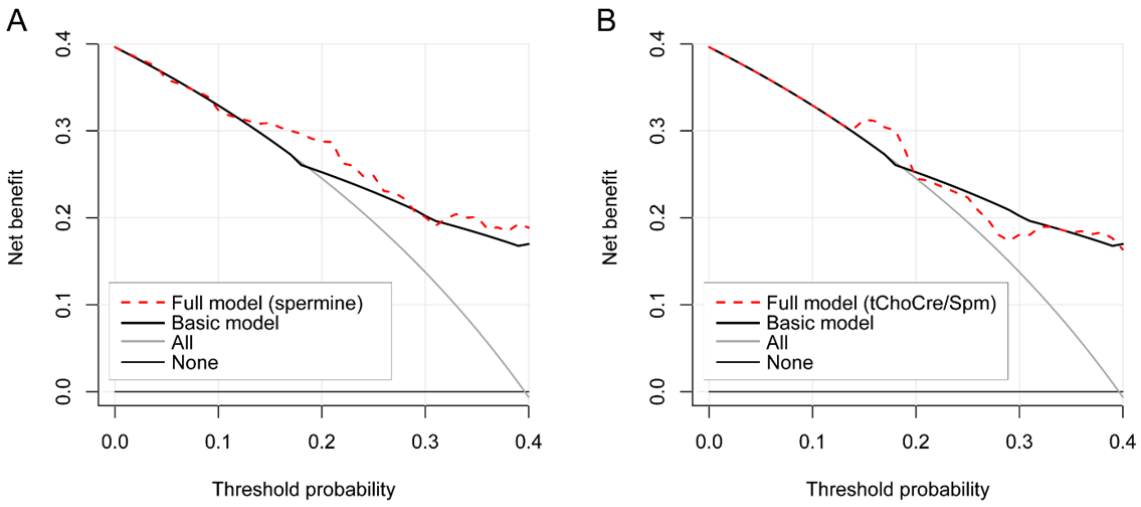
Supplementary Figures



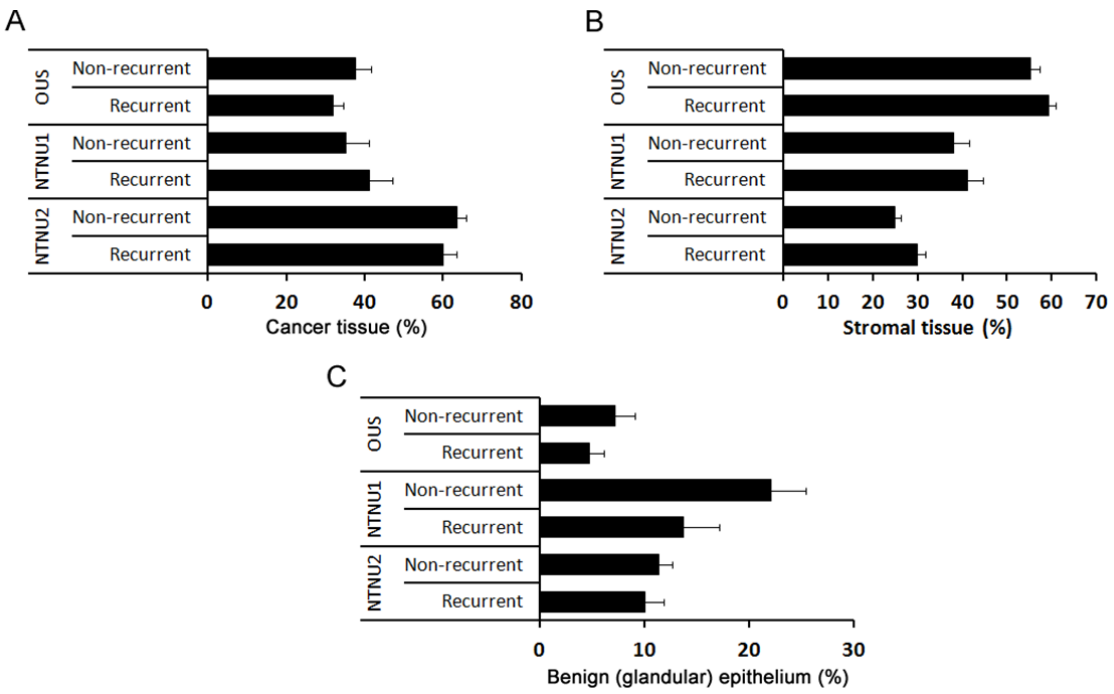
Supplementary Fig. S1. Correlation matrix of spermine (log_2), citrate (log_2), post-HR-MAS MRS tissue pathology examination parameters (glandular benign epithelium (%), cancer (%), and stroma (%)), and Grade Group (radical prostatectomy). Spearman's rank correlation coefficients and *P*-values are shown.



