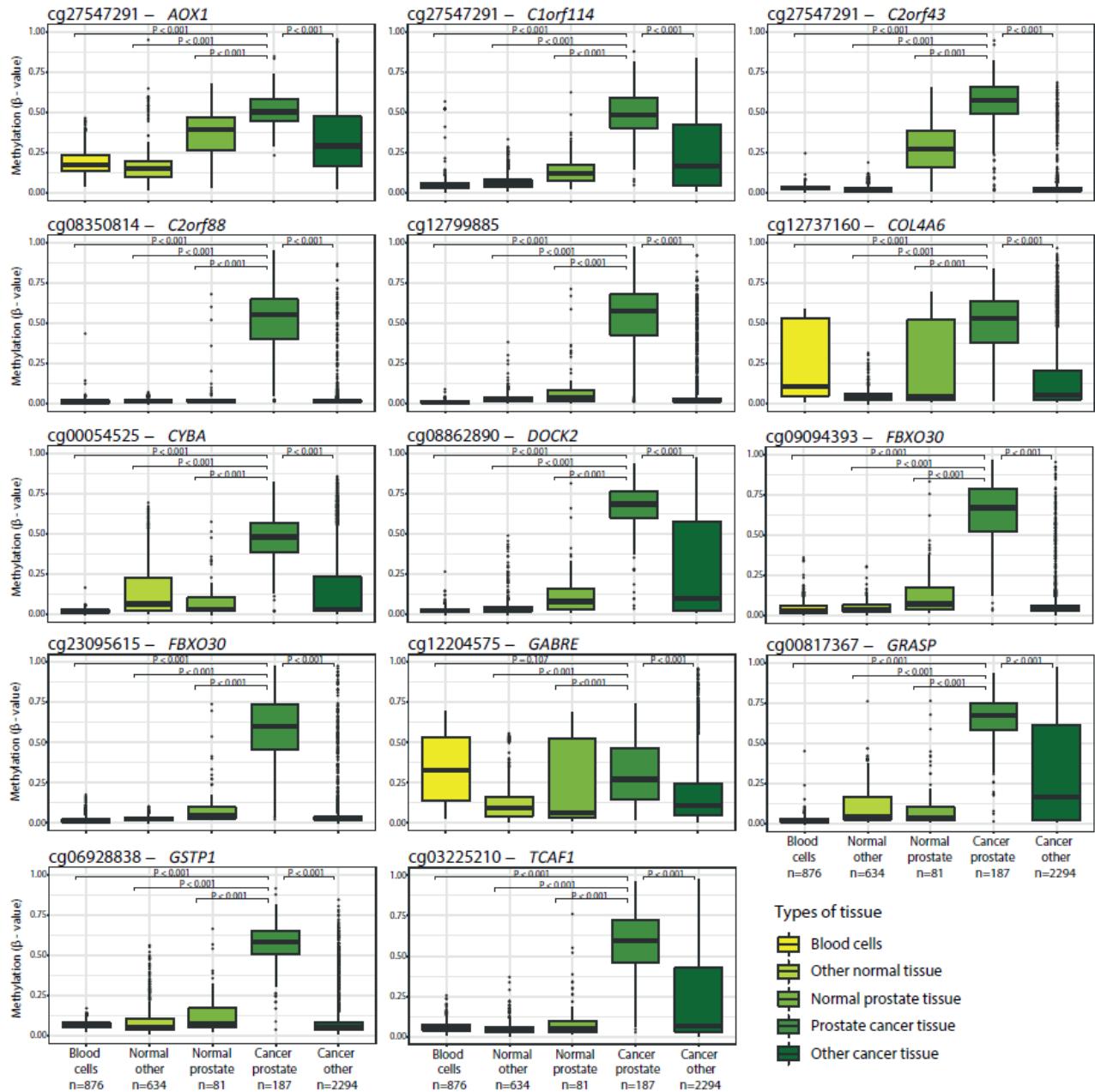


## Supplementary figures

**Figure S1-part1:** In silico analyses of 27 biomarker candidates using the Marmal-aid database. The candidates were identified in previous studies: Bjerre *et al.* [14] (*C2orf88*, *C2orf43*, *cg12799885*, *DOCK2*, *FBXO30*-*cg09094393*, *FBXO30*-*cg23095612*, *GRASP*, *HIF3A*, *MOB3B*, *PFKP*, *TPM4*), Strand *et al.* [12] (*COL4A6*, *CYBA*, *HLF*, *LOC149113*, *LRRC4*, *PROM1*, *RHCG*, *TCAF1*); Haldrup *et al.* [10] (*AOX1*, *C1orf114*, *HAPLN3*, *KLF*, *ST6GALNAC3*, *ZNF660*); Kristensen *et al.* [7] (*GABRE*); and Goering *et al.* [26] (*GSTP1*).



