

# Sample parameters affecting the clinical relevance of RNA biomarkers in translational breast cancer research

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**Abstract** In the frame of translational breast cancer research, eligibility criteria for formalin-fixed paraffin-embedded tissue (FFPE) material processing for gene expression studies include tumor cell content (TCC) and sample site (primary vs metastatic tumors). Herein we asked whether the observed differences in gene expression between paired samples with respect to TCC and sample site also have different clinical significance. We assessed *ESR1*, *ERBB2*, *MAPT*, *MMP7*, and *RACGAP1* mRNA expression with real time PCR in paired samples before (NMD) and after macrodissection (MD) from 98 primary tumors ( $P_{MD}$ ,  $P_{NMD}$ ) and 72 metastatic lymph nodes ( $LN_{MD}$ ,  $LN_{NMD}$ ), as well as from 93 matched P (mP) and LN (mLN). TCC range

was 2.5–75 % in the NMD series and 28–98 % in the MD and in the mP/mLN series. The prognostic effect of these markers, individually or in clusters, remained stable between paired  $P_{MD/NMD}$ . In comparison, cluster classification failed in the  $LN_{NMD}$  group with lower TCC. In the mP/mLN cohort, *RACGAP1* mRNA expression was of prognostic significance when tested in mLN samples ( $p < 0.001$ ). Similarly, luminal B, HER2, and triple negative tumors were of dismal prognosis when classified in the LN component of the same series (mLN, overall survival:  $p = 0.013$ ,  $p = 0.034$ , and  $p = 0.007$ , respectively). In conclusion, the clinical relevance of the RNA markers examined may be affected by TCC in metastatic LN samples but not in primary tumors,

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while it differs between primary tumors and matched metastases. These data will facilitate the design of translational studies involving FFPE sample series.

**Keywords** Macrodissection · Tumor cell content · Gene expression · FFPE · Primary tumor · Metastatic lymph node · Breast cancer · Translational study

## Introduction

Translational studies are increasingly performed on RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue material with quantitative real-time PCR (qPCR) because it yields analytically accurate results even with degraded molecular templates, such as those from FFPE tissues [1]. Conceivably, sampling is of utmost importance for obtaining reliable and reproducible results that will be translated into clinical practice. The initial workflow involves pathologists who select tissue material from FFPE tissue banks and evaluate tissue eligibility for RNA extraction processing. Tumor cell content (TCC) and site of tumor sample, e.g., for breast cancer usually primary tumors vs lymph node metastases, are two major parameters that determine tissue sample eligibility for translational studies. In fact, apart from paraffin block availability, these two parameters are major limiting factors for obtaining the large sample series necessitated for the evaluation of the markers of interest.

With respect to TCC, a number of studies have shown that gene expression profiles in normal, cancer, and distinct elements within each tissue compartment from the same section may considerably vary [2–7]. However, the impact of molecular sample TCC on the evaluation of gene expression markers for their effect on patient outcome has mostly not been addressed in translational studies, perhaps with the exception of one [2]. Currently, limiting TCC rates for gene expression assessments broadly vary in the research setting. Minimal TCC ranges from 10 [2], 20 [8], 30–50 [9–15], to 70 % [16, 17]. TCC% cutoffs have been validated individually for diagnostic gene expression applications: 75 % for the classifier PAM50 (<http://www.aruplab.com/guides/ug/tests/2004700.jsp>), 50 % for Oncotype DX (<http://www.oncotypedx.com/en-US/Breast.aspx>), or 30 % for EndoPredict [18]. For large FFPE samples series but also in the diagnostic setting, the usually applied method for increasing TCC is macrodissection, i.e., procurement of tissue fragments from unstained sections with a scalpel [19]. In comparison to the more precise but costly and time-consuming laser microdissection [20], macrodissection is an almost no-cost approach. However, it is still an extra step in the whole procedure of extracting DNA/RNA from FFPE sections, meaning extra time and labor to spend in the course of a large-scale project.

In addition, although several studies have reported variable rates on the concordance of classic breast cancer parameters (hormone receptor and HER2 status) in primary tumors and metastatic lymph nodes with slide-based methods (IHC, mRNA ISH, FISH, CISH) [21–28], knowledge regarding mRNA expression in the same context is limited. In translational studies, however, tissue material from metastatic lymph nodes may occasionally be the only source for tumor geno/phenotyping.

With the above questions still open, the present study emerged as a necessity for understanding whether TCC and assessment in primary tumors vs metastatic lymph nodes would affect the prognostic significance of gene expression markers in the frame of translational research. Focused on these issues, we reevaluated the clinical impact of selected gene expression markers previously published [10, 13, 29–31] or currently under investigation by our group. Paired samples were prepared from whole sections (non-macrodissected, NMD) and from procured tissue fragments (macrodissected, MD) from routinely processed breast carcinoma tissues. The mRNA markers assessed were *ESR1* (6q25.1, estrogen receptor- $\alpha$  [ER]); *ERBB2* (17q21.1, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, [HER2]); *MAPT* (17q21.1, microtubule-associated protein tau); *MMP7* (11q21-q22, metalloproteinase-7); and *RACGAP1* (12q13.12, Rac GTPase-activating protein 1). The role of *ESR1* and *ERBB2* in breast cancer has been extensively studied and the expression of both genes is used in molecular breast cancer subtyping [32] and in prognostic multigene signatures [33, 34]. In breast cancer, *MAPT* expression seems to be an independent favorable prognostic parameter [31] influenced by ER and may be predictive of response to taxanes [35]. *MMP7*, one out of many matrix metalloproteases that are involved in the breakdown of extracellular matrix in normal physiological processes and in wound healing, has been shown to promote breast cancer cell invasiveness in vitro [36]. *RACGAP1*, a GTPase-activating protein, is essential for the induction of cytokinesis [37] and may therefore promote cancer growth. The impact of the same mRNA markers on patient outcome was further examined in paired primary and metastatic lymph node samples.

## Materials and methods

### Patients and tissues

For the purposes of the present study, the clinical records and tissue material from 369 patients that had participated in the clinical trial HE10/97 conducted by the Hellenic Cooperative Oncology Group were retrieved. Patient and treatment characteristics have previously been published [38];

briefly, all patients had received dose-dense sequential epirubicin (E) and CMF with or without the addition of paclitaxel (T). From this clinical cohort, 349 patient cases, 442 paraffin blocks, and 527 RNA samples were included in the present study according to (a) availability of gene expression data for all five mRNA markers examined, (b) matched non-macrodissected (NMD) and macrodissected (MD) RNA samples, and (c) matched primary/lymph node RNA samples. Gene expression was analyzed in three series of matched RNA samples: (a) MD vs NMD from primary tumors (P); (b) MD vs NMD from metastatic lymph nodes [39]; and (c) matched P and LN samples (mP, mLN). The outline of these study groups is shown in Fig. 1; detailed patient demographics, clinical data, and standard tumor characteristics for all paired series are presented in ESM\_1 (ESM\_1\_1). All breast carcinomas were centrally assessed with immunohistochemistry (IHC) for ER, PgR (scored according to [40]), and HER2, as well as with FISH for ERBB2 gene amplification (scored according to [41]). All patients had signed an informed consent form permitting the use of their biologic material for research purposes. The

study was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine.

