Breast Cancer Research and Treatment

Effects of TP53 and PIK3CA mutations in early breast cancer: a matter of co-mutation and tumor infiltrating lymphocytes.

--Manuscript Draft--

Manuscript Number:	BREA-D-16-00263R1
Full Title:	Effects of TP53 and PIK3CA mutations in early breast cancer: a matter of co-mutation and tumor infiltrating lymphocytes.
Article Type:	Clinical trial
Keywords:	TP53; PIK3CA; co-mutation; tumor infiltrating lymphocytes; p53 immunohistochemistry; adjuvant.
Corresponding Author:	Vassiliki Kotoula Aristotle University of Thessaloniki School of Medicine Thessaloniki, GREECE
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Aristotle University of Thessaloniki School of Medicine
Corresponding Author's Secondary Institution:	
First Author:	Vassiliki Kotoula
First Author Secondary Information:	
Order of Authors:	Vassiliki Kotoula
	Vasilios Karavasilis
	Flora Zagouri
	George Kouvatseas
	Eleni Giannoulatou
	Helen Gogas
	Sotiris Lakis
	George Pentheroudakis
	Mattheos Bobos
	Kyriaki Papadopoulou
	Eleftheria Tsolaki
	Dimitrios Pectasides
	Georgios Lazaridis
	Angelos Koutras
	Gerasimos Aravantinos
	Christos Christodoulou
	Pavlos Papakostas
	Christos Markopoulos
	George Zografos
	Christos Papandreou
	George Fountzilas
Order of Authors Secondary Information:	

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Abstract:	Purpose: To investigate whether the outcome of breast cancer (BC) patients treated with adjuvant chemotherapy is affected by co-mutated TP53 and PIK3CA according to stromal tumor infiltrating lymphocytes (TILs). Methods: Paraffin tumors of all clinical subtypes from 1661 patients with operable breast cancer who were treated within 4 adjuvant trials with anthracycline-taxanes chemotherapy were informative for TP53 and PIK3CA mutation status (semiconductor sequencing genotyping) and for stromal TILs density. Disease-free survival (DFS) was examined. Results: TP53 mutations were associated with higher ($p<0.001$) and PIK3CA with lower ($p=0.004$) TILs in an ER/PgR-specific manner ($p<0.001$). Mutations did not affect the favorable DFS of patients with lymphocyte predominant (LP) BC. Within non-LPBC, PIK3CA-only mutations conferred best, while TP53-PIK3CA co-mutations (6% of all tumors) worst DFS (HR 0.59; 95%CI 0.44-0.79; $p=0.001$ for PIK3CA-only). TP53-only mutations were unfavorable in patients with lower TILs, while patients with lower TILs performed worse if their tumors carried TP53-only mutations (interaction $p=0.046$). Multivariate analysis revealed favorable PIK3CA-only mutations in non-LPBC (HR 0.64; 95%CI 0.47-0.88; $p=0.007$), and unfavorable TP53 mutations in ER/PgRpos/HER2neg (HR 1.55; 95% CI 1.07-2.24; $p=0.021$). Mutations did not interact with TILs in non-LP triple-negative and HER2-positive patients. Conclusions: TP53 and PIK3CA mutations appear to have diverse effects on the outcome of early BC patients, according to whether these genes are co-mutated or not, and, for TP53 according to TILs density and ER/PgR-status. These findings need to be considered when evaluating the effect of these two most frequently mutated genes in the context of large clinical trials.
Response to Reviewers:	Thessaloniki, June 14, 2016 Dear BREA Editorial Office, Following your email from May 21, 2016, we are submitting the revised version of our work #BREA-D-16-00263, which we modified according to the reviewer's comments. Please find below our detailed answers to these comments. Reviewer #1: I read with interest the present manuscript. Data here presented are of potential interest. The most interesting observation is related to the number of mutant tumors in LPBC that was low. I have some suggestions: 1)Create an histogram with incidence of PIK3CA mutations across breast cancer subtypes defined as per St Gallen 2013 definition. 2)Create an histogram with incidence of p53 mutations across breast cancer subtypes defined as per St Gallen 2013 definition. Answer to comments (1) and (2): We followed the reviewer's suggestion and added in the revised manuscript: - the description of the two Ki67 cut-offs p.7 (Materials and Methods), and - a new supplementary Figure S2 in file ESM_1, Supplemental Methods, with the appropriate citation in the main revised text in p.9 (Results). 3)What can be interesting is to correlate PIK3CA or p53 mutations across subtypes with TILs. An interesting paper published by the German Breast Group (Budczies J et al. The Journal of Pathology: Clinical Research, 2015, Volume 1, Issue 4, pages 225-238) found very interesting correlation between specific mutations in EBC and mutational burden. It would be interesting if authors can correlated mutational status with TILs. Answer to comment (3): We thank the reviewer for this comment. Indeed, we are currently processing the analysis for all mutations in the same patient population with respect to TILs. We have cited the paper by Budczies J, et al, in p.12 (Discussion) of the revised manuscript. We hope that the incorporated revisions are satisfactory and that you will find the revised version of our manuscript suitable for publication.

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

	Sincerely, On behalf of the authors, Vassiliki Kotoula, MD	
Funding Information:	Hellenic Cooperative Oncology Group (HeCOG) (HE TRANS_BR)	Not applicable
	Ministry of Education, Lifelong Learning and Religious Affairs (ESPA-THALIS#266)	Not applicable

COPYRIGHT STATEMENT. This is a post-peer-review, pre-copyedit version of an article published in Breast Cancer Research and Treatment. The final authenticated version is available online at: http://dx.doi.org/10.1007/s10549-016-3883-z.

Manuscript
Click here to download Manuscript BREA-D-16-00263 R1 NoTrCh.doc
Click here to view linked References

Effects of TP53 and PIK3CA mutations in early breast cancer: a matter of co-mutation and tumor infiltrating lymphocytes

Vassiliki Kotoula[1,2], Vasilios Karavasilis[3], Flora Zagouri[4], George Kouvatseas[5], Eleni Giannoulatou[6,7], Helen Gogas[8], Sotiris Lakis[2], George Pentheroudakis[9], Mattheos Bobos[2], Kyriaki Papadopoulou[2], Eleftheria Tsolaki[2], Dimitrios Pectasides[10], Georgios Lazaridis[3], Angelos Koutras[11], Gerasimos Aravantinos[12], Christos Christodoulou[13], Pavlos Papakostas[14], Christos Markopoulos[15], George Zografos[16], Christos Papandreou[17], George Fountzilas[2,18]

[1]Department of Pathology, Aristotle University of Thessaloniki, School of Health Sciences, Faculty of Medicine, Thessaloniki, Greece; [2]Laboratory of Molecular Oncology, Hellenic Foundation for Cancer Research/Aristotle University of Thessaloniki, Thessaloniki, Greece; [3]Department of Medical Oncology, Papageorgiou Hospital, Aristotle University of Thessaloniki, School of Health Sciences, Faculty of Medicine, Thessaloniki, Greece; [4]Department of Clinical Therapeutics, Alexandra Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece; [5]Department of Biostatistics, Health Data Specialists Ltd, Athens, Greece; [6]Victor Chang Cardiac Research Institute, Darlinghurst, NSW, Australia; [7]The University of New South Wales, Kensington, NSW, Australia; [8]First Department of Medicine, Laiko General Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece; [9]Department of Medical Oncology, Ioannina University Hospital, Ioannina, Greece; [10]Oncology Section, Second Department of Internal Medicine, Hippokration Hospital, Athens, Greece; [11]Division of Oncology, Department of Medicine, University Hospital, University of Patras Medical School, Patras, Greece; [12]Second Department of Medical Oncology, Agii Anargiri Cancer Hospital, Athens, Greece; [13]Second Department of Medical Oncology, Metropolitan Hospital, Piraeus, Greece; [14]Oncology Unit, Hippokration Hospital, Athens, Greece; [15]Second Department of Prop. Surgery, Laiko General Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece; [16]Breast Unit, National and Kapodistrian University of Athens School of Medicine, Athens, Greece; [17]Department of Medical Oncology, University Hospital of Larissa, University of Thessaly School of Medicine, Larissa, Greece; [18]Aristotle University of Thessaloniki, Thessaloniki, Greece

Running title: TP53, PIK3CA & TILs in breast cancer

Corresponding author:

Vassiliki Kotoula, MD, PhD

Dept of Pathology, School of Medicine

Aristotle University of Thessaloniki (AUTH)

Faculty of Medicine &

Laboratory of Molecular Oncology

Hellenic Foundation for Cancer Research/ AUTH

University Campus

54006 Thessaloniki – GREECE

Tel: +30 2310 999 050

Fax: +30 2310 999 229

vkotoula@auth.gr

ABSTRACT

Purpose: To investigate whether the outcome of breast cancer (BC) patients treated with adjuvant chemotherapy is affected by co-mutated TP53 and PIK3CA according to stromal tumor infiltrating lymphocytes (TILs).

Methods: Paraffin tumors of all clinical subtypes from 1661 patients with operable breast cancer who were treated within 4 adjuvant trials with anthracycline-taxanes chemotherapy were informative for TP53 and PIK3CA mutation status (semiconductor sequencing genotyping) and for stromal TILs density. Disease-free survival (DFS) was examined.

Results: TP53 mutations were associated with higher ($p<0.001$) and PIK3CA with lower ($p=0.004$) TILs in an ER/PgR-specific manner ($p<0.001$). Mutations did not affect the favorable DFS of patients with lymphocyte predominant (LP) BC. Within non-LPBC, PIK3CA-only mutations conferred best, while TP53-PIK3CA co-mutations (6% of all tumors) worst DFS (HR 0.59; 95%CI 0.44-0.79; $p=0.001$ for PIK3CA-only). TP53-only mutations were unfavorable in patients with lower TILs, while patients with lower TILs performed worse if their tumors carried TP53-only mutations (interaction $p=0.046$). Multivariate analysis revealed favorable PIK3CA-only mutations in non-LPBC (HR 0.64; 95%CI 0.47-0.88; $p=0.007$), and unfavorable TP53 mutations in ER/PgRpos/HER2neg (HR 1.55; 95% CI 1.07-2.24; $p=0.021$). Mutations did not interact with TILs in non-LP triple-negative and HER2-positive patients.

Conclusions: TP53 and PIK3CA mutations appear to have diverse effects on the outcome of early BC patients, according to whether these genes are co-mutated or not, and, for TP53 according to TILs density and ER/PgR-status. These findings need to be considered when evaluating the effect of these two most frequently mutated genes in the context of large clinical trials.

Key words: TP53; PIK3CA; co-mutation; tumor infiltrating lymphocytes; p53 immunohistochemistry; adjuvant

INTRODUCTION

Breast cancer is characterized by the presence of few recurrently mutated genes, with mutations in PIK3CA and TP53 constantly reported as the most frequent alterations in a hormone receptor (ER/PgR) specific manner [1-5]. PIK3CA mutations are found in more than 40% of luminal A/B but in less than 15% of triple negative (TNBC) tumors; by contrast, TP53 mutations are prevalent in TNBC and in HER2-positive but their incidence is low in luminal A tumors. Whether this diversity and inverse correlation is the cause or the effect of hormone dependency in breast cancer remains unclear. PIK3CA mutations have been intensely investigated because of the possibility for therapeutic interventions against activated PI3K. PIK3CA mutations have been associated with better outcome of breast cancer patients in general [3]; without significant effect in the adjuvant setting [6-8] or with only short-term benefit, as shown in the FinHER trial [4]; with resistance to trastuzumab in the neoadjuvant [9,10] and metastatic settings [11]. TP53 mutations have subtype specific impact on response to chemotherapy [12] and outcome [13]. TP53 mutations but not p53 IHC were found predictive for response to neoadjuvant chemotherapy and trastuzumab [14], while p53 immunopositivity predicted for early and late recurrence in adjuvantly treated ER-positive disease [15,16]. Co-mutation of these genes is not a rare occasion in breast carcinomas, but the effect of this condition has not yet been addressed.