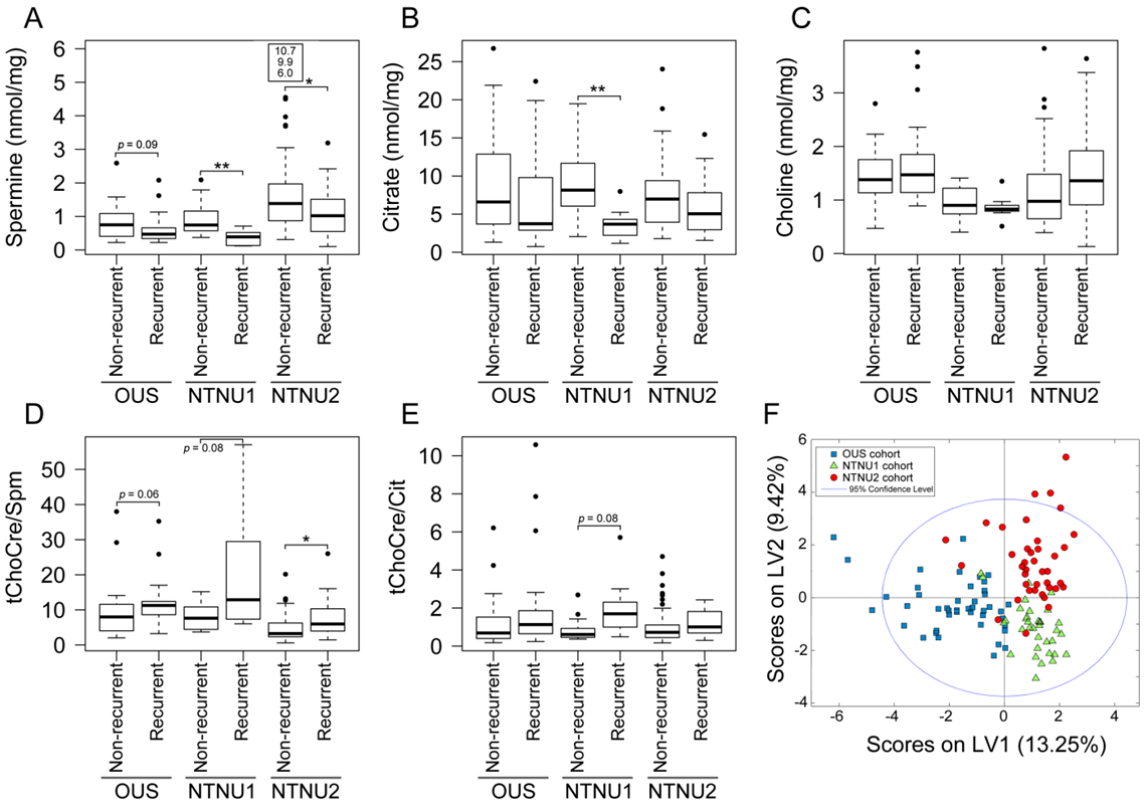
Supplementary Fig. S2. Histograms showing distributions of hazard ratios (A and B) and *P*-values (C and D) for spermine and tChoCre/Spm in multivariate Cox proportional hazards modeling computed 1000 times with random selections of samples where more than one sample was available per patient. The vertical red lines in A and B indicate the hazard ratios obtained in the results section (random selection of one sample per patient where applicable). The vertical red lines in C and D indicate *P*-value = 0.05.