Tissue sections were macrodissected where possible in cases with <75 % tumor cells in the whole section in order to increase tumor cell content (TCC) in the molecular sample. Samples were thus distinguished as MD (macrodissected) and NMD (non-macrodissected, whole sections) and are referred to as such throughout this manuscript. Histologic components were recorded as continuous variables (ESM\_1\_2). More details on manual macrodissection are described in ESM\_1.

#### RNA extraction and mRNA expression investigations

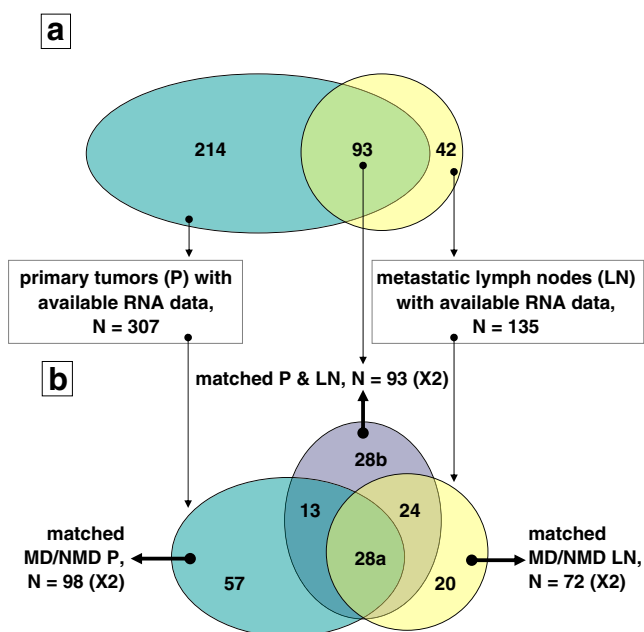
RNA extraction from 527 tissue samples was performed using a fully automated silica-coated magnetic bead-based method in combination with a liquid handling robot (VER-SANT Tissue Preparation System, Siemens Healthcare Diagnostics), as previously described [8, 42]. Details on RNA extraction and processing with reverse transcription quantitative real time PCR (RT-qPCR) are described in ESM\_1. The assays used for *ESR1*, *ERBB2*, *MAPT*, *MMP7*, and *RACGAP1* mRNA expression and their performance characteristics are shown in ESM\_1\_3. Relative quantification (RQ) values were assessed linearly as  $(40 - dCT)$ , whereby  $dCT = \text{triplicate mean}(CT_{\text{target}} - CT_{\text{RPL37A}})$ .

#### Statistics

This study involved paired sample analyses regarding TCC%, sample site, and RQ values.  $TCC_{\text{NMD}}$  represents the percentage of neoplastic cells in the whole section and  $TCC_{\text{MD}}$ , the percentage of neoplastic cells in the dissected tissue area. deltaTCC variables were calculated as  $TCC_{\text{MD}} - TCC_{\text{NMD}}$  for matched  $P_{\text{MD/NMD}}$  and  $LN_{\text{MD/NMD}}$  samples, and as  $TCC_{\text{mLN}} - TCC_{\text{mP}}$  for the corresponding matched samples.

RQ values were used as continuous variables throughout this study. For comparisons of individual mRNA expression between paired samples, deltaRQ variables were calculated as follows:  $\text{deltaRQ}_P = (RQ_{P_{\text{MD}}}) - (RQ_{P_{\text{NMD}}})$ ;  $\text{deltaRQ}_{LN} = (RQ_{LN_{\text{MD}}}) - (RQ_{LN_{\text{NMD}}})$ ; and  $\text{deltaRQ}_{P/LN} = (RQ_{\text{mLN}}) - (RQ_{\text{mP}})$ .

TCC percentage, deltaTCC, percentage of normal glandular breast structures, epithelial hyperplasia, and in situ carcinoma component were correlated with RQ and deltaRQ values using regression analysis. RQ values were compared against nominal values (ER/PgR IHC and HER2 status) using the Mann–Whitney test and for bivariate correlations with the Spearman's test. RQ values were also compared for the same gene in paired samples with the Wilcoxon signed ranks test. deltaRQ variables were evaluated for changes in transcript levels between paired sample series with one-sample *t* test by taking into account the two-sided 95 % CI.



**Fig. 1** Outline of paired sample groups. *P*, primary tumor; *LN*, metastatic lymph node; *MD*, macrodissected; *NMD*, non-macrodissected. **a** The entire HE10/97 sample series with available RNA expression data are shown (in total, 442 FFPE tumor blocks from 349 patients). Matched P and LN samples were available in 93 cases. **b** Paired sample groups and overlapping are shown. The matched MD/NMD P series included 92 and the matched MD/NMD LN series included 72 sample pairs. Out of the MD/NMD P series, 41 (13+28a) MD samples were included in the P component of the matched P and LN series. Similarly, out of the matched MD/NMD LN series, 48 (20+28a) MD samples were included in the LN component of the matched P and LN series. Four samples ( $P_{\text{MD}}$ ,  $P_{\text{NMD}}$ ,  $LN_{\text{MD}}$ , and  $LN_{\text{NMD}}$ ) were available in 28 cases (28a). A subset of P ( $N=157$ ) and LN samples ( $N=22$ ) shown in **a** was not eligible for paired sample analysis, as shown in **b**

All above analyses concerned individual markers of gene expression. However, (a) molecules act in concert in biological systems, hence their ratios are important; and (b) changes in RQ values in paired samples might be due to changes in the expression of the reference gene rather than of the target gene. Therefore, RQ values of all markers were profiled for each sample group with hierarchical clustering by using the JMP v8.0.2 software (SAS). The number of clusters was selected based on the joint assessment of (a) the ability of the clusters to form meaningful biological patterns and (b) the cubic clustering criterion and the pseudo *F*-statistic. In order to describe clustered RQ values in matched paired groups, we used canonical discriminant analysis measuring the distance between clusters for each sample group and the contribution of each variable in the clustering process. Based on these results, clustering concordance was evaluated with simple Kappa statistics.

The main question addressed in this study was whether assessing mRNA expression markers in the above-described different sample series would yield a different prognostic impact for these markers. For this purpose, individual continuous *ESR1*, *ERBB2*, *MAPT*, *MMP7*, and *RACGAP1* RQ variables from each sample group were initially submitted to univariate Cox analysis for correlations with patient disease-free (DFS) and overall survival (OS) that were calculated as previously described [30, 31, 38, 43].

Next, the discriminatory ability of the clusters of the above RQ values regarding DFS and OS was assessed by applying the C-index along with the 95 % CI [44, 45]. Clusters were compared against each other in each group with univariate Cox for assessing the predicted risk of events with 95 % CI's.