Morphologically assessed stromal tumor infiltrating lymphocytes (TILs) density has recently been featured as a significant favorable prognostic marker in breast cancer, in the neoadjuvant [17-20] and adjuvant [21-25] setting, especially in TNBC and HER2-positive disease. TILs presumably accumulate as a response by the host to tumor neoantigens [26]. TILs density is higher in TNBC and HER2-positive, and low in luminal A/B tumors [22,25]. This pattern is in line with the described high incidence of TP53 mutations in ER-negative and of PIK3CA in ER-positive tumors but it is still unclear whether mutations in these genes are related to TILs density. However, evidence from statistical models suggests the opposite, i.e., that TP53 wild type and a functional p53-pathway is needed for cytotoxic immune cells to accumulate in ER-negative tumors [27]. Patients with high TILs TNBC have better prognosis [21-23,17], but whether TILs density is related to benefit from chemotherapy according to the presence of TP53 and/or PIK3CA mutations with respect to breast cancer subtypes and nodal status remains unexplored.

In a series of early breast cancer patients treated with adjuvant chemotherapy we have previously shown that PIK3CA mutations were not related to outcome [7]. In the present

study we examined TP53 and PIK3CA mutation data in an extended cohort of patients with early breast cancer treated within the context of four adjuvant clinical trials by the Hellenic Cooperative Oncology Group that had been previously evaluated for the effect of TILs density [22]. All tumor parameters, targeted next generation sequencing included, were obtained from previously diagnosed routinely processed paraffin tissues. Mutations in association with TILs density were investigated according to clinical subtypes and nodal status.

PATIENTS, TUMORS AND METHODS

This study was conducted retrospectively on retrospectively/prospectively collected tumor tissue material (formalin-fixed paraffin-embedded, FFPE). Tumors derived from patients with operable breast cancer who had been treated with adjuvant chemotherapy within 4 clinical trials conducted by HeCOG during 1997 – 2010, as previously described [22]. All patients had received anthracycline-based chemotherapy. In the first 2 trials patients had not received trastuzumab for HER2-positive disease (pre-trastuzumab era) (**Figure 1,** REMARK). Tumors had been locally subtyped with standard ER/PgR/HER2 immunohistochemistry (IHC) and HER2 fluorescent in situ hybridization (FISH) for HER2 2+ IHC cases, as Luminal A/B, HER2-positive, and triple negative (TNBC). Hormonal therapy in all trials and trastuzumab in the last 2 trials (post-trastuzumab era) were administered based on local diagnoses. Patients had provided written consent for the use of their biologic material for research purposes and the study was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Health Sciences, Faculty of Medicine (#77/10June2014) and by the Institutional Review Board of the Papageorgiou Hospital of Thessaloniki (#725/10May2013).

Central tumor testing

Tumors were further centrally reviewed at the Laboratory of Molecular Oncology (Hellenic Foundation for Cancer Research and Aristotle University of Thessaloniki, Thessaloniki, Greece) for histology; clinical subtypes with ER/PgR/HER2 IHC and HER2 FISH as previously described [28]; tumor cell content (TCC%); and, stromal tumor infiltrating lymphocytes (TILs) density based on Salgado et al [29] as previously described for the entire series including the present cohort [22]. IHC and FISH were performed on in-house low-density tissue microarrays (TMA) that contained two 1.5mm cores per tumor. Ki67 cut-off at 20% [30] and at 14% as previously practiced [31] was applied for distinguishing between Luminal A and Luminal B tumors. CK5 and EGFR IHC (cut-off at 1% for positive/negative) were used for calling basal-like (basal) phenotypes. IHC for p53 protein expression with the DO7 antibody and 10% nuclear staining as a cut-off for positive/negative was also applied (more details in file **ESM_1, Supplemental Methods**).

TP53 and PIK3CA genotyping and study population

DNA was extracted upon manual macrodissection with magnetic beads (VERSANT Tissue Prep Kit, Siemens Healthcare, Erlangen, Germany); quantity was measured with the Qubit

fluorometer (Life Technologies, Paisley, UK); and, amplification performance was evaluated by two different control qPCR assays. Samples were processed for genotyping if ≥2ng/ul DNA amplifiable at Ct≤32 for both control assays was available. Genotyping was accomplished in an Ion Proton Sequencer with a previously validated custom highly-multiplexed panel [32]. The panel targeted the entire coding region of TP53 and hot spot exons of PIK3CA (coding exons 9 and 20). Samples were accepted for analysis if all amplicons corresponding to the above regions had been read individually >100 times. Variants obtained from Ion Reporter v.4 were filtered out upon multiple quality control steps. For the samples analyzed in the present study, all TP53 and PIK3CA variant positions were read >100 and variants >40 times. Coding mutations corresponded to amino acid changing variants in coding regions for which no minor allele frequency (MAF) was reported or, if registered SNPs, with MAF <0.1%. More details on genotyping are presented in file **ESM_1.**

As shown in **Figure 1**, informative genotyping data were obtained in 1766 samples. Out of these, stromal TILs density had been successfully assessed in 1661; 568 in the pre-, and 1093 in the post-trastuzumab era.

Statistics

Patient and tumor characteristics, as well as patient follow-up data for cases with informative genotypes are shown in **Table 1**. Due to the relatively short follow-up period for the trials in the post-trastuzumab era, only disease-free survival (DFS) was examined as clinical end-point. DFS was assessed from the date of diagnosis until investigator determined disease relapse or death, whichever occurred first, or loss from follow-up. For outcome analyses, tumor subtypes classified upon local testing were examined, since hormonal therapy and trastuzumab were administered based on local diagnoses. Survival status was updated in June 2014.

TILs density was examined in a continuous mode and as a categorical variable. Because mutations in fact accumulated in tumors with ≥5% TILs, while 50% appeared as a natural cut-off for TILs density in the entire cohort (**Figure 2a**), TILs were examined at <5%, 5-50% and >50%. Tumors were distinguished into lymphocyte-predominant (LPBC) with TILs >50% and non LPBC. TP53 and PIK3CA mutations were examined as individual variables or in combination as a 4-scale variable including tumors without mutations; with mutations in both genes (co-mutated); mutations in TP53 only (TP53-only); and, mutations in PIK3CA

only (PIK3CA-only). Mutations were also examined as missense, nonsense, frameshift indels; and, for the corresponding domain per gene.

The analysis was fully compliant with the reporting recommendations for tumor marker prognostic studies [33]. SAS for Windows, version 9.3 (SAS Institute Inc., Cary, NC) was used for all descriptive, log-rank, univariate and multivariate Cox analyses, the latter involving backward selection of the parameters modelled for adjustment. Detailed information on statistical methods and multivariate models is presented in file **ESM_1.**

RESULTS

Out of the 1661 tumors with informative data for TP53 and PIK3CA genotyping, 894 (53.8%) were mutated; 436 (26.2%) in PIK3CA, 357 (21.5%) in TP53, and 101 (6.0%) tumors were co-mutated. TILs density as a continuous variable (mean±SD) was significantly lower in PIK3CA mutant (10.1±12.0) as compared to PIK3CA wild type tumors (13.2±16.1) (p=0.004); by contrast, it was significantly higher in tumors with TP53 mutations (16.7±17.3) as compared to those without (11.2±14.3) (p<0.001). Close to 1/3 of tumors had TILs density <5%, while only 71 (4.3%) were LPBC (**Figure 2a; Table 1**). The rate of mutations was similar in LPBC and in tumors with <5% TILs (41%), while it reached 60% in tumors with 30-49% TILs (**Figure 2b**). TP53 mutations were significantly more frequent in LPBC (29.6%) and in tumors with 5-49% TILs (25.1%) than in tumors with <5% TILs (14.2%), while PIK3CA mutations were significantly more frequent in non-LPBC (26.9%) as compared to LPBC (11.3%) (**Figure 2c**). Since missense outweighed all other mutation types, the observed differences for both genes were statistically significant for this type of mutations only; the same applied for DNA binding TP53 mutations when compared to all other domain-specific mutations (file **ESM_2, Table S1**). No particular mutant amino acid was associated with increased TILs density for either gene, while tumors with nonsense TP53 mutations were also present among LPBC (**Figure S1 in file ESM_1**).

The number of mutant tumors in LPBC was low, not allowing for meaningful statistics. Similarly, fragmentation of the cohort according to mutation types and domain-specific mutations yielded very small groups for most categories (file **ESM_2, Table S1**). Hence, associations of TP53 and PIK3CA mutations were applied for the 1590 tumors with TILs density <50% (non-LPBC) (file **ESM_2, Table S2**). These were analyzed at 5% TILs for high/low density, which appeared as a natural cut-off (**Figure 2a**). TILs according to mutations were compared with standard clinicopathological characteristics (**Figure 2d and Figure S2 in file ESM_1**). High TILs and TP53 mutations were more frequent in HER2-positive and TNBC; low TILs and PIK3CA mutations were prominent in Luminal A tumors, with either 14% or 20% Ki67 cut-off. The incidence of PIK3CA-only mutations in ER-negative and non-basal tumors was very low (**Figure 2e**). Mutations in both genes were associated with higher Ki67 labeling (file **ESM_2, Table S2**) and with specific histological types (file **ESM_1, Supplemental Results**).

Immunopositivity for p53 protein (n=848/1585 assessable tumors; 54%) showed poor agreement with TP53 mutations (Cohen's kappa 0.18), but 90% of missense mutations and

80% of mutations in the DNA binding domain were IHC positive. Unlike TP53 mutations, p53 immunopositivity was not associated with TILs density. TILs and mutations were not significantly associated with nodal status, tumor size, patient age or menopausal status.

Effects of TILs density and mutations on patient outcome

Patient follow-up and survival data are shown in **Table 1**. For the entire cohort, mean DFS was 125.6 months in the pre-trastuzumab and 74.7 months in the post-trastuzumab era. Among the 71 patients with LPBC of all subtypes (**Figure 3a**) only 5 relapsed (7%), 2 of them within a period of 9 months from treatment start. All 5 patients had ductal carcinomas of the non-specific type, 2 were HER2-positive in the pre-trastuzumab era, 2 Luminal B and 1 TNBC. Three did not have TP53 or PIK3CA mutations, 1 had PIK3CA only, and the last one had mutations in both genes. In all, none of the clinicopathological characteristics of these patients could be related to their outcome. The low relapse rate of LPBC in the present cohort was concordant with our previous observations, justifying the distinction of LPBC as a distinct entity [22]. The effect of TP53 and PIK3CA mutations on patient outcome was therefore assessed in non-LPBC. Further distinction of TILs density into high/low with the 5% cut-off was not related to patient DFS (**Figure 3a**). In the entire cohort, the absence of TP53 and the presence of PIK3CA mutations were marginally associated with favorable DFS (**Figure 3b and 3c**). When co-mutations were taken into account, the presence of PIK3CA-only mutations was significantly associated with favorable outcome; in this setting, patients with TP53 mutated tumors had similar DFS as patients without TP53 or PIK3CA mutations (**Figure 3d**).