**Figure S1-part2:** In silico analyses of 27 biomarker candidates using the Marmal-aid database. The candidates were identified in previous studies: Bjerre *et al.* [14] (*C2orf88*, *C2orf43*, *cg12799885*, *DOCK2*, *FBXO30*-*cg09094393*, *FBXO30*-*cg23095612*, *GRASP*, *HIF3A*, *MOB3B*, *PFKP*, *TPM4*), Strand *et al.* [12] (*COL4A6*, *CYBA*, *HLF*, *LOC149113*, *LRRC4*, *PROM1*, *RHCG*, *TCAF1*); Haldrup *et al.* [10] (*AOX1*, *C1orf114*, *HAPLN3*, *KLF*, *ST6GALNAC3*, *ZNF660*); Kristensen *et al.* [7] (*GABRE*); and Goering *et al.* [26] (*GSTP1*).

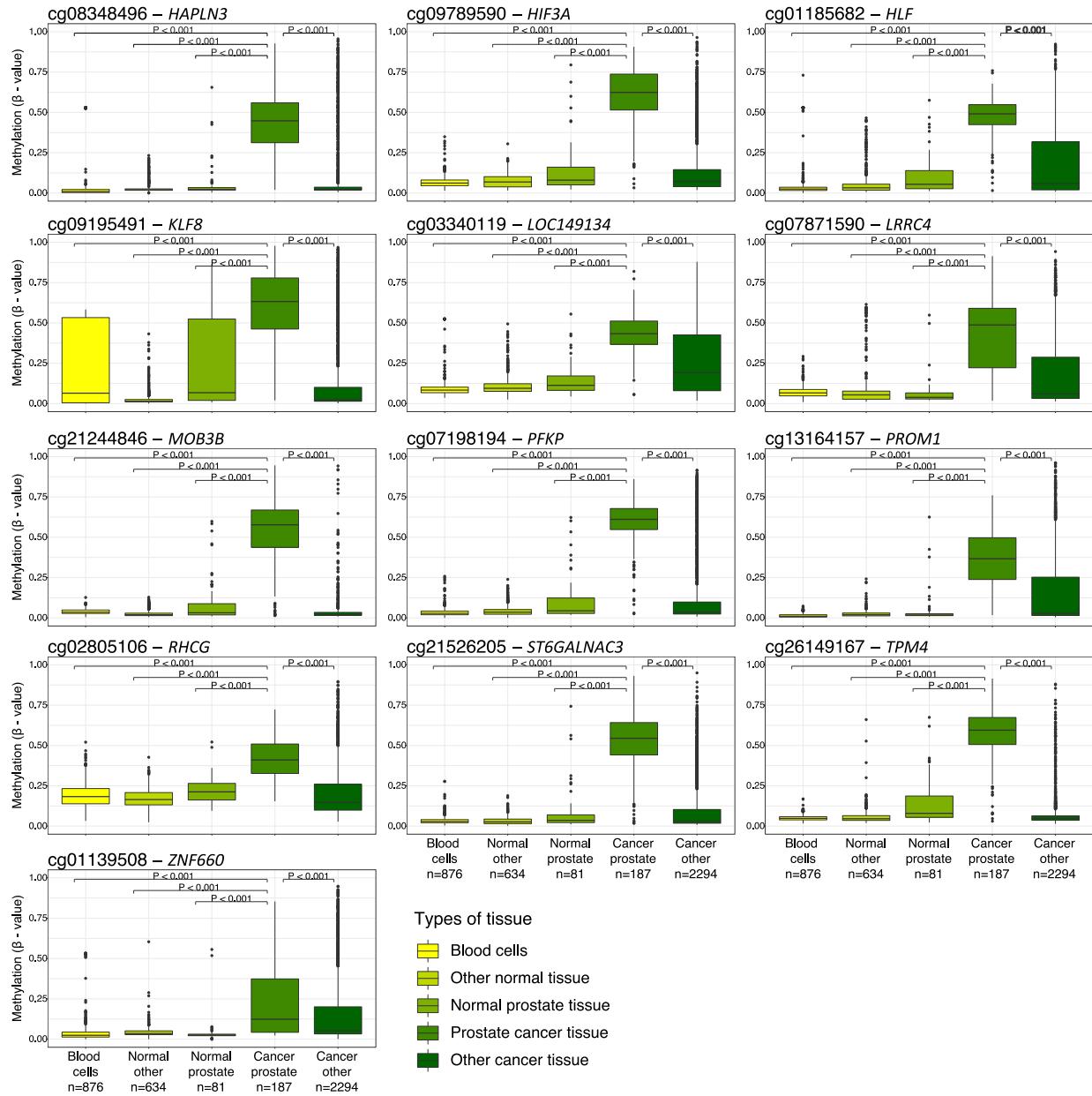


Figure S2-part1: Small-scale experimental validation using quantitative methylation-specific PCR (qMSP)