## Results

### TCC% in paired sample series and impact on individual mRNA marker expression

The distribution of TCC% in all sample series examined is shown in Table 1 and in ESM\_2 (ESM\_2\_1). The higher efficiency of MD in LN as compared to P samples was expected based on the more diffuse growth patterns of primary tumors as compared to metastatic foci in lymph nodes (examples are shown in ESM\_2\_2). Matched P and LN (mP and mLN) series included both MD and NMD samples. In comparison to the samples of the entire cohort (Table 1), TCC was >25 % in P<sub>MD</sub> and LN<sub>MD</sub>, as well as in mP and mLN samples.

Variations of relative quantification (RQ) values between paired samples are shown in Fig. 2 and in ESM\_3 (ESM\_3\_1 and ESM\_3\_2). deltaRQ values appeared higher or lower up to more than 6 units corresponding to 6 cycles

**Table 1** Tumor cell content (TCC%) in the various study cohorts

	P	LN
TCC%, NMD, <i>n</i> samples	98	72
Mean	27.0	30.1
Median	25.0	35.0
SD	14.8	18.0
Minimum	2.5	2.5
Maximum	70.0	75.0
TCC%, MD, <i>n</i> samples	98	72
Mean	68.1	82.0
Median	67.5	90.0
SD	20.5	18.2
Minimum	35.0	27.5
Maximum	97.5	95.0
TCC%, matched P and LN, <i>n</i> samples	93	93
Mean	79.9	63.3
Median	88.0	60.0
SD	16.6	18.0
Minimum	28.0	35.0
Maximum	95.0	98.0
TCC%, entire cohort, <i>n</i> samples <sup>a</sup>	307	135
Mean	61.4	76.6
Median	60.0	85.0
SD	18.8	19.0
Minimum	3.0	10.0
Maximum	98.0	95.0

NMD non-macrodissected, MD macro-dissected, P primary, LN metastatic lymph

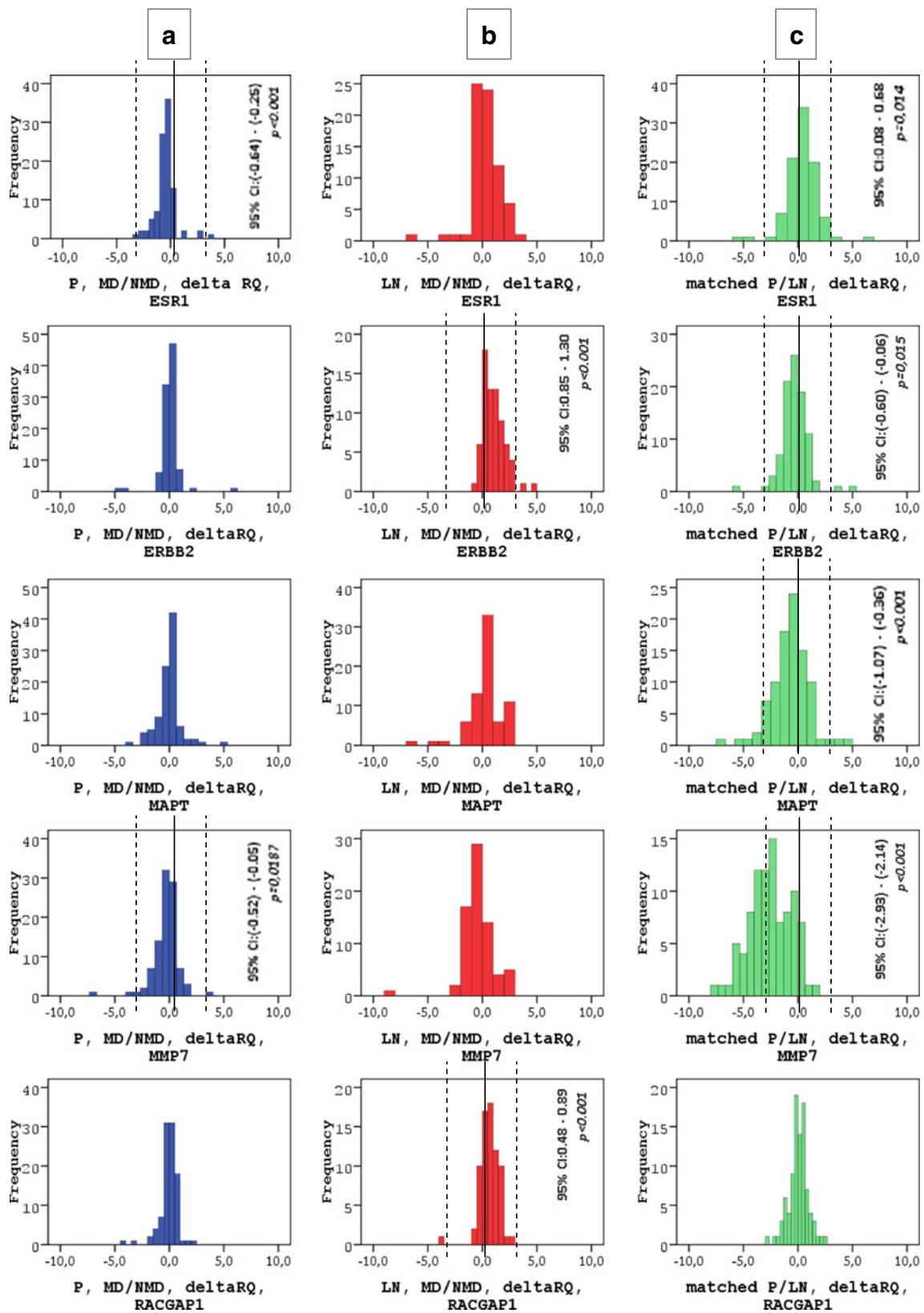
<sup>a</sup> Mixed MD and NMD samples

because RQ values were calculated linearly. Considering that 3 cycles correspond to a difference of tenfold in gene expression, the differences observed in individual matched pairs reached relative gene expression differences up to more than a hundredfold, in both directions.

Macrodissection efficiency in increasing TCC% was related to higher RQ values in LN<sub>MD</sub> samples for *ESR1*, *ERBB2*, and *MAPT*, as well as in P<sub>MD</sub> samples for *ERBB2*