Next, TILs and mutation parameters were examined in the 1590 non-LPBC patients with respect to nodal status and clinical subtypes. These results are shown in **Table 2** for the entire series and in file **ESM_2, Table S3** for the above mentioned subgroups. The absence of TP53 mutations, the presence of PIK3CA, and particularly PIK3CA-only mutations were all associated with favorable DFS in all non-LPBC patients. TILs up to 50%, either as a continuous or as a scaled variable had no significant effect (**Table 2**). High nodal burden (≥4 positive nodes), Luminal B, TNBC and HER2-positive tumors in the pre-trastuzumab era, all conferred significantly aggravated DFS. The favorable effect of PIK3CA mutations and the unfavorable effect of TP53 mutations were restricted or particularly pronounced in patients with <5% TILs tumors. The same parameters were not associated with DFS in patients with 5-50% TILs. In patients with high nodal burden (file **ESM_2, Table S3**), the unfavorable effect of TP53 mutations was restricted to <5% TILs tumors, while the presence of PIK3CA–

only mutations was favorable independently of TILs density. In patients with low nodal burden, higher TILs were particularly favorable, while favorable PIK3CA–only was restricted to <5% TILs density. In this group, TP53 mutations did not affect patient outcome, while higher Ki67 and absence of p53 protein expression were associated with unfavorable DFS. In Luminal A/B tumors, TP53 and PIK3CA mutations were unfavorable and favorable, respectively, effects that were partially related to TILs density. Significant interactions were observed between TILs density and TP53 mutations, particularly TP53-only (**Table 3**). In all non-LPBC and in patients with Luminal A/B tumors, TP53 mutations conferred poor outcome if present in tumors with <5% TILs, while in the presence of TP53 mutations, high TILs conferred better prognosis.

Mutation status was not associated with DFS in TNBC and HER2-positive patients, the latter irrespectively of trastuzumab treatment. In trastuzumab-treated HER2-positive patients, p53 protein positivity and higher TILs conferred favorable outcome. In all subtypes, patients with high nodal burden had unfavorable prognosis.

Multivariate adjustments were applied in the non-LPBC cohort only. TILs density and mutation status were adjusted independently in the multivariate models applied in the examined patient groups (file **ESM_1, Supplemental Methods**). Thus, in all non-LPBC patients, PIK3CA–only mutations remained a strong favorable prognosticator, along with low nodal burden, small tumor size, younger patient age, higher TILs, and, lower Ki67 (**Figure 4**). With respect to nodal status, PIK3CA-only mutations and TILs retained their favorable significance in patients with low nodal burden only. In Luminal A/B patients, TP53 mutations were independently unfavorable, along with high nodal burden, large tumor size and high grade. In HER2-positive patients in the pre-trastuzumab era, low nodal burden, higher TILs, younger age and postmenopausal status were favorable prognosticators; in the post-trastuzumab era, p53 protein positivity was a strong independent favorable parameter, along with higher TILs. Lastly, mutation and TILs status did not remain significant for TNBC patient outcome.

DISCUSSION

The main concept of this study was to associate TILs with mutations in the most frequently affected genes in breast cancer, TP53 and PIK3CA. Based on the high TILs density in TNBC and in HER2-positive tumors [22,23,25,34], in parallel with the high incidence of TP53 mutations in these tumors [35,36], with the long known antigenicity of TP53 mutant peptides [37,38], and with the high number of mutations observed in TP53 mutant tumors [39], the latter were expected to be TILs-rich. Indeed, the incidence of TP53 mutations increased in parallel with TILs density in the present series. The TP53mutant-TILs-rich pattern was observed in all subtypes except for Luminal A. This finding appears in contrast to the reported *in silico* model suggesting that wild type TP53 is needed for cytotoxic T cell marker expression in ER-negative tumors [27]. Reasons for this discrepancy include different classifiers for ER-positive and negative disease, which shows considerable discordance [40], and the non-specificity of morphologically assessed TILs with respect to their cytotoxic potential [34]. Further, neoantigens may be produced by proteins that are unrelated to tumor development [41] and TILs are not necessarily attracted in the tumor environment due to the exposure of p53 mutant epitopes. In comparison to TP53, tumors with PIK3CA mutations were mostly TILs-poor, especially when excluding TP53 co-mutations. The pattern with PIK3CA-only mutations and low TILs was more prominent in ER-positive tumors, which seems in line with the recently described hormone-related subtype of lobular carcinomas that is characterized by PIK3CA mutations and absence of immunocompetence [42]. The present data overall confirm PIK3CA mutations associated with low TILs as the typical pattern for ER-positive tumors and TP53 mutations associated with high TILs for ER-negative tumors.

Although it has been suggested that LPBC should not been examined separately and TILs should only be analyzed in a continuous mode [34], it was deemed necessary to distinguish this group for outcome analyses based on the very favorable prognosis of these patients, as previously shown [22] and as recently applied in the same context, although with 60% as a cut-off [24]. LPBC were particularly poor in PIK3CA-only mutations, in line with the pattern described above. However, given the over-representation of TILs- and TP53-mutation-rich TNBC and HER2-positive tumors in LPBC, the incidence of TP53 mutant tumors in this tumor subset was lower than expected. In order to demonstrate the role of TP53 mutations in LPBC, pooled analyses from large studies will be necessitated, given the low overall incidence of such tumors in breast cancer. It will also be interesting to clarify whether mutations in genes not examined here are attributable for the high TILs density in LPBC or

whether immune cells are attracted as an infectious inflammatory response to these tumors, since microbial and viral sequences have been demonstrated for example in TNBC [43].

In patients with non-LPBC, higher TILs and PIK3CA-only mutations conferred longer DFS irrespectively of subtype. The same effects remained significant in patients with favorable nodal status. The favorable effect of higher TILs was expected but, to our knowledge, the finding concerning favorable PIK3CA mutations in the absence of TP53 mutations has not been described before. Co-mutated TP53 and PIK3CA represent more than 6% of breast cancers, accounting for more than 20% of PIK3CA mutated tumors, for approximately 30% of TP53 mutated tumors, and they are found in similar rates in the various subtypes except for Luminal A, where they are very rare. As shown here, patients with co-mutated tumors have an aggressive disease course, while those with PIK3CA-only fare better. It is possible that the lack of association of PIK3CA mutations with patient outcome in the adjuvant setting [44-46,8] be partly due to the fact that these mutations have been examined as a single parameter whilst they may have not been so. PIK3CA mutations result in cellular remodeling in non-tumorigenic breast epithelial cells [47] but the activation of the PI3K pathway through these mutations is reported as mild (reviewed in [48]). Although the mechanism underlying the favorable prognostic effect of PIK3CA-only mutations needs to be clarified, it appears important to distinguish these from mutations occurring in the presence of TP53 for outcome analyses irrespectively of breast cancer subtype. Further, based on the present favorable prognosis data associated with PIK3CA-only mutations, inhibition of this molecule and the PI3K-pathway may not be clinically relevant in the adjuvant setting.

TP53 mutations and p53 immunopositivity are considered to reflect aberrations of the p53-pathway although they only vaguely correlate with each other [12]. The present findings on unfavorable TP53 mutations in Luminal A/B tumors and the possible predictive role of p53 protein expression for trastuzumab benefit in the adjuvant setting were previously presented for the same patient cohort [49]. The novelty here is the significant interaction between TP53 mutations and TILs density. This finding explains the observed unfavorable effect of TP53 mutations in patients with Luminal A/B tumors, which is in line with TP53 mutations conferring increased risk for late relapse in ER-positive disease [50] and with the poor outcome of patients with TP53 mutated Luminal B tumors that were typed with PAM50 [13]. Taking together the favorable effect of p53 immunopositivity in HER2-positive disease, which is rich in TILs, this study provides preliminary evidence that aberrations in the p53-pathway have diverse effects on patient outcome according to the immunocompetence of the

host against tumor cells. Further (pre)clinical validation of the present hypothesis-generating data will enlighten our understanding with respect to the type and timing for efficient immunomodulation in patients with or without TP53 mutations in their tumors.

In conclusion, this study confirms two patterns regarding TILs and the most frequently mutated genes in breast cancer, i.e., TP53 mutations and higher TILs in HER2-positive and TNBC, as compared to PIK3CA-only mutations and low TILs in ER-positive tumors. Other than expected though, the majority of LPBC did not bear mutations in any of these genes. In non-LPBC, the prognostic effects of mutations were related to TILs density, particularly with respect to TP53 mutations and low TILs tumors. These findings prompt for a detailed characterization of mutant protein antigenicity and immune cell infiltrations at the tissue level, especially if aiming at a rational use of immunomodulators. Lastly, the finding that PIK3CA mutations are favorable prognosticators in the absence of TP53 mutations is worthy considering for the clinical relevance of these mutations in the adjuvant setting.

Acknowledgements

The authors wish to thank Mrs. Emily Daskalaki for excellent technical assistance with MPS and Ms. Maria Moschoni and Mrs. Stella Dallidou for secretarial assistance.

Funding

This study was supported by an internal Hellenic Cooperative Oncology Group (HeCOG) translational research grant (HE TRANS_BR). The funders played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

This study was also partly supported by the Greek General Secretary for Research and Technology (GSRT) Program, Research in Excellence II, funded by 75 % from the European Union and the Operational Program ‘‘Education & Lifelong Learning’’ ESPA-THALIS#266 of the Ministry of Education, Lifelong Learning & Religious Affairs.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Cancer Genome Atlas N (2012) Comprehensive molecular portraits of human breast tumours. Nature 490 (7418):61-70. doi:10.1038/nature11412

2. Cizkova M, Susini A, Vacher S, Cizeron-Clairac G, Andrieu C, Driouch K, Fourme E, Lidereau R, Bieche I (2012) PIK3CA mutation impact on survival in breast cancer patients and in ERalpha, PR and ERBB2-based subgroups. Breast cancer research : BCR 14 (1):R28. doi:10.1186/bcr3113

3. Kalinsky K, Jacks LM, Heguy A, Patil S, Drobnjak M, Bhanot UK, Hedvat CV, Traina TA, Solit D, Gerald W, Moynahan ME (2009) PIK3CA mutation associates with improved outcome in breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 15 (16):5049-5059. doi:10.1158/1078-0432.CCR-09-0632

4. Loi S, Michiels S, Lambrechts D, Fumagalli D, Claes B, Kellokumpu-Lehtinen PL, Bono P, Kataja V, Piccart MJ, Joensuu H, Sotiriou C (2013) Somatic mutation profiling and associations with prognosis and trastuzumab benefit in early breast cancer. Journal of the National Cancer Institute 105 (13):960-967. doi:10.1093/jnci/djt121

5. Ng CK, Schultheis AM, Bidard FC, Weigelt B, Reis-Filho JS (2015) Breast cancer genomics from microarrays to massively parallel sequencing: paradigms and new insights. Journal of the National Cancer Institute 107 (5)

6. Sabine VS, Crozier C, Brookes CL, Drake C, Piper T, van de Velde CJ, Hasenburg A, Kieback DG, Markopoulos C, Dirix L, Seynaeve C, Rea DW, Bartlett JM (2014) Mutational analysis of PI3K/AKT signaling pathway in tamoxifen exemestane adjuvant multinational pathology study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 32 (27):2951-2958

7. Papaxoinis G, Kotoula V, Alexopoulou Z, Kalogeras KT, Zagouri F, Timotheadou E, Gogas H, Pentheroudakis G, Christodoulou C, Koutras A, Bafaloukos D, Aravantinos G, Papakostas P, Charalambous E, Papadopoulou K, Varthalitis I, Efstratiou I, Zaramboukas T, Patsea H, Scopa CD, Skondra M, Kosmidis P, Pectasides D, Fountzilas G (2015) Significance of PIK3CA Mutations in Patients with Early Breast Cancer Treated with Adjuvant Chemotherapy: A Hellenic Cooperative Oncology Group (HeCOG) Study. PloS one 10 (10):e0140293. doi:10.1371/journal.pone.0140293