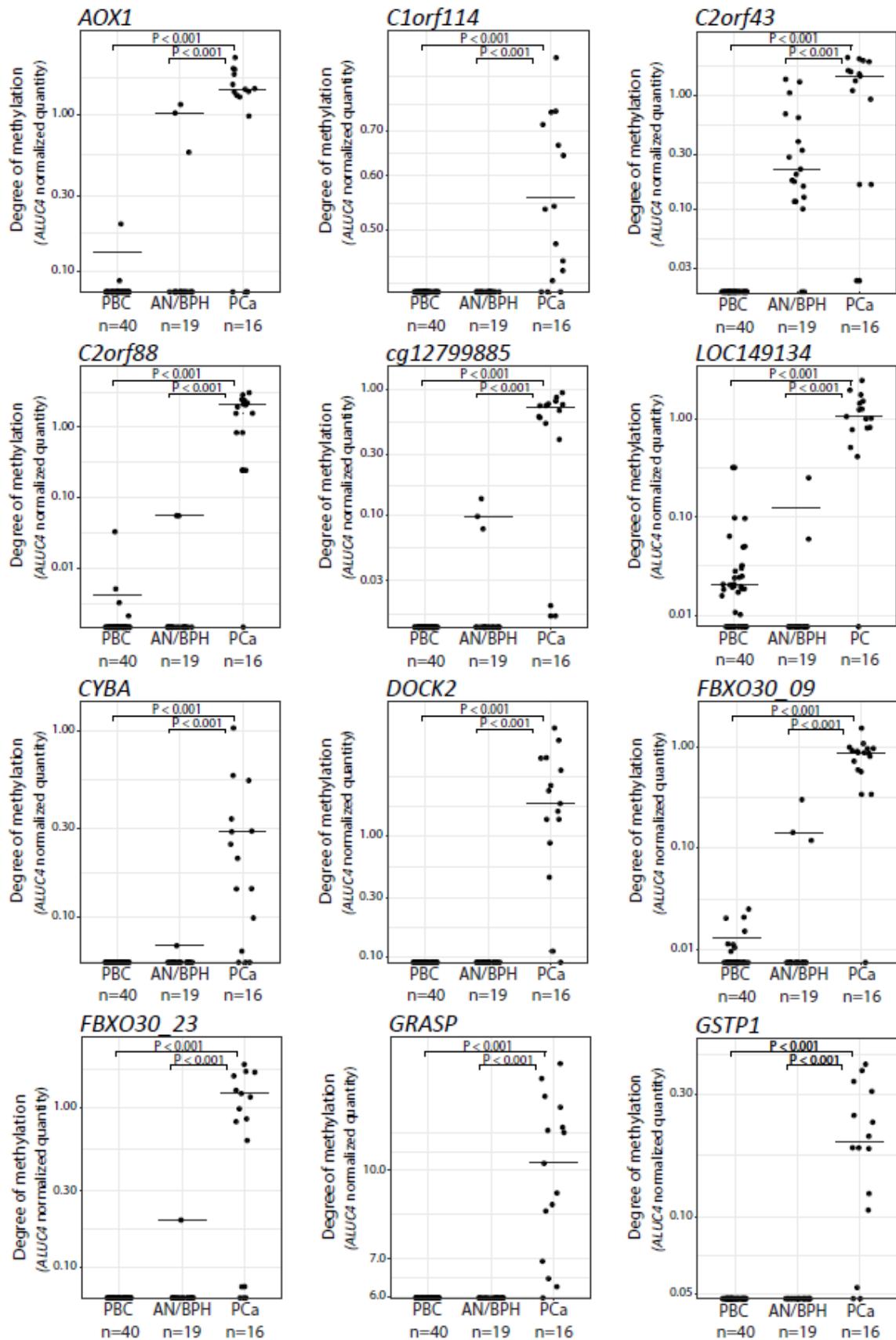
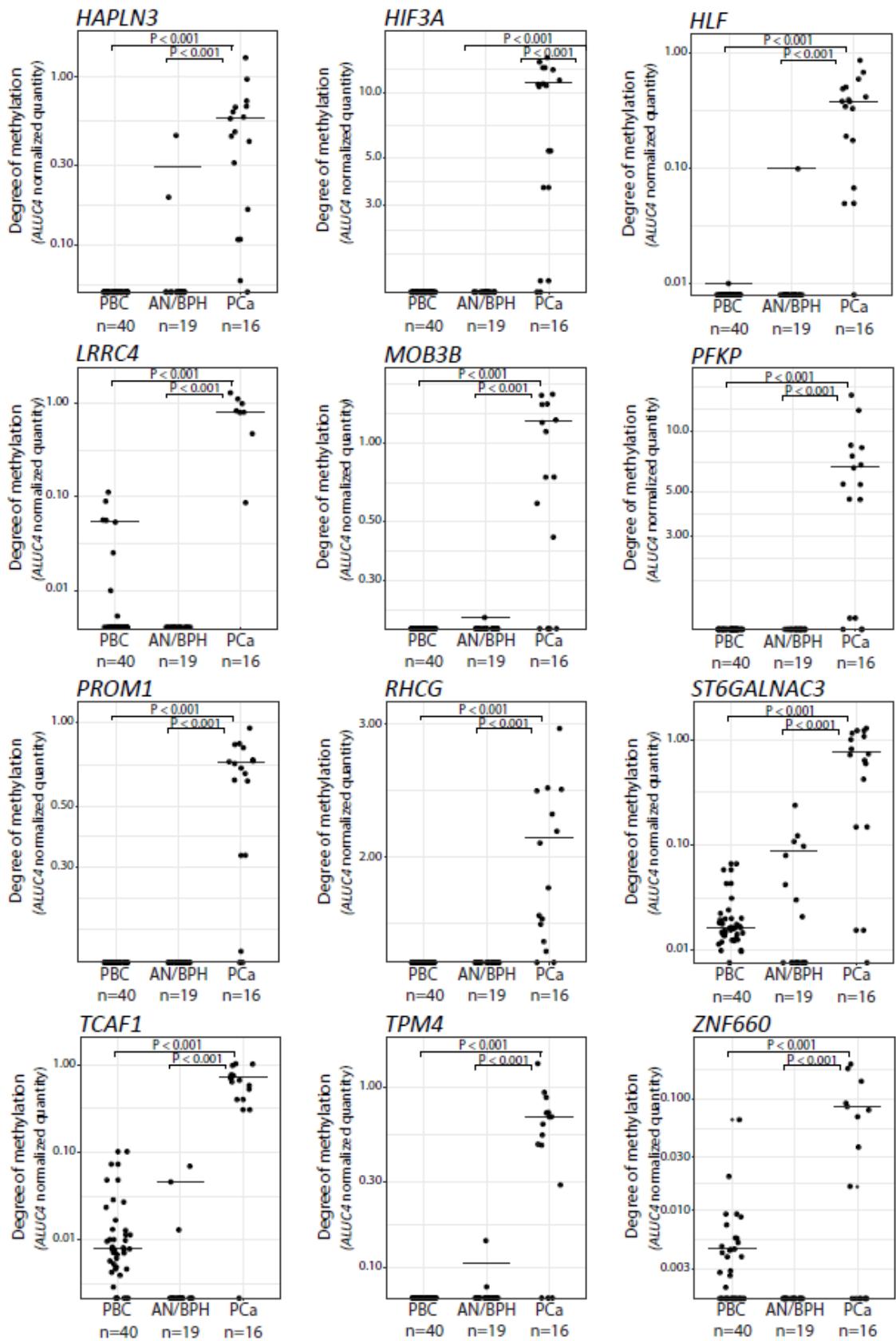