**Fig. 2** Difference in the expression of individual *ESR1*, *ERBB2*, *MAPT*, *MMP7*, and *RACGAP1* mRNA values in paired sample series. deltaRQ values are shown. P, primary tumor; LN, metastatic lymph node; MD, macrodissected; NMD, non-macrodissected. For P and LN MD/NMD pairs, deltaRQ = RQ<sub>MD</sub> - RQ<sub>NMD</sub>; for P/LN pairs, deltaRQ = RQ<sub>mLN</sub> - RQ<sub>mP</sub>. Although outliers were found in both the P and LN series, relative *ESR1* and *MMP7* mRNA expression was generally lower in P<sub>MD</sub> than in P<sub>NMD</sub> samples (a), while relative *ESR1*, *ERBB2*, and *RACGAP1* expression appeared generally increased in LN<sub>MD</sub> as compared to LN<sub>NMD</sub> samples (b). In matched P/LN samples (c), *MMP7* was expressed greater than tenfold lower in P as compared to matched LN metastases, while approximately 1/4 of *ERBB2* and 1/3 of *MAPT* RQ values were more than twofold lower in matched P as well. One-sample *t* test 95 % CI and significant variability in deltaRQ values are shown





mRNA (ESM\_3\_3). In addition, in primary tumors, the extent of non-neoplastic breast tissue that was removed

upon MD influenced *ERBB2* and *RACGAP1*, while the extent of in situ carcinoma areas influenced *MMP7* RQ

values in  $P_{MD}$  samples (ESM\_3\_4). The associations of individual marker mRNA expression with classic IHC parameters (ER, PgR, and HER2) were not altered in  $P_{MD}$  as compared to  $P_{NMD}$  samples (ESM\_3\_5) but varied between  $LN_{MD}$  and  $LN_{NMD}$  samples (ESM\_3\_6) and also between mP and mLN samples (ESM\_3\_7). When comparing all results from ESM\_3\_5, \_3\_6, and \_3\_7, it would be expected that marker associations be preserved in the  $P_{MD}$  and mP, as well as in the  $LN_{MD}$  and mLN sample groups. Such associations were indeed preserved for *ESR1* and *ERBB2* expression but not for *MAPT*, *MMP7*, and *RACGAP1*, indicating that the above cohorts were not comparable with each other.

The strongest positive correlations of RQ values were observed between *ESR1* and *MAPT* in all matched sample groups (all Spearman's  $r > 0.5$ ), while positive correlations between *ERBB2* and *RACGAP1* were encountered in LN samples only (all  $r > 0.35$ ) (ESM\_3\_8). Negative correlations were observed between the RQ values of *MMP7* and *ESR1*, as well as *MAPT*, with  $r$  values ranging between  $-0.23$  and  $-0.35$ .

#### Clustering of *ESR1*, *ERBB2*, *MAPT*, *MMP7*, and *RACGAP1* RQ values

Hierarchical clustering of the five mRNA markers was applied as described in the "Methods" section in the entire primary tumor and lymph node samples of the HE10/97 project and revealed four distinct categories of tumors in each series, with distinct characteristics (Fig. 3). Based on the above findings, for the biological characterization of clusters, except for the established roles of *ESR1* and *ERBB2* in breast cancer, we considered *MAPT* as a marker reflecting estrogen receptor activity [35] and *RACGAP1* as a marker of proliferating cells [46]. Clusters were designated according to the established molecular subtypes of breast cancer as luminal A (LumA), luminal B (LumB), HER2-enriched (HER2), and triple negative (TN) (Fig. 3). These clusters were obtained for  $P_{MD}$  and  $P_{NMD}$  samples, for  $LN_{MD}$ , and for mP and mLN samples. In the  $LN_{NMD}$  series, the LumB cluster could not be formed, since it was represented by only one sample. Cluster discrimination in the paired sample series is shown in Fig. 4. Clusters LumA and LumB showed considerable overlapping in all groups, while HER2 and TN clusters were sharply distinguished. *MMP7* RQ values had the lowest determinant role in cluster formation, while the role of *RACGAP1* varied in the different sample groups. Detailed cluster statistics showing analogies between standardized values, driver genes, and discrimination ability are shown in ESM\_4 (ESM\_4\_1 to ESM\_4\_4).

Cluster concordance in paired sample groups is shown in ESM\_4\_5. Importantly, although cluster concordance for paired groups ranged from only from 64 to 80 %, it did

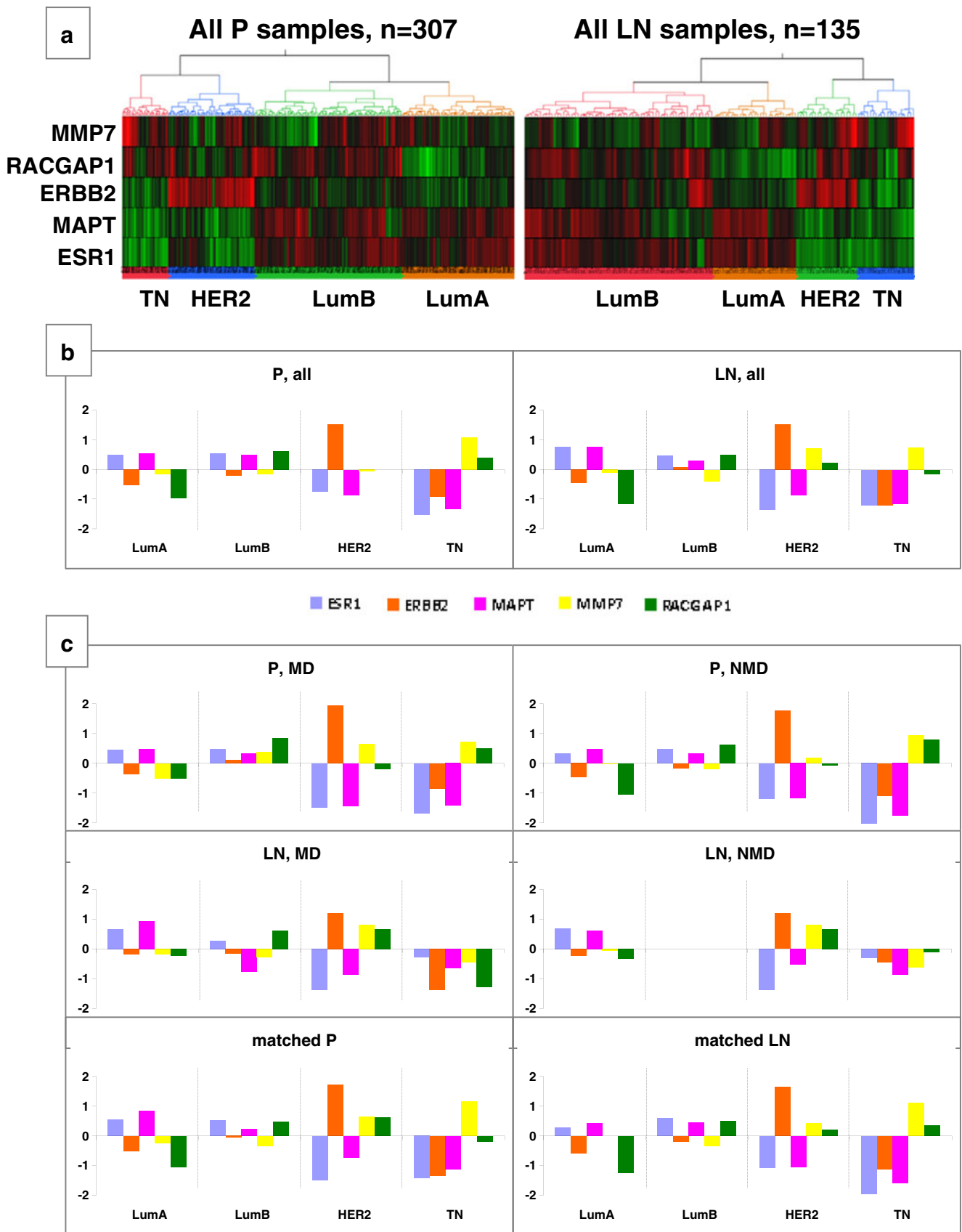
not statistically differ in samples with TCC  $< 20$  % vs those with original TCC  $\geq 20$  % within the same paired sample series (ESM\_4\_6). Finally, cluster associations with standard breast cancer markers, such as ER IHC and HER2 status determined in primary tumors only, were statistically significant for all comparisons (ESM\_4\_7).