8. Pogue-Geile KL, Song N, Jeong JH, Gavin PG, Kim SR, Blackmon NL, Finnigan M, Rastogi P, Fehrenbacher L, Mamounas EP, Swain SM, Wickerham DL, Geyer CE, Jr., Costantino JP, Wolmark N, Paik S (2015) Intrinsic Subtypes, PIK3CA Mutation, and the Degree of Benefit From Adjuvant Trastuzumab in the NSABP B-31 Trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 33 (12):1340-1347

9. Loibl S, von Minckwitz G, Schneeweiss A, Paepke S, Lehmann A, Rezai M, Zahm DM, Sinn P, Khandan F, Eidtmann H, Dohnal K, Heinrichs C, Huober J, Pfitzner B, Fasching PA, Andre F, Lindner JL, Sotiriou C, Dykgers A, Guo S, Gade S, Nekljudova V, Loi S, Untch M, Denkert C (2014) PIK3CA mutations are associated with lower rates of pathologic complete response to anti-human epidermal growth factor receptor 2 (her2) therapy in primary HER2-overexpressing breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 32 (29):3212-3220

10. Majewski IJ, Nuciforo P, Mittempergher L, Bosma AJ, Eidtmann H, Holmes E, Sotiriou C, Fumagalli D, Jimenez J, Aura C, Prudkin L, Diaz-Delgado MC, de la Pena L, Loi S, Ellis C, Schultz N, de Azambuja E, Harbeck N, Piccart-Gebhart M, Bernards R, Baselga J (2015) PIK3CA mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2-targeted therapies in breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 33 (12):1334-1339. doi:10.1200/JCO.2014.55.2158

11. Razis E, Bobos M, Kotoula V, Eleftheraki AG, Kalofonos HP, Pavlakis K, Papakostas P, Aravantinos G, Rigakos G, Efstratiou I, Petraki K, Bafaloukos D, Kostopoulos I, Pectasides D, Kalogeras KT, Skarlos D, Fountzilas G (2011) Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. Breast cancer research and treatment 128 (2):447-456. doi:10.1007/s10549-011-1572-5

12. Bertheau P, Lehmann-Che J, Varna M, Dumay A, Poirot B, Porcher R, Turpin E, Plassa LF, de Roquancourt A, Bourstyn E, de Cremoux P, Janin A, Giacchetti S, Espie M, de The H (2013) p53 in breast cancer subtypes and new insights into response to chemotherapy. Breast 22 Suppl 2:S27-29. doi:10.1016/j.breast.2013.07.005

13. Silwal-Pandit L, Vollan HK, Chin SF, Rueda OM, McKinney S, Osako T, Quigley DA, Kristensen VN, Aparicio S, Borresen-Dale AL, Caldas C, Langerod A (2014) TP53 mutation spectrum in breast cancer is subtype specific and has distinct prognostic relevance. Clinical cancer research : an official journal of the American Association for Cancer Research 20 (13):3569-3580

14. Gluck S, Ross JS, Royce M, McKenna EF, Jr., Perou CM, Avisar E, Wu L (2012) TP53 genomics predict higher clinical and pathologic tumor response in operable early-stage breast cancer treated with docetaxel-capecitabine +/- trastuzumab. Breast cancer research and treatment 132 (3):781-791. doi:10.1007/s10549-011-1412-7

15. Yamamoto M, Hosoda M, Nakano K, Jia S, Hatanaka KC, Takakuwa E, Hatanaka Y, Matsuno Y, Yamashita H (2014) p53 accumulation is a strong predictor of recurrence in estrogen receptor-positive breast cancer patients treated with aromatase inhibitors. Cancer science 105 (1):81-88. doi:10.1111/cas.12302

16. Coates AS, Millar EK, O'Toole SA, Molloy TJ, Viale G, Goldhirsch A, Regan MM, Gelber RD, Sun Z, Castiglione-Gertsch M, Gusterson B, Musgrove EA, Sutherland RL (2012) Prognostic interaction between expression of p53 and estrogen receptor in patients with node-negative breast cancer: results from IBCSG Trials VIII and IX. Breast cancer research : BCR 14 (6):R143. doi:10.1186/bcr3348

17. Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, Pfitzner BM, Salat C, Loi S, Schmitt WD, Schem C, Fisch K, Darb-Esfahani S, Mehta K, Sotiriou C, Wienert S, Klare P, Andre F, Klauschen F, Blohmer JU, Krappmann K, Schmidt M, Tesch H, Kummel S, Sinn P, Jackisch C, Dietel M, Reimer T, Untch M, Loibl S (2015) Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without Carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 33 (9):983-991. doi:10.1200/JCO.2014.58.1967

18. Salgado R, Denkert C, Campbell C, Savas P, Nucifero P, Aura C, de Azambuja E, Eidtmann H, Ellis CE, Baselga J, Piccart-Gebhart MJ, Michiels S, Bradbury I, Sotiriou C, Loi S (2015) Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and Trastuzumab: A Secondary Analysis of the NeoALTTO Trial. JAMA oncology 1 (4):448-454. doi:10.1001/jamaoncol.2015.0830

19. Seo AN, Lee HJ, Kim EJ, Kim HJ, Jang MH, Lee HE, Kim YJ, Kim JH, Park SY (2013) Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer. British journal of cancer 109 (10):2705-2713. doi:10.1038/bjc.2013.634

20. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, Budczies J, Darb-Esfahani S, Kronenwett R, Hanusch C, von Torne C, Weichert W, Engels K, Solbach C, Schrader I, Dietel M, von Minckwitz G (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. Journal of

clinical oncology : official journal of the American Society of Clinical Oncology 28 (1):105-113. doi:10.1200/JCO.2009.23.7370

21. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, Martino S, Wang M, Jones VE, Saphner TJ, Wolff AC, Wood WC, Davidson NE, Sledge GW, Sparano JA, Badve SS (2014) Prognostic Value of Tumor-Infiltrating Lymphocytes in Triple-Negative Breast Cancers From Two Phase III Randomized Adjuvant Breast Cancer Trials: ECOG 2197 and ECOG 1199. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. doi:10.1200/JCO.2013.55.0491

22. Kotoula V, Chatzopoulos K, Lakis S, Alexopoulou Z, Timotheadou E, Zagouri F, Pentheroudakis G, Gogas H, Galani E, Efstratiou I, Zaramboukas T, Koutras A, Aravantinos G, Samantas E, Psyrri A, Kourea H, Bobos M, Papakostas P, Kosmidis P, Pectasides D, Fountzilas G (2015) Tumors with high-density tumor infiltrating lymphocytes constitute a favorable entity in breast cancer: a pooled analysis of four prospective adjuvant trials. Oncotarget. doi:10.18632/oncotarget.6231

23. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, Kellokumpu-Lehtinen PL, Bono P, Kataja V, Desmedt C, Piccart MJ, Loibl S, Denkert C, Smyth MJ, Joensuu H, Sotiriou C (2014) Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 25 (8):1544-1550. doi:10.1093/annonc/mdu112

24. Perez EA, Ballman KV, Tenner KS, Thompson EA, Badve SS, Bailey H, Baehner FL (2016) Association of Stromal Tumor-Infiltrating Lymphocytes With Recurrence-Free Survival in the N9831 Adjuvant Trial in Patients With Early-Stage HER2-Positive Breast Cancer. JAMA oncology 2 (1):56-64. doi:10.1001/jamaoncol.2015.3239

25. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, Rouas G, Francis P, Crown JP, Hitre E, de Azambuja E, Quinaux E, Di Leo A, Michiels S, Piccart MJ, Sotiriou C (2013) Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 31 (7):860-867. doi:10.1200/JCO.2011.41.0902

26. Schumacher TN, Schreiber RD (2015) Neoantigens in cancer immunotherapy. Science 348 (6230):69-74. doi:10.1126/science.aaa4971

27. Quigley D, Silwal-Pandit L, Dannenfelser R, Langerod A, Vollan HK, Vaske C, Siegel JU, Troyanskaya O, Chin SF, Caldas C, Balmain A, Borresen-Dale AL, Kristensen V (2015) Lymphocyte Invasion in IC10/Basal-Like Breast Tumors Is Associated with Wild-Type TP53. Molecular cancer research : MCR 13 (3):493-501. doi:10.1158/1541-7786.MCR-14-0387

28. Fountzilas G, Dafni U, Bobos M, Batistatou A, Kotoula V, Trihia H, Malamou-Mitsi V, Miliaras S, Chrisafi S, Papadopoulos S, Sotiropoulou M, Filippidis T, Gogas H, Koletsa T, Bafaloukos D, Televantou D, Kalogeras KT, Pectasides D, Skarlos DV, Koutras A, Dimopoulos MA (2012) Differential response of immunohistochemically defined breast cancer subtypes to anthracycline-based adjuvant chemotherapy with or without paclitaxel. PloS one 7 (6):e37946. doi:10.1371/journal.pone.0037946

29. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, Wienert S, Van den Eynden G, Baehner FL, Penault-Llorca F, Perez EA, Thompson EA, Symmans WF, Richardson AL, Brock J, Criscitiello C, Bailey H, Ignatiadis M, Floris G, Sparano J, Kos Z, Nielsen T, Rimm DL, Allison KH, Reis-Filho JS, Loibl S, Sotiriou C, Viale G, Badve S, Adams S, Willard-Gallo K, Loi S (2015) The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014.

Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 26 (2):259-271. doi:10.1093/annonc/mdu450
30. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, Senn HJ, Panel m (2013) Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 24 (9):2206-2223. doi:10.1093/annonc/mdt303
31. Cheang MC, Chia SK, Voduc D, Gao D, Leung S, Snider J, Watson M, Davies S, Bernard PS, Parker JS, Perou CM, Ellis MJ, Nielsen TO (2009) Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. Journal of the National Cancer Institute 101 (10):736-750. doi:10.1093/jnci/djp082
32. Kotoula V, Lyberopoulou A, Papadopoulou K, Charalambous E, Alexopoulou Z, Gakou C, Lakis S, Tsolaki E, Lilakos K, Fountzilas G (2015) Evaluation of two highly-multiplexed custom panels for massively parallel semiconductor sequencing on paraffin DNA. PloS one 10 (6):e0128818. doi:10.1371/journal.pone.0128818
33. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, Statistics Subcommittee of NCIEWGoCD (2006) REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast cancer research and treatment 100 (2):229-235. doi:10.1007/s10549-006-9242-8
34. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, Loi S (2015) Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nature reviews Clinical oncology. doi:10.1038/nrclinonc.2015.215
35. Comprehensive molecular portraits of human breast tumours (2012). Nature 490 (7418):61-70
36. Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, Nik-Zainal S, Martin S, Varela I, Bignell GR, Yates LR, Papaemmanuil E, Beare D, Butler A, Cheverton A, Gamble J, Hinton J, Jia M, Jayakumar A, Jones D, Latimer C, Lau KW, McLaren S, McBride DJ, Menzies A, Mudie L, Raine K, Rad R, Chapman MS, Teague J, Easton D, Langerod A, Oslo Breast Cancer C, Lee MT, Shen CY, Tee BT, Huimin BW, Broeks A, Vargas AC, Turashvili G, Martens J, Fatima A, Miron P, Chin SF, Thomas G, Boyault S, Mariani O, Lakhani SR, van de Vijver M, van 't Veer L, Foekens J, Desmedt C, Sotiriou C, Tutt A, Caldas C, Reis-Filho JS, Aparicio SA, Salomon AV, Borresen-Dale AL, Richardson AL, Campbell PJ, Futreal PA, Stratton MR (2012) The landscape of cancer genes and mutational processes in breast cancer. Nature 486 (7403):400-404. doi:10.1038/nature11017
37. Lubin R, Schlichtholz B, Bengoufa D, Zalcman G, Tredaniel J, Hirsch A, Caron de Fromentel C, Preudhomme C, Fenaux P, Fournier G, Mangin P, Laurent-Puig P, Pelletier G, Schlumberger M, Desgrandchamps F, Le Duc A, Peyrat JP, Janin N, Bressac B, Soussi T, et al. (1993) Analysis of p53 antibodies in patients with various cancers define B-cell epitopes of human p53: distribution on primary structure and exposure on protein surface. Cancer research 53 (24):5872-5876
38. Yanuck M, Carbone DP, Pendleton CD, Tsukui T, Winter SF, Minna JD, Berzofsky JA (1993) A mutant p53 tumor suppressor protein is a target for peptide-induced CD8+ cytotoxic T-cells. Cancer research 53 (14):3257-3261
39. Budczies J, Bockmayr M, Denkert C, Klauschen F, Lennerz JK, Györffy B, Dietel M, Loibl S, Weichert W, Stenzinger A (2015) Classical pathology and mutational load of breast cancer – integration of two worlds. . The Journal of Pathology: Clinical Research 1:225–238. doi:doi: 10.1002/cjp2.25
40. Cheang MC, Martin M, Nielsen TO, Prat A, Voduc D, Rodriguez-Lescure A, Ruiz A, Chia S, Shepherd L, Ruiz-Borrego M, Calvo L, Alba E, Carrasco E, Caballero R, Tu D, Pritchard KI, Levine MN, Bramwell VH, Parker J, Bernard PS, Ellis MJ, Perou CM, Di Leo

A, Carey LA (2015) Defining breast cancer intrinsic subtypes by quantitative receptor expression. The oncologist 20 (5):474-482. doi:10.1634/theoncologist.2014-0372
41. Gubin MM, Artyomov MN, Mardis ER, Schreiber RD (2015) Tumor neoantigens: building a framework for personalized cancer immunotherapy. The Journal of clinical investigation 125 (9):3413-3421. doi:10.1172/JCI80008
42. Michaut M, Chin SF, Majewski I, Severson TM, Bismeijer T, de Koning L, Peeters JK, Schouten PC, Rueda OM, Bosma AJ, Tarrant F, Fan Y, He B, Xue Z, Mittempergher L, Kluin RJ, Heijmans J, Snel M, Pereira B, Schlicker A, Provenzano E, Ali HR, Gaber A, O'Hurley G, Lehn S, Muris JJ, Wesseling J, Kay E, Sammut SJ, Bardwell HA, Barbet AS, Bard F, Lecerf C, O'Connor DP, Vis DJ, Benes CH, McDermott U, Garnett MJ, Simon IM, Jirstrom K, Dubois T, Linn SC, Gallagher WM, Wessels LF, Caldas C, Bernards R (2016) Integration of genomic, transcriptomic and proteomic data identifies two biologically distinct subtypes of invasive lobular breast cancer. Scientific reports 6:18517. doi:10.1038/srep18517
43. Banerjee S, Wei Z, Tan F, Peck KN, Shih N, Feldman M, Rebbeck TR, Alwine JC, Robertson ES (2015) Distinct microbiological signatures associated with triple negative breast cancer. Scientific reports 5:15162. doi:10.1038/srep15162
44. Mukohara T (2015) PI3K mutations in breast cancer: prognostic and therapeutic implications. Breast cancer 7:111-123. doi:10.2147/BCTT.S60696
45. Loi S (2013) Tumor-infiltrating lymphocytes, breast cancer subtypes and therapeutic efficacy. Oncoimmunology 2 (7):e24720. doi:10.4161/onci.24720
46. Cescon DW, Bedard PL (2015) PIK3CA genotype and treatment decisions in human epidermal growth factor receptor 2-positive breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 33 (12):1318-1321. doi:10.1200/JCO.2014.59.3160
47. Hart JR, Zhang Y, Liao L, Ueno L, Du L, Jonkers M, Yates JR, 3rd, Vogt PK (2015) The butterfly effect in cancer: A single base mutation can remodel the cell. Proceedings of the National Academy of Sciences of the United States of America 112 (4):1131-1136. doi:10.1073/pnas.1424012112
48. Zardavas D, Phillips WA, Loi S (2014) PIK3CA mutations in breast cancer: reconciling findings from preclinical and clinical data. Breast cancer research : BCR 16 (1):201. doi:10.1186/bcr3605
49. Fountzilas G, Giannoulatou E, Alexopoulou Z, Zagouri F, Timotheadou E, Papadopoulou K, Lakis S, Bobos M, Poulios C, Sotiropoulou M, Lyberopoulou A, Gogas H, Pentheroudakis G, Pectasides D, Koutras A, Christodoulou C, Papandreou C, Samantas E, Papakostas P, Kosmidis P, Bafaloukos D, Karanikiotis C, Dimopoulos MA, Kotoula V (2016) TP53 mutations and protein immunopositivity may predict for poor outcome but also for trastuzumab benefit in patients with early breast cancer treated in the adjuvant setting. Oncotarget. doi:10.18632/oncotarget.9022
50. Yates LK, S.; Martincorena, I.;, M. Gerstung, M.; Stratton, M.; Lonning, P.E.; Campbell, P. (2015) The driver landscape of breast cancer metastasis and relapse. http://www.europeancancercongress.org/Scientific-Programme/Abstract-search?abstractid=21851.

TABLE LEGENDS

Table 1 Patient and tumor characteristics. Cohort split into non-LPBC and LPBC

Table 2 Univariate Cox analysis in all non-LPBC (N patients: 1590)

Table 3 Interaction tests between TILs density and TP53 / PIK3CA mutations in non-LPBC

FIGURE LEGENDS

Figure 1 REMARK diagram for the study patients and tumors

Figure 2 Association between TILs status and mutations in TP53 and PIK3CA

a. Distribution of TILs density values; 5% and 50% appear as natural cut-offs for distinguishing main TILs subgroups.

b. Distribution of TP53 and PIK3CA mutations distinguished in the affected functional protein domains. PIK3CA helical mutations are very rare and TP53 oligomerization domain mutations are more frequent in LPBC as compared to non-LPBC.

c. Association between mutation status and main TILs categories. LPBC are mutation poor but if mutated, it is primarily in TP53.

d. Distribution of TP53 and PIK3CA mutations according to TILs density in clinical subtypes, ER/PgR and nodal status, and p53 protein expression (cut-off for immunohistochemical [IHC] positivity at 10%).

e. TP53 and PIK3CA co-mutated tumors were mostly found in ER-negative, basal-like tumors.

Figure 3 Impact of main study parameters on patient DFS in the entire cohort

a. TILs density, 3-scale: LPBC (≥50% TILs); 5-50% TILs; and, <5% TILs. LPBC fared best, while the remaining two categories conferred similar outcome.

b. PIK3CA mutations

c. TP53 mutations

d. Mutation status in 4 categories: none (no mutation); both genes mutated; TP53-only; PIK3CA-only. PIK3CA mutated-only fared best, co-mutated worst, and TP53-only did not differ from tumors without mutations in the two genes.

Figure 4 Forest plots for multivariate models

a. Non-subtype specific models; **b.** Subtype specific models. Non co-mutated PIK3CA remained as a favourable prognostic parameter in the entire cohort and in patients with low nodal burden. Note that the favourable effect of TILs was marginally significant in most of the cases where this parameter was retained in the final model.