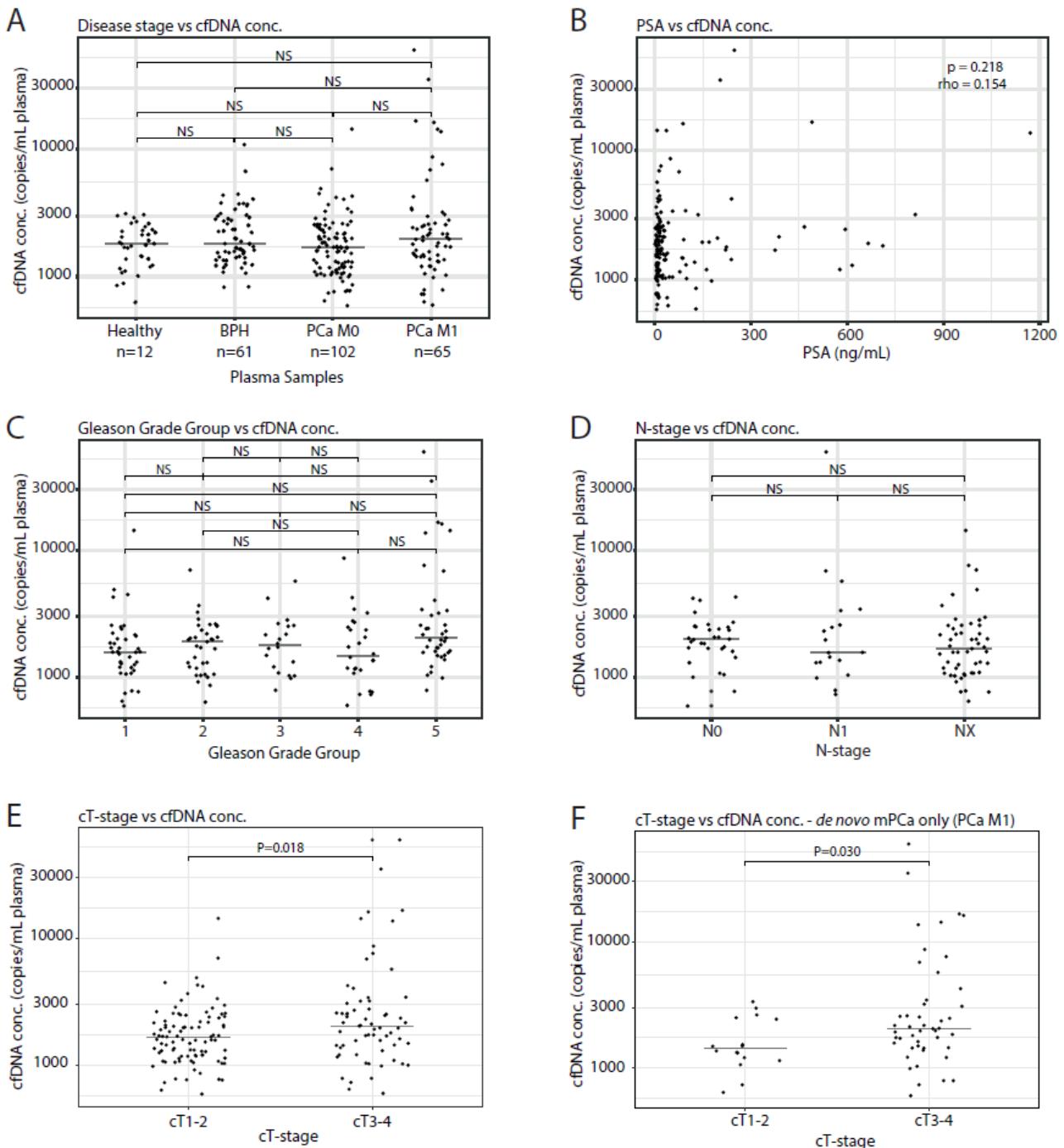