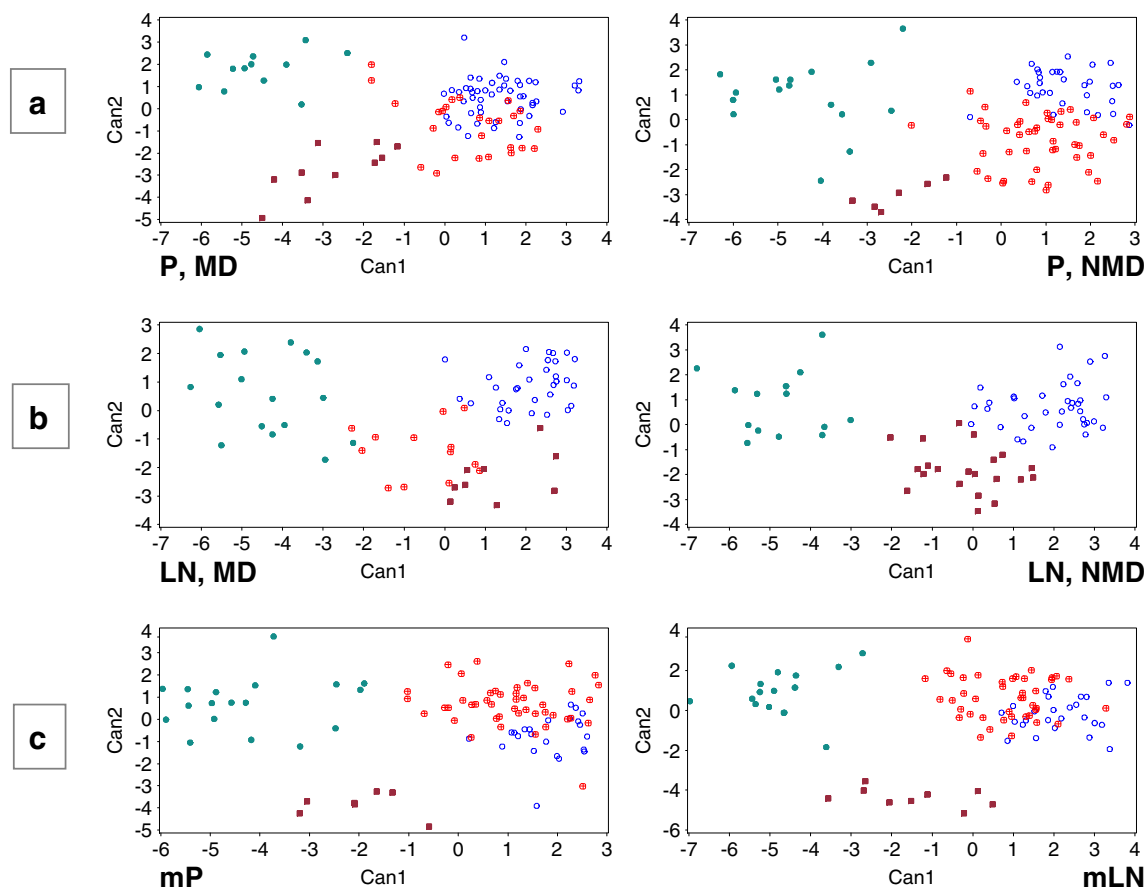
#### Comparison of *ESR1*, *ERBB2*, *MAPT*, *MMP7*, and *RACGAP1* mRNA expression, individually and clustered, with patient outcome

As shown in Table 2, no strongly significant differences were observed with respect to TCC% for *ESR1*, *ERBB2*, *MAPT*, *MMP7*, and *RACGAP1* mRNA, when these markers were analyzed individually as continuous variables in paired  $P_{MD/NMD}$  and  $LN_{MD/NMD}$  samples. The only weak difference concerned the unfavorable prognostic effect of relatively high *MMP7* in  $P_{MD}$ , which was not observed in paired  $P_{NMD}$  samples. This may be explained because *MMP7* is a stromal marker, and  $P_{MD}$  samples are expected to contain more tumor–stroma-specific mRNA than  $P_{NMD}$  samples. However, hazard ratios and 95 % confidence intervals for *MMP7* were similar in  $P_{MD}$  and in  $P_{NMD}$ . Thus, the observed difference in *MMP7* statistical significance between  $P_{MD}$  and  $P_{NMD}$  samples was not considered as clinically significant. Similarly, the difference observed for relatively high *MAPT* as a favorable prognosticator for patient OS in mLN, but not in mP samples, was also not considered as clinically relevant. In this mP/mLN paired series, relatively high *RACGAP1* was strongly associated with unfavorable DFS and OS only when examined in mLN samples (Table 2). However, these differences appeared to be paired sample cohort-specific, since, when examined in the entire HE10/97 population, *RACGAP1* was an unfavorable prognostic parameter when examined in both primary tumor and in metastatic lymph node series.

For cluster analysis with respect to patient outcome, cluster discrimination based on the C-index did not reveal any differences for both DFS and OS between paired groups, as shown in ESM\_5 (ESM\_5\_1). Accordingly, no difference was observed in the prognostic relevance of the four clusters in  $P_{MD}$  as compared to the  $P_{NMD}$  group (Table 3 and Fig. 5a). The paired  $LN_{MD/NMD}$  groups were practically

**Fig. 3** Biological relevance of the four-cluster model. Hierarchical clustering was set to define four clusters corresponding to *LumA* (*ESR1* and *MAPT* high), *LumB* (*ESR1* high and high *RACGAP1*/*MAPT*), *HER2* (*HER2* high/*ESR1* and *MAPT* low), and TN (*ESR1*, *HER2* and *MAPT* low) breast cancer subtypes, as shown in **a** and **b**. These clusters were initially identified in the entire series of primary tumor (*P*) and metastatic lymph node (*LN*) of the HE10/97 cohort. **c** The major analogies observed in **b** were preserved in paired sample series for *LumA*, *LumB*, *HER2*, and *TN*. LumB could not be identified in  $LN_{NMD}$  samples. Standardized values correspond to mean of 0 and a standard deviation of 1





**Fig. 4** LumA, LumB, HER2, and TN clusters in paired primary and lymph node samples. Canonical discriminant structure is shown for macrodissected/non-macrodissected (MD/NMD) primary tumors (**a**), metastatic lymph nodes (LN) MD/NMD (**b**), and matched primary

tumors and metastatic lymph nodes (mP/mLN) (**c**). *Blue*, LumA; *red*, LumB; *green*, HER2; *brown*, TN. Clusters in A and C followed the same pattern of discrimination and overlapping, which differed in the LN MD/NMD series

not comparable for cluster performance, since LumB could not be formed in the LN<sub>NMD</sub> samples. Instead, LumA and especially TN tumors were overrepresented in this group, yielding statistically significant results (Table 3 and Fig. 5b). In comparison, the more accurately classified HER2 clusters were associated with worst prognosis in both LN<sub>MD</sub> and LN<sub>NMD</sub> series. Cluster comparisons for patient outcome in the mP/mLN series revealed the expected worse performance of LumB, HER2, and TN in comparison to LumA tumors only in the mLN series (Table 3 and Fig. 5c). Finally, in the entire HE10/97 P and LN cohorts, the same prognostic significance was revealed for the four clusters in P samples unrelated to TCC%, while comparable significance was observed in LN samples with higher TCC% only (ESM\_5\_2).