Figure 1

Click here to download Figure Fig 1.tif

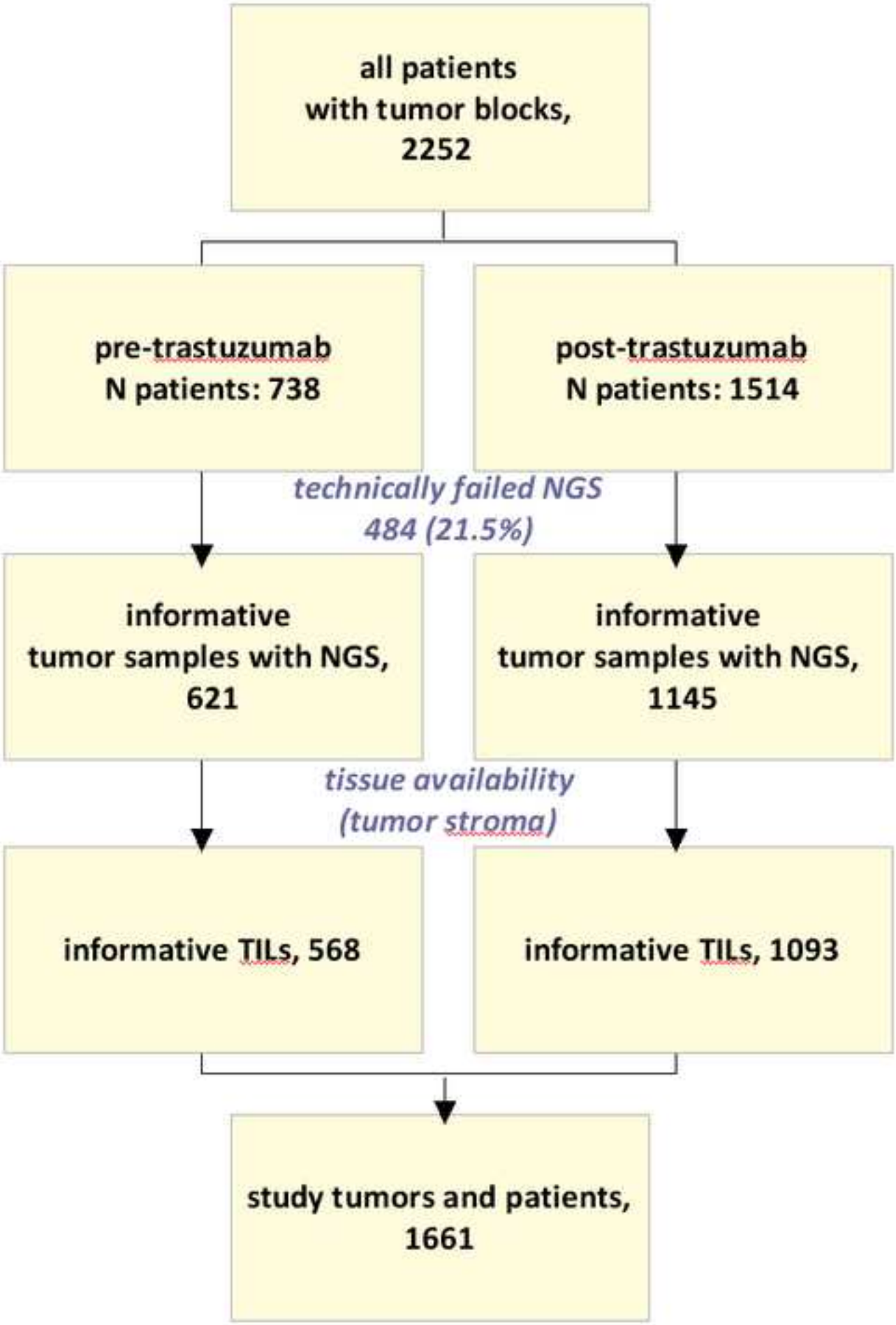


Figure 2

Click here to download Figure Fig 2.tif

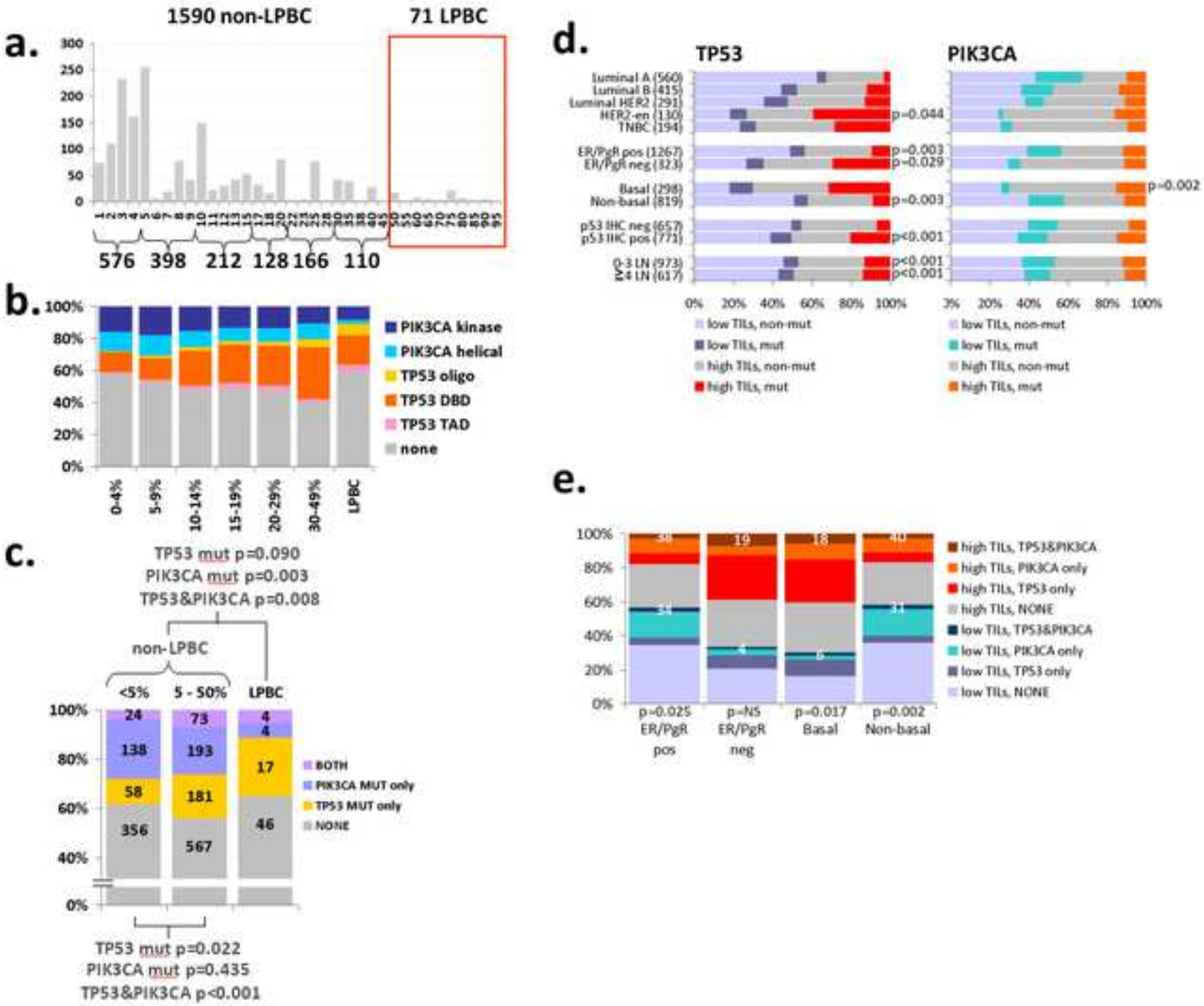
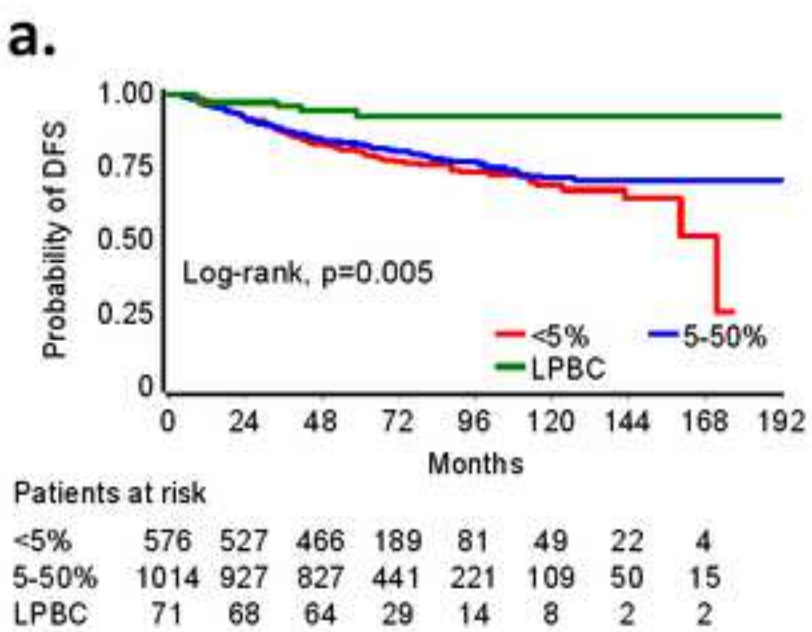
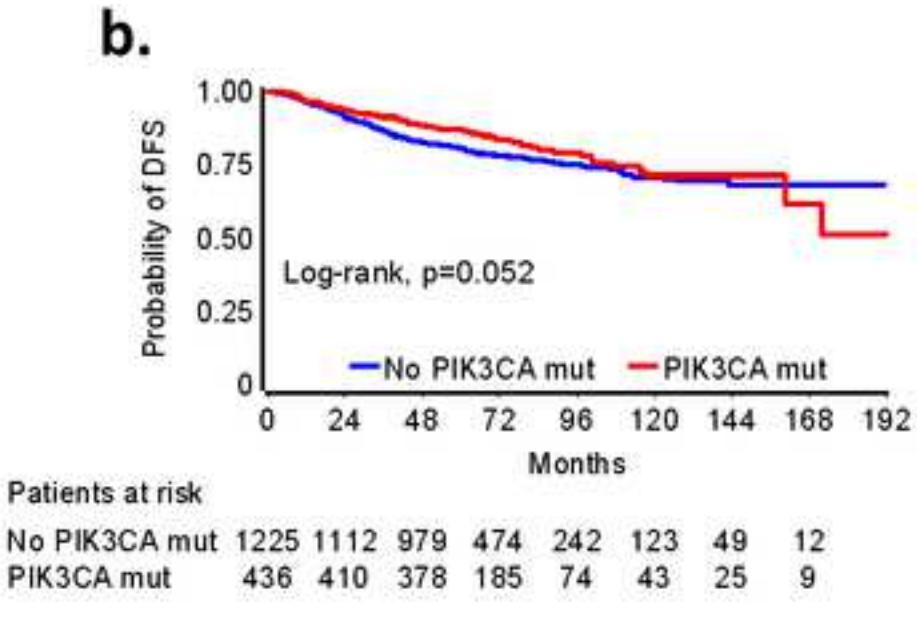
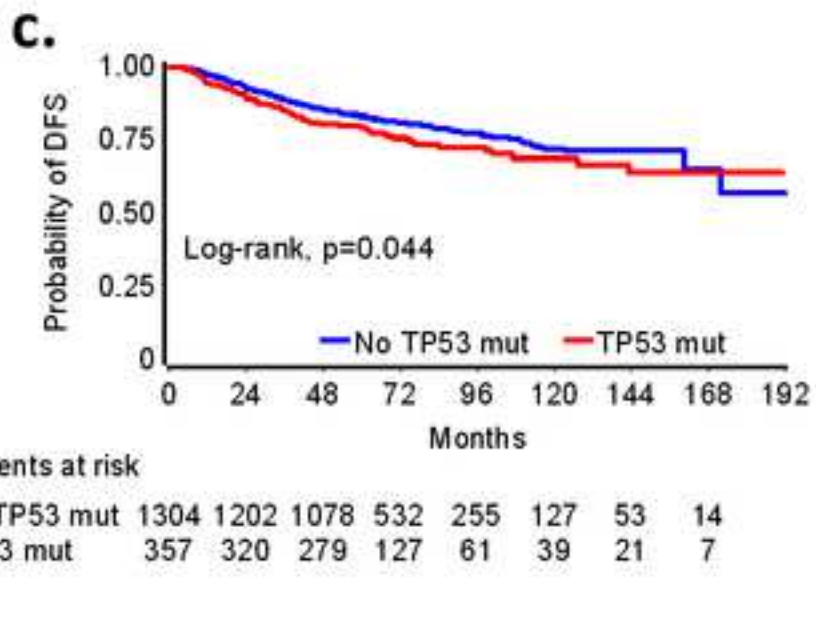