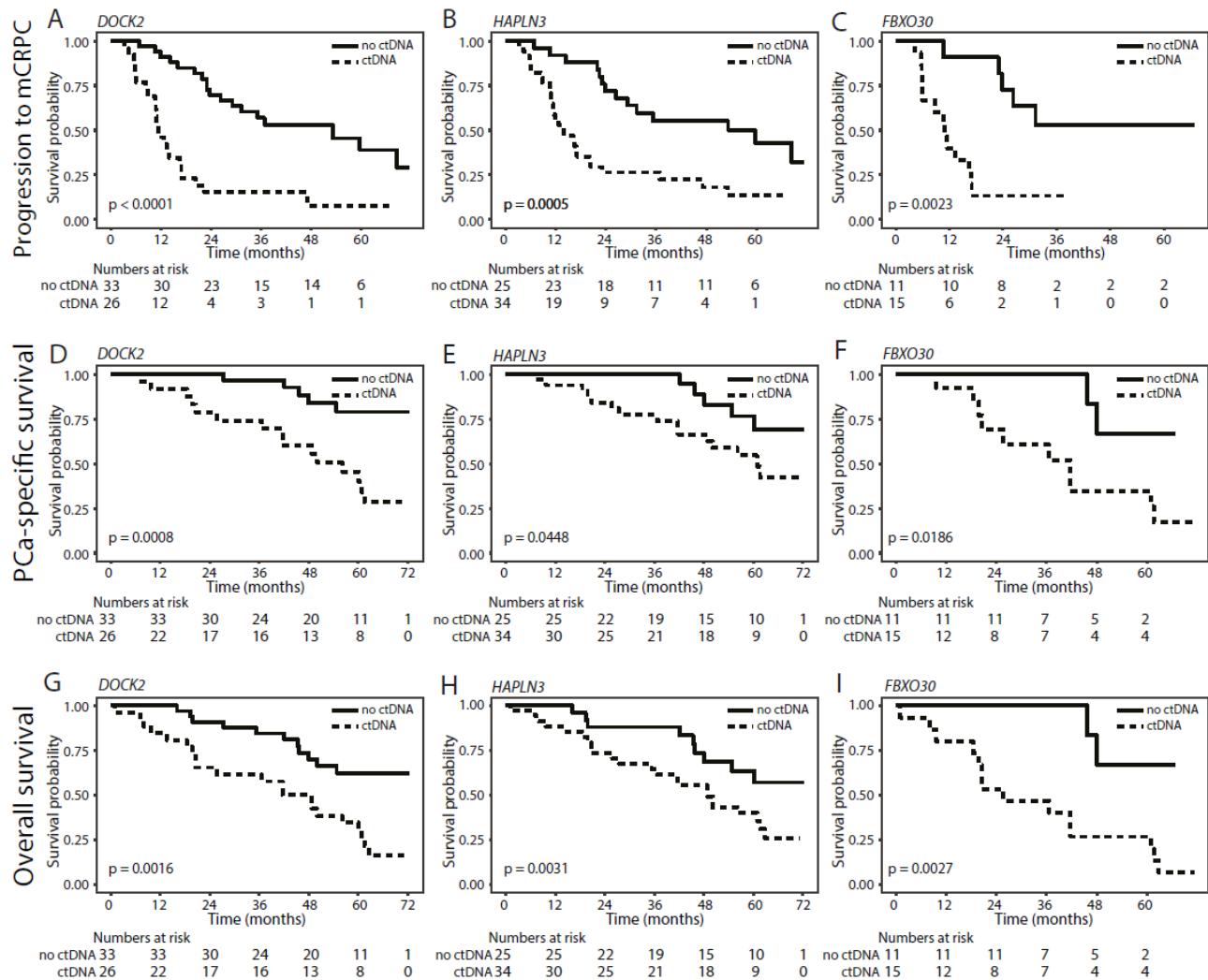
Figure S2-part2: Small-scale experimental validation using quantitative methylation-specific PCR (qMSP)