Multivariate COX analysis was applied in each one of the paired groups and in the entire HE10/97 sample cohorts for observing the interference of the obtained clusters with standard clinicopathologic parameters (age, menopausal status, grade, tumor size, number of metastatic lymph nodes, chemotherapy regimen, hormonal therapy, ER and PgR

IHC, and HER2 status) in paired sample series. The statistically significant results from this analysis are presented in ESM\_5\_3. The clinical significance of these findings should be assessed with caution, because small sample numbers for several categories and possible cohort specificity of the clusters yielded large confidence intervals, implying that the observed hazard ratios may not replicate in a future study. Besides cohort specificity of the findings, it should be noticed that calling a tumor as HER2-positive by IHC/FISH and as HER2-enriched by RQ-value clustering was not necessarily identical (ESM\_4\_7). Overall, though, hazard ratios for the clusters in the univariate (Table 3) and in the adjusted multivariate analysis (ESM\_5\_3) were either close to each other, or they were at least in the same direction (favorable or unfavorable).

## Discussion

The purpose of this study was to investigate whether TCC in molecular samples affects the clinical relevance of broadly



**Table 2** Impact of TCC% and tumor site on the clinical relevance of gene expression markers in paired sample groups (univariate Cox analysis with RNA markers as continuous variables)

Sample group	Marker	Disease-free survival			Overall survival		
		HR	CI 95 %	Wald's <i>p</i>	HR	CI 95 %	Wald's <i>p</i>
Primary tumors, matched MD/NMD pairs, <i>N</i> =98							
MD	ESR1	0.98	0.86–1.11	0.7034	0.98	0.85–1.14	0.8225
	ERBB2	1.07	0.90–1.26	0.4577	1.04	0.84–1.27	0.7301
	MAPT	0.87	0.75–1.01	0.0604	0.88	0.74–1.05	0.1605
	MMP7 <sup>a</sup>	1.26	1.03–1.53	0.0226	1.26	1.01–1.59	0.0432
	RACGAP1	1.37	1.01–1.86	0.0415	1.86	1.29–2.67	0.0008
NMD	ESR1	1.01	0.88–1.15	0.9194	1.02	0.87–1.19	0.8425
	ERBB2	1.07	0.88–1.29	0.5067	1.03	0.82–1.30	0.7967
	MAPT	0.89	0.75–1.06	0.1803	0.92	0.75–1.13	0.4419
	MMP7	1.10	0.90–1.33	0.3592	1.13	0.90–1.41	0.2957
	RACGAP1	1.31	0.99–1.73	0.0569	1.66	1.22–2.27	0.0013
Metastatic lymph nodes, matched MD/NMD pairs, <i>N</i> =72							
MD	ESR1	0.91	0.81–1.03	0.1314	0.88	0.76–1.03	0.1076
	ERBB2	1.23	1.06–1.44	0.0079	1.18	0.96–1.44	0.1212
	MAPT	0.84	0.71–1.00	0.0530	0.77	0.62–0.96	0.0183
	MMP7	1.16	0.99–1.35	0.0615	1.21	0.99–1.46	0.0564
	RACGAP1	2.12	1.40–3.22	0.0004	2.77	1.51–5.08	0.0010
NMD	ESR1	0.89	0.76–1.04	0.1537	0.87	0.72–1.05	0.1442
	ERBB2	1.27	1.08–1.48	0.0035	1.11	0.91–1.37	0.3051
	MAPT	0.89	0.71–1.11	0.3098	0.71	0.53–0.96	0.0283
	MMP7	1.15	0.97–1.38	0.1151	1.07	0.86–1.32	0.5397
	RACGAP1	1.76	1.34–2.30	0.0001	1.40	1.05–1.86	0.0223
Primary tumors and matched metastatic lymph nodes, <i>N</i> =93							
Primary tumours	ESR1	0.98	0.87–1.10	0.6956	0.93	0.80–1.07	0.2891
	ERBB2	0.98	0.83–1.16	0.8369	0.92	0.74–1.14	0.4393
	MAPT	0.94	0.83–1.07	0.3526	0.93	0.80–1.09	0.3881
	MMP7	1.01	0.84–1.23	0.8890	1.05	0.83–1.31	0.7039
	RACGAP1	1.19	0.88–1.61	0.2652	1.45	0.99–2.11	0.0541
Metastatic lymph nodes	ESR1	0.95	0.85–1.06	0.3337	0.93	0.82–1.06	0.2648
	ERBB2	1.07	0.93–1.23	0.3399	1.05	0.88–1.25	0.5719
	MAPT <sup>a</sup>	0.91	0.80–1.03	0.1344	0.84	0.72–0.98	0.0275
	MMP7	1.05	0.92–1.19	0.4793	1.06	0.90–1.24	0.5030
	RACGAP1 <sup>b</sup>	1.82	1.29–2.57	0.0006	2.27	1.43–3.58	0.0005
Entire HE 10/97 cohort							
Primary tumors, <i>N</i> =315	ESR1	0.94	0.88–1.00	0.0656	0.90	0.84–0.97	0.0071
	ERBB2	1.10	1.01–1.20	0.0256	1.09	0.98–1.20	0.1098
	MAPT	0.91	0.85–0.98	0.0081	0.89	0.82–0.97	0.0095
	MMP7	1.06	0.97–1.16	0.1980	1.06	0.95–1.18	0.2870
	RACGAP1	1.30	1.09–1.54	0.0026	1.48	1.20–1.82	0.0002
Metastatic lymph nodes, <i>N</i> =135	ESR1	0.93	0.85–1.02	0.1416	0.91	0.82–1.01	0.0841
	ERBB2	1.07	0.95–1.20	0.2629	1.08	0.94–1.24	0.2749
	MAPT	0.90	0.80–1.00	0.0509	0.85	0.75–0.97	0.0183
	MMP7	1.05	0.94–1.17	0.3772	1.05	0.91–1.20	0.5234
	RACGAP1	1.63	1.22–2.17	0.0010	1.86	1.30–2.67	0.0008

MD macrodissected (higher TCC%), NMD non-macrodissected (lower TCC%), CI confidence intervals, HR hazard ratios

<sup>a</sup>Weak differences between paired sample groups, as indicated by the respective CI 95 % and HR

<sup>b</sup>Significant difference for RACGAP1 expression as an unfavorable marker in metastatic lymph nodes but not in the paired primary tumors