Figure 3

Click here to download Figure Fig 3.tif







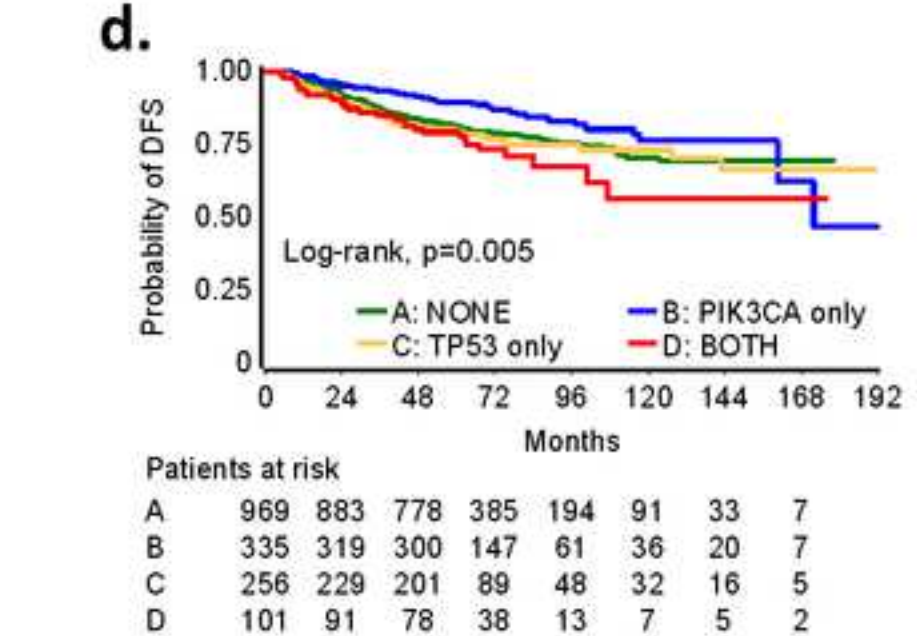


Figure 4a

Click here to download Figure Fig 4a.tif

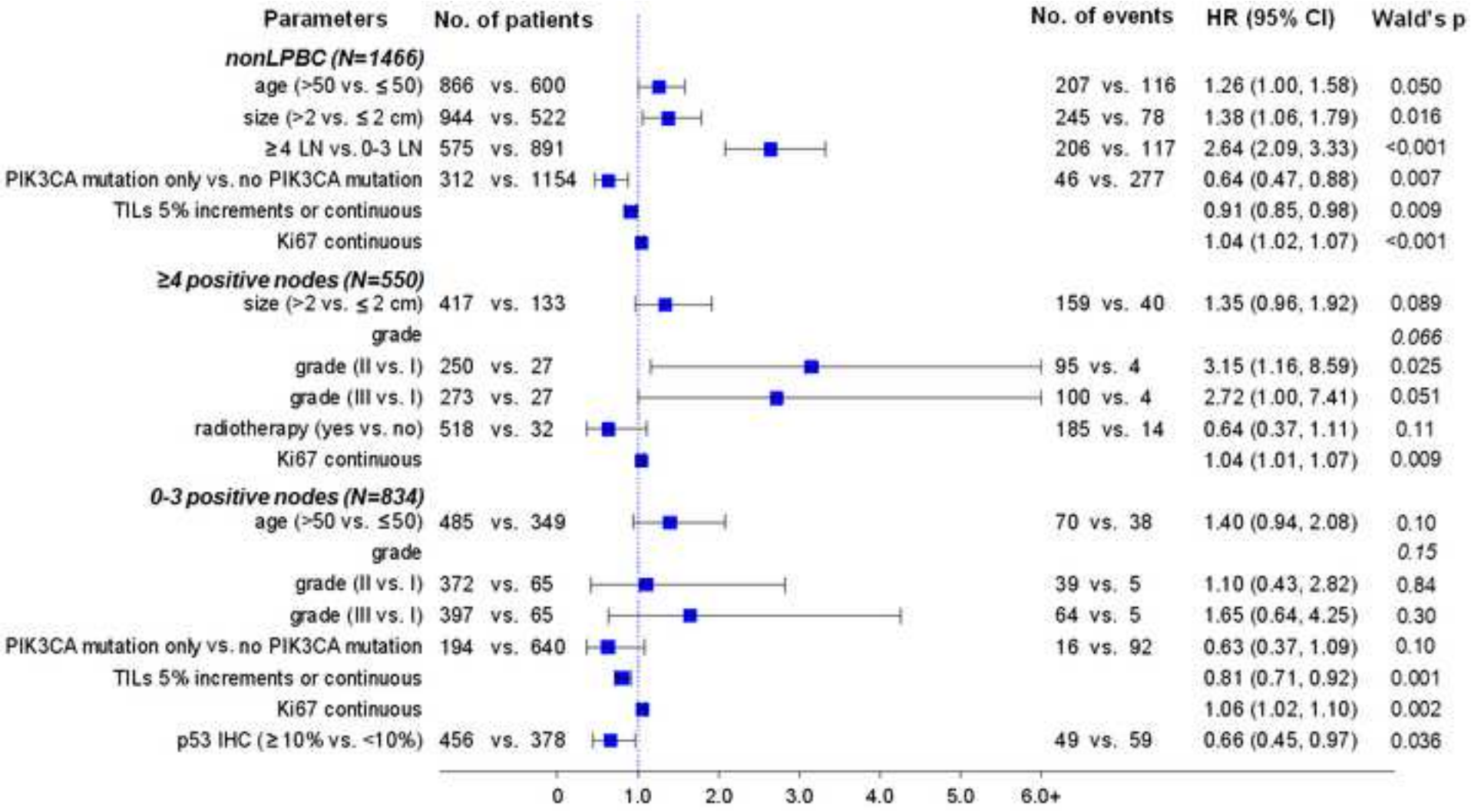


Figure 4b

Click here to download Figure Fig 4b.tif

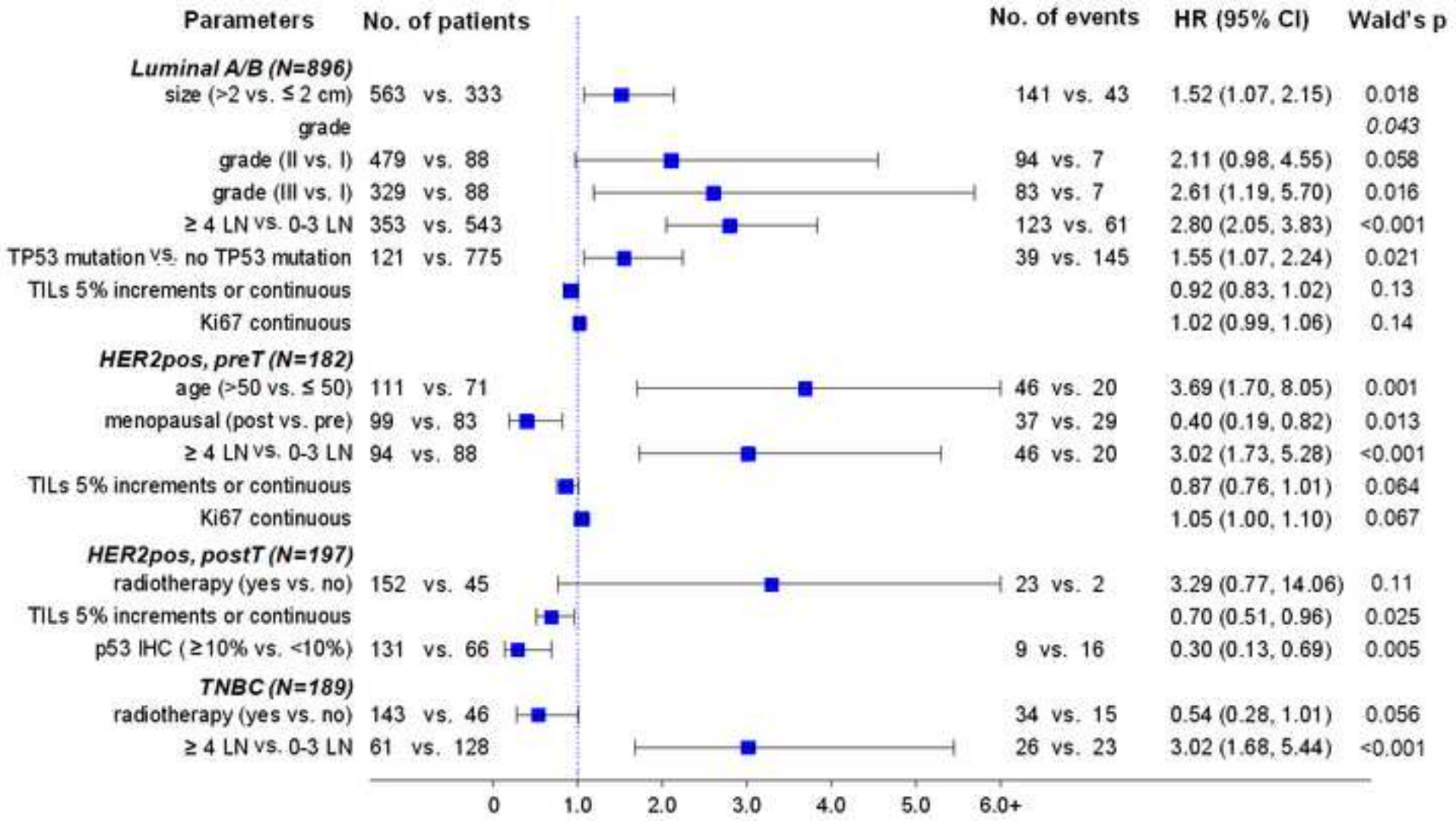


Table 1 Click here to download Table Table1.doc

	study groups			
	entire population	**non-LPBC**	**LPBC**	**p-value***
Patients				
N	1661	1590	71	
Age (years)				
Mean (SD)	53.1 (11.5)	53.2 (11.5)	51.5 (11.3)	0,25
Median	52,8	52,8	52,6	
Min-Max	21-83	21-83	32-76	
Tumor size				
Mean (SD)	2.9 (1.6)	2.9 (1.7)	2.7 (1.2)	0,86
Median	2,5	2,5	2,5	
Min-Max	0-15	0-15	0-8	
Positive lymph nodes				
Mean (SD)	4.6 (6.4)	4.7 (6.3)	4.2 (7.1)	0,16
Median	2	2	2	
Min-Max	0-54	0-54	0-43	
Ki67				
Mean (SD)	29.9 (26.8)	28.8 (26.2)	53.0 (29.2)	<0.001
Median	20	20	55	
Min-Max	0-100	0-100	0-100	
CEN17 copies				
Mean (SD)	2.4 (1.4)	2.4 (1.4)	2.5 (1.4)	0,61
Median	2	2	2,1	
Min-Max	1-18	1-18	1-8	
	N (%)	**N (%)**	**N (%)**	
Age (years)				
≤50	688 (41.4)	657 (41.4)	31 (43.6)	0,70
>50	973 (58.6)	933 (58.6)	40 (56.4)	
Menopausal status				
Postmenopausal	893 (53.8)	854 (53.8)	39 (55.0)	0,97
Premenopausal	768 (46.2)	736 (46.2)	32 (45.0)	
Tumor size (N=1660)				
≤2	594 (35.8)	572 (36.0)	22 (31.0)	0,39
>2	1066 (64.2)	1017 (64.0)	49 (69.0)	
Positive lymph nodes				
0-3	1019 (61.4)	973 (61.2)	46 (64.8)	0,54
≥4	642 (38.6)	617 (38.8)	25 (35.2)	
Histological grade (N=1654)				
I	109 (6.6)	108 (6.8)	1 (1.4)	<0.001
II	746 (45.1)	730 (46.1)	16 (22.9)	
III	799 (48.3)	746 (47.1)	53 (75.7)	
Histological type				
IC-NST^	1354 (81.6)	1299 (81.6)	55 (77.4)	<0.001

Invasive lobular	150 (9.0)	148 (9.4)	2 (2.8)	
Mixed	77 (4.6)	77 (4.8)	0 (0.0)	
Medullary	29 (1.8)	16 (1.0)	13 (18.4)	
Other	51 (3.0)	50 (3.2)	1 (1.4)	
Surgery (binary)				
MRM	947 (57.0%)	908 (57.2%)	39 (55.0%)	0,72
PM	714 (43.0%)	682 (42.8%)	32 (45.0%)	
Hormonotherapy (N=1657)				
No	371 (22.4)	333 (21.0)	38 (53.6)	<0.001
Yes	1286 (77.6)	1253 (79.0)	33 (46.4)	
Radiotherapy (N=1615)				
No	389 (24.1)	370 (24.0)	19 (26.8)	0,59
Yes	1226 (75.9)	1174 (76.0)	52 (73.2)	
Subtypes entire cohort				
Luminal A	563 (33.8)	560 (35.2)	3 (4.2)	<0.001
Luminal B	429 (25.8)	415 (26.2)	14 (19.8)	
Luminal HER2	305 (18.4)	291 (18.4)	14 (19.8)	
HER2-Enriched	143 (8.6)	130 (8.2)	13 (18.4)	
TNBC	221 (13.4)	194 (12.2)	27 (38)	
ERPgR central (N=1597)				
Negative	293 (18.3)	263 (17.2)	30 (44.8)	<0.001
Positive	1304 (81.7)	1267 (82.8)	37 (55.2)	
ERPgR local (N=1658)				
Negative	368 (22.2)	327 (20.6)	41 (57.7)	<0.001
Positive	1290 (77.8)	1260 (79.2)	30 (42.3)	
HER2 IHC central (N=1595)				
non-positive	1373 (86.1)	1327 (86.8)	46 (68.7)	<0.001
positive	222 (13.9)	201 (13.2)	21 (31.3)	
HER2 IHC local (N=1633)				
no overexpression	1188 (72.7)	1145 (73.3)	43 (61.4)	0,030
overexpression	445 (27.3)	418 (26.7)	27 (38.6)	
HER2-status central (N=1609)				
non-positive	1232 (76.6)	1192 (77.3)	40 (59.7)	0,001
positive	377 (23.4)	350 (22.7)	27 (40.3)	
CK5 central (N=1588)				
Negative	1370 (86.3)	1331 (87.3)	39 (61.9)	<0.001
Positive	218 (13.7)	194 (12.7)	24 (38.1)	
EGFR central (N=1590)				
Negative	1334 (83.9)	1292 (84.7)	42 (64.6)	<0.001
Positive	256 (16.1)	233 (15.3)	23 (35.4)	
Basal central (N=1582)				
Basal	334 (21.1)	298 (19.6)	36 (57.1)	<0.001
non-Basal	1248 (78.9)	1221 (80.4)	27 (42.9)	
Randomization Group				

