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**Patterns of genomic change in residual disease after neo-adjuvant chemotherapy for
estrogen receptor positive and HER2 negative breast cancer**

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Key words: breast cancer, neoadjuvant chemotherapy, next generation sequencing, clonal evolution

Abstract

Background

Treatment of patients with residual disease after neo-adjuvant chemotherapy for breast cancer is an unmet clinical need. We hypothesised that tumour subclones showing expansion in residual disease after chemotherapy would contain mutations conferring drug resistance.

Methods

We studied estrogen receptor and/or progesterone receptor-positive, HER2-negative tumours from 42 patients in the EORTC 10994/BIG 00-01 trial who failed to achieve pathological complete response. Genes commonly mutated in breast cancer were sequenced in pre and post-treatment samples.

Results

Oncogenic driver mutations were commonest in PIK3CA (38% of tumours), GATA3 (29%), CDH1 (17%), TP53 (17%) and CFBF (12%); and amplification was commonest for CCND1 (26% of tumours) and FGFR1 (26%). The variant allele fraction frequently changed after treatment, indicating that subclones had expanded and contracted, but there were changes in both directions for all of the commonly mutated genes.

Conclusions

We found no evidence that expansion of clones containing recurrent oncogenic driver mutations is responsible for resistance to neoadjuvant chemotherapy. The persistence of classic oncogenic mutations in pathways for which targeted therapies are now available highlights their importance as drug targets in patients who have failed chemotherapy but provides no support for a direct role of driver oncogenes in resistance to chemotherapy.

ClinicalTrials.gov: EORTC 10994/BIG 1-00 Trial registration number NCT00017095.

1 **Background**

2 Neoadjuvant chemotherapy (NAC) before loco-regional treatment is widely used in patients with
3 large operable or locally advanced breast cancers. Several trials have demonstrated that patients
4 without residual invasive disease in the surgical specimen (primary breast tumour and lymph
5 nodes) after neoadjuvant chemotherapy have a low risk of relapse ¹⁻³ and trials aiming to de-
6 escalate therapy in this group of patients are ongoing. On the other hand, patients with residual
7 invasive tumour after neoadjuvant chemotherapy have at least a 25% risk of invasive relapse
8 within 5 years ¹⁻⁴. This high risk of relapse is observed in all molecular breast cancer subtypes
9 despite the systematic use of adjuvant hormonal therapy in luminal tumours and trastuzumab in
10 HER2-positive tumours ¹⁻⁴.

11
12 Based on these data, treatment of patients with residual invasive disease after NAC should be
13 considered as an unmet clinical need. Several studies have been conducted or are ongoing to
14 assess the clinical impact of additional post-neoadjuvant treatments in these patients after
15 standard neoadjuvant treatment. This approach has been successful in two recently published
16 phase III studies. A first study conducted in luminal HER2-negative and triple negative breast
17 cancers (TNBC) compared observation to capecitabine for six to eight cycles ⁵. This trial
18 demonstrated a survival advantage in the overall population which was more pronounced in the
19 TNBC group. A second study conducted in HER2-positive tumours compared standard treatment
20 with trastuzumab for 1 year to trastuzumab emtansine (T-DM1) for the same duration ⁶. This study
21 demonstrated a halving of the risk of invasive relapse with T-DM1 as compared to continued
22 trastuzumab.

23
24 Although this "one size fits all" post-neoadjuvant chemotherapy strategy in patients with residual
25 disease has been successful in these two phase III studies, a large proportion of patients will still
26 relapse. A personalised post-neoadjuvant chemotherapy strategy based on genomic analysis of
27 residual disease could have two important advantages. First, tumour cells in the residual tumour
28 after neoadjuvant chemotherapy are by definition resistant to this treatment. We would expect
29 genomic aberrations conferring resistance to be more easily identified in samples taken after
30 treatment had eliminated the drug-sensitive cells present in the primary tumour. Second, genomic
31 analysis of tumour cells in residual disease could identify de novo mutations that were not present
32 before chemotherapy. These mutations might create new therapeutic opportunities. A previous
33 study focusing on residual disease after NAC for TNBC identified genetic alterations which could
34 potentially be targeted in 90% of the tumours ⁷ but there was no evidence that these were de novo
35 mutations selected by the treatment. Another study on TNBC and one including multiple subtypes
36 also failed to identify any recurrent changes after treatment ^{8,9}.