**Table 3** Univariate COX comparison of cluster prognostic value in paired sample groups (Wald’s *p*)

Sample group	Comparison	Disease-free survival			Overall survival		
		HR	95 % CI	<i>p</i>	HR	95 % CI	<i>p</i>
P, MD	Lum B vs Lum A	4.63	1.96–10.95	0.0005	6.02	2.11–17.12	0.0008
	HER2 vs Lum A	2.81	0.92–8.58	0.0705	2.43	0.58–10.20	0.2248
	TN vs Lum A	4.28	1.40–13.12	0.0110	5.11	1.37–19.06	0.0152
	Lum B vs HER2	1.65	0.60–4.55	0.3318	2.48	0.70–8.80	0.1612
	Lum B vs TN	1.08	0.39–2.98	0.8779	1.18	0.38–3.66	0.7770
	HER2 vs TN	0.66	0.19–2.27	0.5050	0.48	0.11–2.13	0.3317
P, NMD	Lum B vs Lum A	3.34	1.25–8.97	0.0165	13.26	1.76–100.1 <sup>a</sup>	0.0122
	HER2 vs Lum A	2.68	0.82–8.78	0.1043	8.12	0.91–73.34 <sup>a</sup>	0.0601
	TN vs Lum A	4.20	1.00–17.67	0.0502	20.20	2.09–195.1 <sup>a</sup>	0.0094
	Lum B vs HER2	1.25	0.50–3.13	0.6348	1.62	0.54–4.85	0.3887
	Lum B vs TN	0.80	0.23–2.70	0.7147	0.66	0.19–2.26	0.5051
	HER2 vs TN	0.64	0.16–2.56	0.5253	0.41	0.09–1.82	0.2382
LN, MD	Lum B vs Lum A	2.15	0.74–6.22	0.1583	2.71	0.68–10.83	0.1596
	HER2 vs Lum A	3.1	1.28–7.9	0.0176	3.71	1.08–12.72	0.037
	TN vs Lum A	0.42	0.05–3.4	0.4184	0.95	0.11–8.63	0.9669
	Lum B vs HER2	0.69	0.25–1.92	0.4802	0.73	0.21–2.5	0.6154
	Lum B vs TN	5.08	0.61–42.2	0.1325	2.83	0.31–25.58	0.3533
	HER2 vs TN	7.33	0.93–57.5	0.0581	3.89	0.48–31.74	0.2051
LN, NMD	Lum B vs Lum A						
	HER2 vs Lum A	4.86	1.79–13.16	0.0019	6.21	1.54–24.97	0.0101
	TN vs Lum A	2.42	0.88–6.69	0.088	4.6	1.19–17.82	0.027
	Lum B vs HER2						
	Lum B vs TN						
	HER2 vs TN	2.01	0.77–5.22	0.154	1.35	0.45–4.03	0.5922
Matched P	Lum B vs Lum A	2.15	0.86–5.35	0.101	3.01	0.86–10.54	0.0841
	HER2 vs Lum A	2.17	0.75–6.28	0.1512	2.55	0.61–10.69	0.202
	TN vs Lum A	1.3	0.26–6.49	0.7431	3.23	0.53–19.53	0.2025
	Lum B vs HER2	0.99	0.43–2.24	0.9758	1.18	0.42–3.33	0.7492
	Lum B vs TN	1.64	0.38–7.04	0.5041	0.93	0.21–4.13	0.9287
	HER2 vs TN	1.66	0.35–7.84	0.5204	0.79	0.15–4.13	0.7793
Matched LN	Lum B vs Lum A	<b>3.47</b>	<b>1.29–9.32</b>	<b>0.0137</b>	<b>13.32</b>	<b>1.71–103.5<sup>a</sup></b>	<b>0.0133</b>
	HER2 vs Lum A	<b>3.83</b>	<b>1.25–11.78</b>	<b>0.019</b>	<b>10.21</b>	<b>1.19–87.75<sup>a</sup></b>	<b>0.0343</b>
	TN vs Lum A	3.24	0.86–12.12	0.0811	<b>20.6</b>	<b>2.24–189.4<sup>a</sup></b>	<b>0.0075</b>
	Lum B vs HER2	0.9	0.40–2.07	0.8125	1.31	0.44–3.88	0.6315
	Lum B vs TN	1.07	0.36–3.15	0.901	0.65	0.21–1.98	0.445
	HER2 vs TN	1.18	0.36–3.94	0.7835	0.5	0.13–1.96	0.3167

Bold values indicate significant differences observed between comparable paired sample groups; italicized values indicate significant difference between LN MD/NMD paired samples due to overrepresentation of the TN cluster in the LN<sub>NMD</sub> group, where the luminal B cluster could not be formed

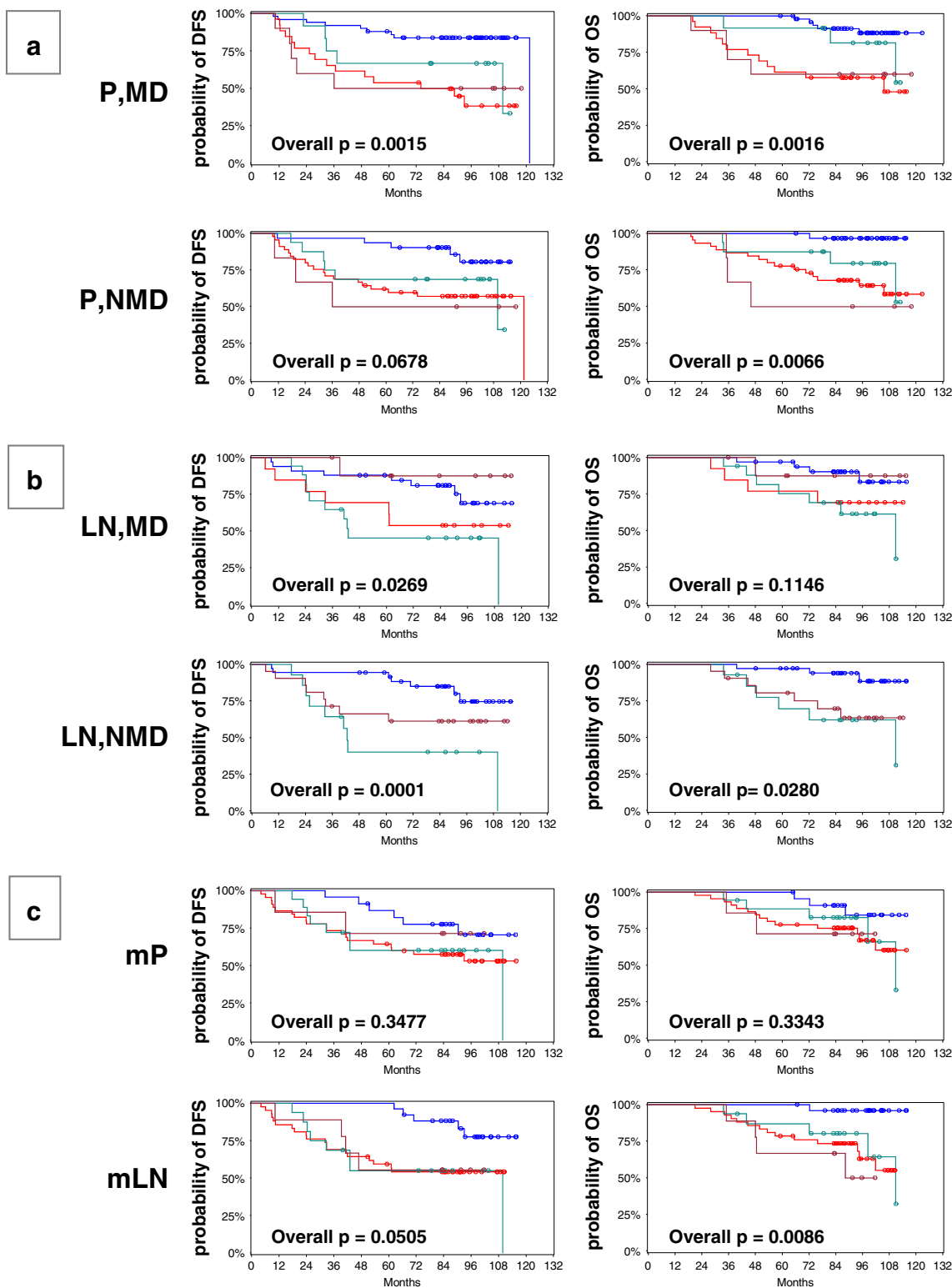
HR hazard ratio, CI confidence interval, P primary tumor, LN metastatic lymph node, MD macrodissected, NMD non-macrodissected, TN triple negative