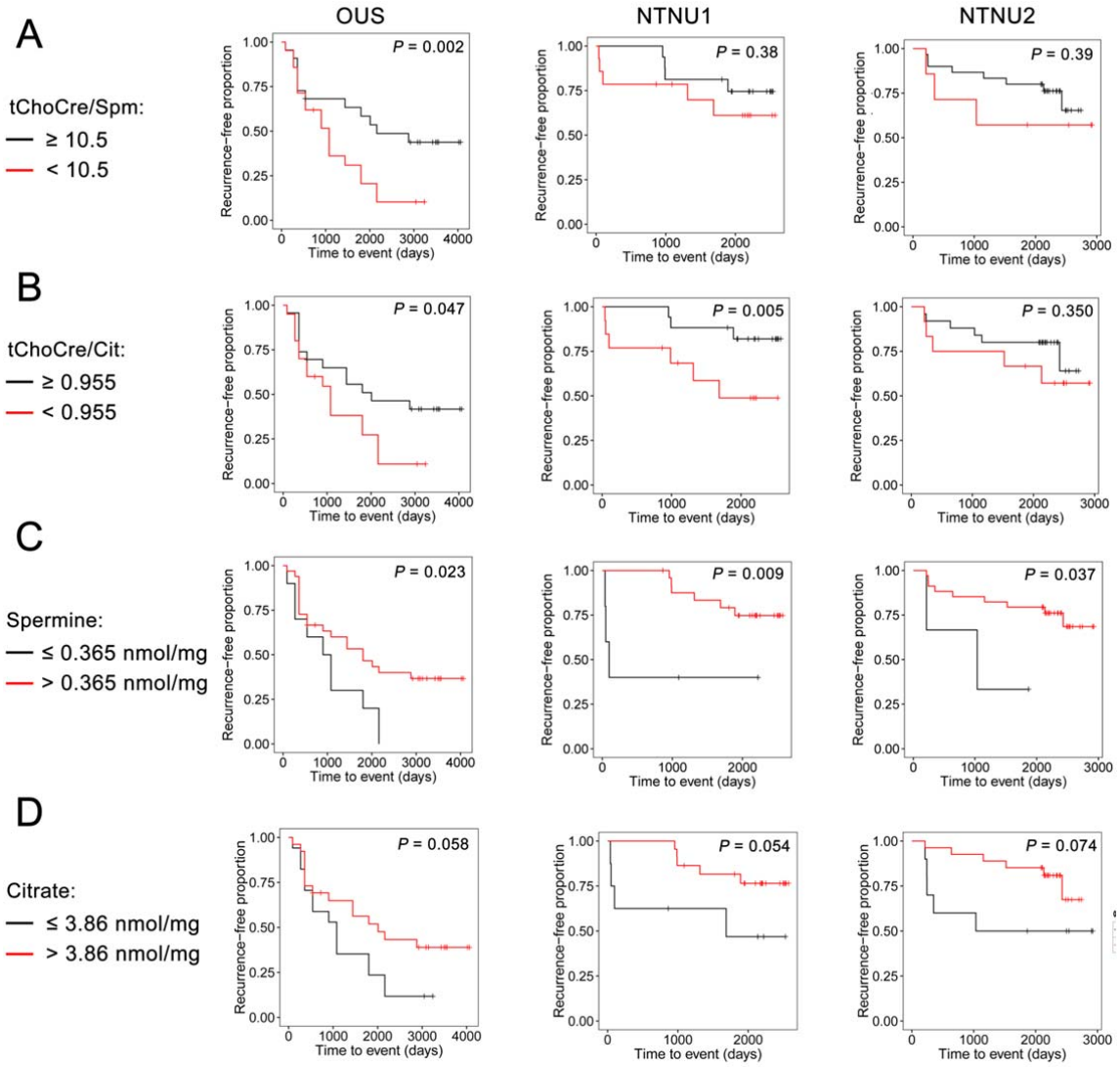
Supplementary Fig. S3. Decision curve analyses. Net benefits are plotted against the threshold probabilities for basic models (Grade Groups, extraprostatic extension and seminal vesicle invasion), full models (**A**: basic model + spermine (continuous), and **B**: basic model + tChoCre/Spm (continuous)), all (all "treated", thin gray line) and none ("treat none", thin black line). The "all treated" line assumes that all patients will experience recurrence, whereas the horizontal line assumes that none of the patients will experience recurrence.