**Figure S3:** Association between cfDNA concentration and clinicopathological parameters in the clinical cohort (see also Table 1). P values from Mann-Whitney test (A, C-E) or spearman's rho test (B). NS, not significant.



**Figure S4:** Kaplan-Meier survival analyses in *de novo* mPCa patients for *DOCK2*, *HAPLN3*, and *FBXO30* assays individually. Patients were stratified by detection of methylated ctDNA based on our assays *DOCK2* (A, E, G), *HAPLN3* (B, F, H), and *FBXO30* (C, G, I) using time to mCRPC (A-C), PCa-specific survival (D-F), or overall survival (G-I) as end-points. P-values were calculated using log-rank tests.



**Table S1:** Overview of samples used for small-scale experimental evaluation (qMSP), technical evaluation (MS-ddPCR), and the clinical cohort (matched tissue and plasma (MS-ddPCR)).

	Small-scale experimental evaluation (qMSP)					Technical evaluation (MS-ddPCR)		Clinical cohort - Matched tissue and plasma (MS-ddPCR)	
Samples	PCa (n=20)	AN (n=13)	BPH (n=7)	PBC (n=20)	PBC (n=20)	PBC (n=52)	Healthy blood donors (n=70)	PCa (n=55)	AN (n=51)
Material	FFPE tissue, RP punch biopsy	FFPE tissue, RP punch biopsy	FFPE tissue, TUR-P punch biopsy	Whole blood	Buffy coat	Buffy coat	Plasma	FFPE tissue, RP punch biopsy	FFPE tissue, RP punch biopsy
Volume (mL), median (min-max)	-	-	-	-	-	-	7.7 (5.9-7.7)	-	-
Age (years), median (min-max)	66 (56-71)	61 (57-79)	70 (57-81)	67 (56-71)	57 (51-63)	57 (51-67)	< 45	64 (48-75)	64 (48-75)
Samples BS converted, n (%)	20 (100)	13 (100)	7 (100)	20 (100)	20 (100)	46 (88)	67 (95)	48 (87)	36 (71)
Samples analysed, n (%)	16 (80)	13 (100)	6 (86)	20 (100)	20 (100)	44 (85)	64 (91)	41 (75)	11 (22)

qMSP, quantitative methylation-specific PCR. MS-ddPCR, methylation specific droplet digital PCR. PCa, prostate cancer. AN, adjacent normal. BPH, benign prostatic hyperplasia. PBC, peripheral blood cells. FFPE, formalin-fixed paraffin-embedded. RP, radical prostatectomy. TUR-P, transurethral resection of the prostate. BS, bisulfite.