<sup>a</sup>Broad 95 % CI’s were observed in these cases because of the limited number of events in the luminal A patient population

applied RNA markers in breast cancer research. Our data show that, independently of molecular sample TCC rate, RNA clusters with the markers examined yield the same prognostic information. This appears as a paradox but it is not, since clusters are, basically, ratios between marker measurements. Thus, although individual marker measurements do vary between matched samples with low and high TCC, as previously established [2–7], their analogies in such samples from the same tumor seem to be preserved. These results are in concordance with the only relevant

published study so far [2], which employed fresh tissues from a limited number of patients, multiple sites from the same section, and microarray gene profiling. Our findings are also in line with a more recent study [47] showing that normal tissues in the presence of breast cancer may express the same ER-positive or ER-negative gene profiles as the hosted tumor, in a broad sense of field cancerization.

It is impossible and inapplicable to suggest a safe TCC cutoff for assessing RNA markers in primary tumor samples based on the results of the present study. For establishing an



**Fig. 5** Comparison of overall patient outcome according to LumA, LumB, HER2 and TN classification of primary tumors and their metastases in lymph nodes. **a** Clusters are compared in paired groups from primary tumors (*P*), macrodissected (*MD*), and non-

macrodissected (*NMD*). **b** Clusters are compared in paired metastatic lymph node (*LN*) *MD* and *NMD* samples. **c** Matched *P* and *LN* samples. Log-rank test significance is shown. *Blue*, LumA; *red*, LumB; *green*, HER2; *brown*, TN

optimal cutoff, multiple RNA samples should have been prepared from every single histological sample with various

but precise TCC rates (for example, 10, 30, 50, 70, and 100 %), involving the same tumor site (for example, tumor

front). Further, in order to obtain adequate statistical power, the major breast cancer subtypes *and* the multiplicity of non-cancerous histological elements and tumor microenvironment, which would be contained in the 100–TCC% of the sample, should be considered in large numbers. To our knowledge, a study taking into account all of the above parameters has not yet been performed. Our data show that the 10 % TCC previously described [2] may not be irrelevant for assessing RNA markers in primary breast tumor samples. A safe conclusion from the present study may be that the commonly published 70 or 75 % TCC cutoff as an eligibility criterion for primary tumors seems overrated and results in the exclusion of large numbers of samples from translational study cohorts, thereby lowering the statistical power of such studies. Clearly, the low TCC allowance for RNA investigations should not apply for DNA studies [48].

In comparison to primary tumors, TCC seemed to affect the clinical relevance of clusters in metastatic lymph nodes, although not of single markers. This condition may be *RACGAPI*-related, since this marker is expressed in lymphocytes as well [46] and will not be tumor-specific in a lymph node environment. However, the clinical relevance of these clusters in the LN<sub>MD</sub> and in the mLN groups was not the same, suggesting sample cohort bias, which is expected in fragmented sample series. Hence, TCC alone did not seem to determine the clinical relevance of the markers examined in the present study in metastatic lymph node samples.

Our data also suggest that it is ineligible to substitute for primary tumor samples with lymph node metastases and *vice versa* for translational study purposes, since the same RNA markers may have different clinical relevance when examined in each setting. Regional lymph node metastases are usually diagnosed simultaneously with the primary tumor and are, hence, not considered as a metachronous disease development. These regional metastases may not share the phenotypic characteristics of the primary tumor [22, 24–27, 49], one of the reasons being the evolution of different metastatic clones from a heterogeneous genetic background in the primary tumor [50]. Although, again, cohort bias may underlie the presented results from the mP and mLN series, our data indicate that when histologic material from both primary tumor and metastatic lymph nodes is available, it may be more informative, for example, to evaluate the HER2-enriched subtype in lymph nodes than in primary tumors. Whether such an approach should be integrated into practice for clinical decision-making is a question to be answered in prospective studies.

Concerning individual markers, *RACGAPI* has recently been revealed as a proliferation marker associated with prognosis in breast cancer [51, 52]. Herein we show that its expression may undergo changes similar to those described for Ki-67 in metastatic lymph nodes vs primary

tumors [24], which, at least in the present series, seemed *ERBB2*-related. In addition, *MMP7* expression, a marker of epithelial–mesenchymal transition in colorectal cancer [53] and of invasiveness of breast cancer cells *in vitro* [36], may be associated with adverse outcome in a subset of primary breast carcinomas that needs to be defined.

Overall, in line with the previously described intrinsic characteristics of breast cancer [32, 54–56], the major genes determining the molecular subtypes in the four clusters were *ESR1* and *ERBB2*, followed by the ER-dependent *MAPT* and by the proliferation marker *RACGAPI*. The stromal factor *MMP7* did not significantly contribute in this rough subtype classification. The present study shows that, when examining RNA markers which are involved in pathways that are drivers in cancer cells but are of low activity in the coexisting non-cancer cells, such as in primary tumors surrounded by non-neoplastic breast tissue elements, TCC% may be of low importance for obtaining clinically relevant results. By contrast, when the same markers are examined in an environment where some of them may be expressed in non-cancerous cells, such as the proliferation pathway in lymph nodes bearing breast cancer metastases, TCC% may influence the prognostic significance of these markers. With the reservation that the results concerning “quantitative” or semiquantitative RNA markers, individually or in profiles, are overall cohort-specific in retrospective studies, our data may contribute to a more efficient and rational design of translational studies on FFPE tissues.

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**Conflicts of interest** We declare that we have no conflict of interest.

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