E-CMF	72 (4.4)	72 (4.6)	0 (0.0)	0,11
E-CMF-Doc	172 (10.4)	169 (10.6)	3 (4.2)	
E-CMF-T	186 (11.2)	178 (11.2)	8 (11.2)	
E-T-CMF	1002 (60.4)	951 (59.8)	51 (71.8)	
ET-CMF	229 (13.8)	220 (13.8)	9 (12.6)	
Survival data				
Median FU in months	72,3	72,4	70,5	
N of valid cases	1661	1590	71	
Deaths, N	253	248	5	0,060
Event free at 3 years, %	95,4	95,3	97,2	
Event free at 5 years, %	89,4	89,1	97,2	
Relapse, N	357	352	5	0,004
Event free at 3 years, %	88,1	87,8	95,8	
Event free at 5 years, %	82,5	82,0	92,6	

Notes: MRM: modified radical mastectomy; PM: partial mastectomy; FU: follow-up; N: number; IC-NST: invasive carcinoma of non-specific type; IHC: Immunohistochemistry *: comparison of variable categories in LPBC and non-LPBC

Table 2

Click here to download Table Table2.doc

Molecular & TILs parameters	N patients	N events	HR	95% CI	Wald's p
TILs (continuous)	-	-	0,96	0.90-1.02	0,15
TILs 5-50% vs. <5%	1014 vs. 576	215 vs. 137	0,84	0.68-1.05	0,12
PIK3CA mutation vs. no PIK3CA mutation	428 vs. 1162	78 vs. 274	0,74	0.58-0.95	0,02
PIK3CA-only vs. other*	331 vs. 1259	50 vs. 302	0,59	0.44-0.79	0,001
TP53 mutation vs. no TP53 mutation	336 vs. 1254	89 vs. 263	1,32	1.04-1.68	0,023
TP53-only vs. other**	239 vs. 1351	61 vs. 291	1,23	0.93-1.62	0,15
PIK3CA mutation vs. no PIK3CA mutation in TILs 5-50%	266 vs. 748	48 vs. 167	0,79	0.57-1.09	0,15
PIK3CA mutation vs. no PIK3CA mutation in TILs <5%	162 vs. 414	30 vs. 107	0,64	0.42-0.96	0,033
PIK3CA-only vs. other in TILs 5-50%	193 vs. 821	28 vs. 187	0,61	0.41-0.90	0,013
PIK3CA-only vs. other in TILs <5%	138 vs. 438	22 vs. 115	0,51	0.32-0.82	0,005
TP53 mutation vs. no TP53 mutation in TILs 5-50%	254 vs. 760	59 vs. 156	1,19	0.88-1.61	0,26
TP53 mutation vs. no TP53 mutation in TILs <5%	82 vs. 494	30 vs. 107	1,88	1.25-2.82	0,002
TP53-only vs. other in TILs 5-50%	181 vs. 833	39 vs. 176	1,06	0.75-1.50	0,73
TP53-only vs. other in TILs <5%	58 vs. 518	22 vs. 115	1,85	1.17-2.92	0,009
Ki67 (continuous)	-	-	1,04	1.02-1.06	<0.001
p53 IHC ≥10% vs. <10%	771 vs. 657	156 vs. 148	0,89	0.71-1.12	0,32
Demographic and clinicopathological parameters					
≥4 LN vs. 0-3 LN	617 vs. 973	220 vs. 132	2,72	2.19-3.37	<0.001
Age >50 vs. ≤50	933 vs. 657	224 vs. 128	1,28	1.03-1.59	0,026
Basal vs. non-Basal	298 vs. 1221	73 vs. 255	1,29	1.00-1.68	0,052
ER/PgR local positive vs. negative	1260 vs. 327	270 vs. 82	0,81	0.63-1.04	0,094
Histological grade					0,004
Histological grade II vs. I	730 vs. 108	156 vs. 11	2,14	1.16-3.94	0,015
Histological grade III vs. I	746 vs. 108	185 vs. 11	2,6	1.42-4.78	0,002
Histological grade III vs. I-II	746 vs. 838	185 vs. 167	1,31	1.06-1.61	0,012

Histological type grouped					0,26
Invasive lobular vs. Invasive ductal	148 vs. 1299	34 vs. 283	0,97	0.68-1.39	0,88
Mixed vs. Invasive ductal	77 vs. 1299	24 vs. 283	1,39	0.92-2.11	0,12
Other vs. Invasive ductal	66 vs. 1299	11 vs. 283	0,7	0.38-1.28	0,25
Hormonotherapy Yes vs. No	1253 vs. 333	263 vs. 87	0,69	0.54-0.88	0,003
Menopausal status Post vs. Pre	854 vs. 736	204 vs. 148	1,21	0.98-1.50	0,077
Radiotherapy Yes vs. No	1174 vs. 370	276 vs. 68	1,25	0.96-1.64	0,094
Subtypes (with respect to trastuzumab [T]-treatment)					<0.001
HER2pos-T vs. LumA	221 vs. 560	29 vs. 99	0,81	0.53-1.22	0,31
HER2pos-noT vs. LumA	200 vs. 560	74 vs. 99	1,9	1.40-2.58	<0.001
LumB vs. LumA	415 vs. 560	99 vs. 99	1,33	1.01-1.76	0,043
TNBC vs. LumA	194 vs. 560	51 vs. 99	1,56	1.11-2.19	0,010
Tumor size >2 vs. ≤2	1017 vs. 572	267 vs. 84	1,84	1.44-2.35	<0.001

PIK3CA-only, TP53-only: no co-mutation

other*: TP53-only OR co-mutation TP53&PIK3CA OR no mutation in either gene

other**: PIK3CA-only OR co-mutation TP53&PIK3CA OR no mutation in either gene

Table 3

Click here to download Table Table3.doc

Interaction	N patients	N events	HR (FIRTH)	95% CI (FIRTH)	Wald's p (FIRTH)
	Non-LPBC (N=1590)				
PIK3CA MUT *TILs					0,57
PIK3CA MUT YES vs NO At TILs < 5%	414 vs. 162	107 vs. 30	0,68	0.45-1.02	
PIK3CA MUT YES vs NO At TILs 5-50%	748 vs. 266	167 vs. 48	0,79	0.57-1.09	
TILs 5-50% vs <5% At NO PIK3CA MUT	414 vs. 748	107 vs. 167	0,81	0.63-1.03	
TILs 5-50% vs <5% At PIK3CA MUT	162 vs. 266	30 vs. 48	0,94	0.60-1.48	
PIK3CA-only *TILs					0,78
TILs 5-50% vs <5% At Other^	438 vs. 821	115 vs. 187	0,81	0.64-1.02	
TILs 5-50% vs <5% At PIK3CA-only	138 vs. 193	22 vs. 28	0,88	0.51-1.53	
PIK3CA-only vs Other At TILs < 5%	438 vs. 138	115 vs. 22	0,56	0.36-0.88	
PIK3CA-only vs Other At TILs 5-50%	821 vs. 193	187 vs. 28	0,61	0.41-0.91	
TP53 MUT *TILs					**0,065**
TP53 MUT YES vs NO At TILs < 5%	494 vs. 82	107 vs. 30	1,9	1.27-2.84	
TP53 MUT YES vs NO At TILs 5-50%	760 vs. 254	156 vs. 59	1,18	0.88-1.60	
TILs 5-50% vs <5% At NO TP53 MUT	494 vs. 760	107 vs. 156	0,9	0.70-1.15	
TILs 5-50% vs <5% At TP53 MUT	82 vs. 254	30 vs. 59	0,56	0.36-0.87	
TP53-only *TILs					**0,046**
TILs 5-50% vs <5% At Other^^	518 vs. 833	115 vs. 176	0,9	0.71-1.14	
TILs 5-50% vs <5% At TP53-only	58 vs. 181	22 vs. 39	0,51	0.30-0.85	
TP53-only vs Other At TILs <5%	518 vs. 58	115 vs. 22	1,89	1.20-2.98	
TP53-only vs Other At TILs 5-50%	833 vs. 181	176 vs. 39	1,06	0.75-1.50	
	Luminal A/B (N=975)				
PIK3CA MUT *TILs					0,29
PIK3CA MUT YES vs NO At TILs < 5%	283 vs. 136	67 vs. 23	0,68	0.42-1.09	
PIK3CA MUT YES vs NO At TILs 5-50%	377 vs. 179	74 vs. 34	0,95	0.63-1.43	
TILs 5-50% vs <5% At NO PIK3CA MUT	283 vs. 377	67 vs. 74	0,78	0.56-1.08	
TILs 5-50% vs <5% At PIK3CA MUT	136 vs. 179	23 vs. 34	1,09	0.64-1.84	

PIK3CA-only *TILs					0,78
TILs 5-50% vs <5% At Other	296 vs. 412	71 vs. 87	0,83	0.60-1.13	
TILs 5-50% vs <5% At PIK3CA-only	123 vs. 144	19 vs. 21	0,91	0.49-1.69	
PIK3CA-only vs Other At TILs < 5%	296 vs. 123	71 vs. 19	0,61	0.37-1.01	
PIK3CA-only vs Other At TILs 5-50%	412 vs. 144	87 vs. 21	0,67	0.42-1.08	
TP53 MUT *TILs					0,34
TP53 MUT YES vs NO At TILs < 5%	379 vs. 40	74 vs. 16	2,4	1.40-4.11	
TP53 MUT YES vs NO At TILs 5-50%	466 vs. 90	82 vs. 26	1,72	1.11-2.67	
TILs 5-50% vs <5% At NO TP53 MUT	379 vs. 466	74 vs. 82	0,86	0.63-1.18	
TILs 5-50% vs <5% At TP53 MUT	40 vs. 90	16 vs. 26	0,62	0.33-1.14	
TP53-only *TILs					**0,082**
TILs 5-50% vs <5% At Other	392 vs. 501	78 vs. 95	0,91	0.68-1.23	
TILs 5-50% vs <5% At TP53-only	27 vs. 55	12 vs. 13	0,44	0.20-0.95	
TP53-only vs Other At TILs <5%	392 vs. 27	78 vs. 12	2,72	1.49-4.97	
TP53-only vs Other At TILs 5-50%	501 vs. 55	95 vs. 13	1,3	0.73-2.31	

PIK3CA-only, TP53-only: no co-mutation

Other^: PIK3CA-only OR co-mutation TP53&PIK3CA OR no mutation in either gene
Other^^: TP53-only OR co-mutation TP53&PIK3CA OR no mutation in either gene
Significant interactions are shown in bold.

Click here to access/download
Supplemental Materials - Not to be Published
ESM1_Supplementary Methods and Figures rev1.doc

Supplemental Tables

Click here to access/download

Supplemental Materials - Not to be Published

ESM2_Supplemental Tables.xls