**Table S2:** Overview of plasma extraction and bisulfite conversion efficiency of plasma samples

	Technical evaluation (MS-ddPCR)	Clinical cohort (MS-ddPCR)			
Samples	Healthy blood donors (n=70)	Healthy male blood donors (n=36)	BPH (n=61)	Localized PCa (n=102)	mPCa (n=68)
Extraction efficiency, mean (1st-3th quartile)	88 (75-100)	83 (73-93)	89 (78-99)	73 (64-83)	71 (59-82)
cfDNA concentration (copies/mL plasma), median (min-max)	2149 (606-5086)	1814 (629-3116)	1811 (827-10823)	1693 (593-14321)	1966 (599-59550)
Samples BS converted, n (%)	67 (95) 16335 (4099-41402)	36 (100) 12266 (4871-20731)	61 (100) 14600 (6468-207570)	102 (100) 11458 (2649-78355)	67 (100) 8131 (1910-708730)
- median cfDNA copies before BS (min-max)					
- Recovery after BS conversion (%), median (min-max)	46(28-66)	56 (39-86)	48 (30-77)	57 (8-99)	58 (33-96)
- Median bisulfite converted cfDNA copies per duplex ddPCR reaction (min-max)	4936 (1445-9900)	3762 (1495-6237)	4734 (1548-88814)	4290 (832-29524)	2710 (752-394460)
Samples included in final analyses, n (%)	64 (91)	36 (100)	61 (100)	102 (100)	65 (97)

MS-ddPCR, methylation specific digital droplet PCR. BPH, benign prostatic hyperplasia. PCa, prostate cancer. mPCa, metastatic prostate cancer. BS, bisulfite.

**Table S3:** Primers and probes used for qMSP.

*Submitted as an excel-file*

**Table S4:** Reference numbers (Qiagen) for LNA primers and probes used for MS-ddPCR.

*Submitted as an excel-file*

**Table S5:** Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE) guidelines.

*Submitted as an excel-file*

**Table S6:** Sensitivity and specificity for the 24 candidate markers subjected to small-scale evaluation using quantitative methylation-specific PCR (qMSP). Sensitivity, specificity, negative and positive predictive values were calculated based on analyses of 16 PCa tissue versus 19 AN/BPH tissue samples. False detection rates are reported for 40 PBC samples analysed. Black highlight: assays that were selected for further validation. Red highlight: parameter by which the assays were excluded from further analyses. Blue highlights: the three top candidate markers selected for the final analyses.

Small-scale evaluation (qMSP)					
Assay	Sensitivity	Specificity	Negative predictive value	Positive predictive value	False detection rate for PBC
<i>AOX1</i>	0.81	0.84	0.84	0.81	<b>0.05</b>
<i>C1orf114</i>	<b>0.75</b>	<b>1.00</b>	<b>0.83</b>	<b>1.00</b>	<b>0.00</b>
<i>C2orf43</i>	0.94	<b>0.11</b>	0.67	0.47	0.00
<i>C2orf88</i>	0.94	0.89	0.94	0.88	<b>0.10</b>
<i>cg12799885</i>	<b>0.94</b>	<b>0.84</b>	<b>0.94</b>	<b>0.83</b>	<b>0.00</b>
<i>CYBA</i>	<b>0.81</b>	<b>0.95</b>	<b>0.86</b>	<b>0.93</b>	<b>0.00</b>
<i>DOCK2</i>	<b>0.94</b>	<b>1.00</b>	<b>0.95</b>	<b>1.00</b>	<b>0.00</b>
<i>FBXO30-cg09094393</i>	0.94	0.84	0.94	0.83	<b>0.20</b>
<i>FBXO30-cg23095612</i>	<b>0.75</b>	<b>0.95</b>	<b>0.82</b>	<b>0.92</b>	<b>0.00</b>
<i>GRASP</i>	0.94	<b>1.00</b>	<b>0.95</b>	<b>1.00</b>	<b>0.00</b>
<i>GSTP1</i>	<b>0.88</b>	<b>1.00</b>	<b>0.90</b>	<b>1.00</b>	<b>0.00</b>
<i>HAPLN3</i>	<b>0.94</b>	<b>0.89</b>	<b>0.94</b>	<b>0.88</b>	<b>0.00</b>
<i>HIF3A</i>	<b>0.88</b>	<b>1.00</b>	<b>0.90</b>	<b>1.00</b>	<b>0.00</b>
<i>HLF</i>	0.94	0.95	0.95	0.93	<b>0.03</b>
<i>LOC149134</i>	0.94	0.89	0.94	0.88	<b>0.60</b>
<i>LRRK4</i>	0.81	<b>1.00</b>	0.86	<b>1.00</b>	<b>0.20</b>
<i>MOB3B</i>	<b>0.75</b>	<b>0.95</b>	<b>0.82</b>	<b>0.92</b>	<b>0.00</b>
<i>PFKP</i>	<b>0.83</b>	<b>1.00</b>	<b>0.86</b>	<b>1.00</b>	<b>0.00</b>
<i>PROM1</i>	<b>0.83</b>	<b>1.00</b>	<b>0.86</b>	<b>1.00</b>	<b>0.00</b>
<i>RHCG</i>	<b>0.83</b>	<b>1.00</b>	<b>0.86</b>	<b>1.00</b>	<b>0.00</b>
<i>ST6GALNAC3</i>	0.88	<b>0.58</b>	0.85	0.64	<b>0.10</b>
<i>TCAF1</i>	0.88	0.84	0.89	0.82	<b>0.85</b>
<i>TPM4</i>	<b>0.81</b>	<b>0.95</b>	<b>0.86</b>	<b>0.93</b>	<b>0.00</b>
<i>ZNF660</i>	0.75	<b>1.00</b>	0.83	<b>1.00</b>	<b>0.98</b>