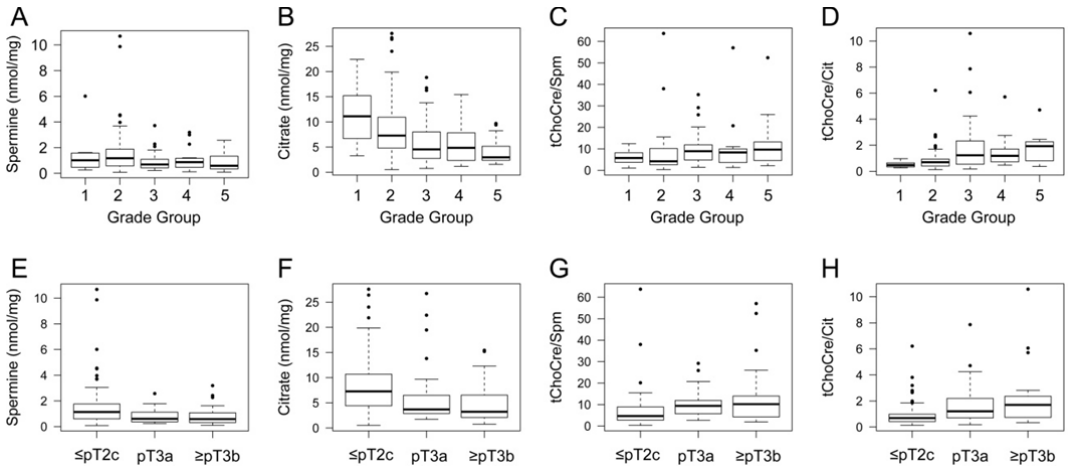
Supplementary Fig. S4. Histopathology on sections from tumor specimens analyzed by HR-MAS MRS. The distributions of cancer tissue % (**A**), stromal tissue % (**B**), and benign (glandular) epithelium % (**C**) is shown as mean ± standard error of the mean (SEM) for the cohorts separately.



Supplementary Fig. S5. Box plots of spermine (**A**), citrate (**B**), choline (**C**), tChoCre/Spm (**D**), and tChoCre/Cit (**E**) in non-recurrent and recurrent tumors (within 5 year follow-up) shown clustered by cohort affiliation. In A, three outliers are shown indented in a box, but were included in the statistical analyses. Non-parametric tests (Mann Whitney U for OUS and NTNU1 cohorts, and generalized linear modeling to account for multiple samples per patient for the NTNU2 cohorts) were performed comparing samples from non-recurrent and recurrent prostate cancer patients *intra*-cohort. *P*-values are not adjusted for multiple testing. Statistical significance is indicated by asterisks (*: $P < 0.05$; **: $P < 0.01$). (**F**) Scores plot of the first two loadings (LV1 and LV2) in a PLS-DA model built to test for systematic differences in the cohorts.



Supplementary Fig. S6. Cut off points yielding lowest log rank *P*-value for (**A**) tChoCre/Spm, (**B**) tChoCre/Cit, (**C**) spermine and (**D**) citrate were calculated in the OUS cohort to yield the largest differences in survival distribution. These cut-off points were used to dichotomize the NTNU1 and NTNU2 cohorts. Recurrence-free proportions were plotted against time to event (days), and a Mantel-Haenszel log rank test was used to test for equality in survival distributions (*P*-values indented).



Supplementary Fig. S7. Associations of metabolites with clinicopathological factors. Median and interquartile ranges of spermine, citrate, tChoCre/Spm and tChoCre/Cit across different Grade Groups (**A-D**, radical prostatectomy) and pT-stages (**E-H**) in the pooled cohort with multiple samples per patient (where applicable) are shown.

Supplementary references

Bertilsson H, Angelsen A, Viset T, Skogseth H, Tessem MB & Halgunset J (2011) A new method to provide a fresh frozen prostate slice suitable for gene expression study and MR spectroscopy. *Prostate* **71**: 461-9, doi:10.1002/pros.21260

Hansen AF, Sandsmark E, Rye MB, Wright AJ, Bertilsson H, Richardsen E, Viset T, Bofin AM, Angelsen A, Selnaes KM, Bathen TF & Tessem MB (2016) Presence of TMPRSS2-ERG is associated with alterations of the metabolic profile in human prostate cancer. *Oncotarget* **7**: 42071-85, doi:10.18632/oncotarget.9817

Sandsmark E, Hansen AF, Selnaes KM, Bertilsson H, Bofin AM, Wright AJ, Viset T, Richardsen E, Drablos F, Bathen TF, Tessem MB & Rye MB (2017) A novel non-canonical Wnt signature for prostate cancer aggressiveness. *Oncotarget* **8**: 9572-9586, doi:10.18632/oncotarget.14161