**Table S7:** Cox regression analyses of *DOCK2*, *HAPLN3*, and *FBXO30* using time to mCRPC, PCa-specific survival, and overall survival as endpoints.

Variable	Characteristics	Univariate		Multivariate		Multivariate		Multivariate	
		HR (95% CI)	p-val	HR (95% CI)	p-val	HR (95% CI)	p-val	HR (95% CI)	p-val
<b>Endpoint: mCRPC</b>									
<i>DOCK2</i>	No ctDNA vs. ctDNA	4.2 (2.2-8.0)	<0.001	4.9 (2.2-11.3)	<0.001	-	-	-	-
<i>HAPLN3</i>	No ctDNA vs. ctDNA	3.1 (1.6-6.1)	0.001	-	-	2.8 (1.2-6.5)	0.024	-	-
<i>FBXO30</i>	No ctDNA vs. ctDNA	4.7 (1.6-13.7)	0.005	-	-	-	-	28.3 (3.2-250.4)	0.003
Tumor volume	Low vs high	2.9 (1.5-5.6)	0.001	1.3 (0.6-2.9)	0.459	1.8 (0.8-3.8)	0.141	0.9 (0.3-2.7)	0.874
<b>Endpoint: PCa-specific survival</b>									
<i>DOCK2</i>	No ctDNA vs. ctDNA	4.8 (1.7-13)	0.003	-	-	-	-	-	-
<i>HAPLN3</i>	No ctDNA vs. ctDNA	2.7 (1.1-8.3)	0.034	-	-	-	-	-	-
<i>FBXO30</i>	No ctDNA vs. ctDNA	5.2 (1.1-24.1)	0.034	-	-	-	-	-	-
Tumor volume	Low vs high	2.4 (0.93-6.2)	0.068	-	-	-	-	-	-
<b>Endpoint: Overall survival</b>									
<i>DOCK2</i>	No ctDNA vs. ctDNA	3.1 (1.5-6.4)	0.003	-	-	-	-	-	-
<i>HAPLN3</i>	No ctDNA vs. ctDNA	2.3 (1.1-5.0)	0.035	-	-	-	-	-	-
<i>FBXO30</i>	No ctDNA vs. ctDNA	7.1 (1.6-32.0)	0.010	-	-	-	-	-	-
Tumor volume	Low vs high	2.1 (0.97-4.3)	0.060	-	-	-	-	-	-

**Table S8:** Sensitivity for prediction of mCRPC progression, PCa-specific survival, and overall survival for *DOCK2*, *HAPLN3*, *FBXO30*, and ctDNA positive (by any of the three assays) vs. negative in *de novo* mPC patients

End-point	Assay	Sensitivity	Specificity
mCRPC progression	<i>DOCK2</i>	54.8%	82.4%
	<i>HAPLN3</i>	66.7%	64.7%
	<i>FBXO30</i>	72.2%	75.0%
	ctDNA pos. (any assay) vs. neg.	69.0%	64.7%
PCa-specific survival	<i>DOCK2</i>	65.6%	81.5%
	<i>HAPLN3</i>	75.0%	51.3%
	<i>FBXO30</i>	83.3%	64.3%
	ctDNA pos. (any assay) vs. neg.	80.0%	51.3%
Overall survival	<i>DOCK2</i>	75.0%	71.8%
	<i>HAPLN3</i>	71.9%	59.3%
	<i>FBXO30</i>	87.5%	90.0%
	ctDNA pos. (any assay) vs. neg.	75.0%	59.3%