37 In this study, we chose to focus on luminal HER2-negative tumours because these tumours are the
38 most frequent and account for the largest number of breast cancer deaths. We therefore believe
39 that the greatest unmet need lies in this group. The genomic landscape of luminal (ER positive and
40 HER2 negative) tumours has been reported in the literature both before any systemic therapy ¹⁰
41 and in the metastatic setting ¹¹. However, to our knowledge, there are no data available from this
42 group of tumours after NAC. We were particularly interested in the possibility that post-treatment
43 samples would show expansion of clones containing mutations that can be targeted by therapies
44 that have shown efficacy in patients with metastatic disease, such as PIK3CA mutations ¹²,
45 BRCA1/2 mutations ¹³ and AKT1 mutations ¹⁴.

46

47 Formally, the primary objective of this study was to discover mechanisms of resistance to
48 neoadjuvant chemotherapy in luminal breast cancer by identifying genetic changes in subclones
49 showing clonal expansion after treatment. To identify clonal expansion we analysed variant allele
50 fractions (VAF) in matched samples before and after chemotherapy from 42 patients with luminal
51 (ER and/or PgR positive) HER2-negative breast cancer who did not achieve a pathological
52 complete response (pCR) after neoadjuvant therapy within the EORTC 10994/BIG1-00 trial ¹⁵.

53

54 The neoadjuvant design of the original 10994 study means we can compare pre-treatment with
55 post-treatment samples to identify changes potentially selected by the treatment. This approach
56 reduces confounding by germline mutations. To reduce the background from passenger mutations
57 we restricted the analysis to a panel of genes that are known to be mutated in breast cancer.

58

59

60 **Patients and methods**

61 **Study design, treatment and eligibility**

62 This is an ancillary study of the EORTC 10994/BIG 1-00 trial which randomized patients between
63 six cycles of a non-taxane regimen (5-fluorouracil, epirubicin, cyclophosphamide) and a taxane-
64 based regimen (docetaxel for three cycles followed by epirubicin + docetaxel for three cycles) all
65 given before primary surgery¹⁵. Eligible patients were women aged less than 71 years with
66 histologically-proven invasive carcinoma of the breast suitable for neoadjuvant chemotherapy, with
67 any large operable or locally advanced/inflammatory breast cancer. The trial was approved by
68 national and/or local ethics committees in all participating centres. Patients gave signed informed
69 consent at the time of enrolment for the original EORTC 10994 study.

70 For the sub-study that is the subject of this report, we have selected patients in a subgroup
71 of the initial population of 1856 based on the following eligibility criteria: (1) patients eligible for the
72 main p53 trial; (2) patients who gave informed consent for optional research on tumour samples;
73 (3) luminal tumours (ER and/or PgR positive) and HER2-negative based on assessment by local
74 laboratory; (4) patients who received at least one cycle of neo-adjuvant chemotherapy and who did
75 not receive radiotherapy before surgery; (5) non pCR after neoadjuvant chemotherapy; (6) Pre-
76 and post-chemotherapy samples with more than 50% invasive tumour cells and 250-300 ng DNA
77 available (for details see Sup Table 1 and the CONSORT diagram in Sup Fig 1).

78

79 **Objectives**

80 The primary objective was to discover mechanisms of resistance to NAC by identifying recurrent
81 genetic changes in subclones showing clonal expansion after treatment. We defined clonal
82 expansion as a significant change by Fisher test in the variant allele fraction (VAF) after treatment.
83 The secondary objectives were: 1. To describe the mutational landscape of all the samples
84 (number of coding mutations, number of driver mutations, type of mutations), 2. To identify
85 changes in the mutational landscape after NAC (VAF changes in driver oncogenes, copy number
86 changes in driver oncogenes), 3. To document the expansion and contraction of subclones after
87 NAC.

88

89 **Tumour samples and pathology assessment**

90 Tumour biopsies were taken with a 14G trucut needle before starting neoadjuvant chemotherapy.
91 Some of these biopsies were fixed in formalin and embedded in paraffin (FFPE) as part of routine
92 assessment, the remaining biopsies were frozen as part of the mandatory procedure in the original
93 EORTC 10994/BIG 1-00 trial. Grade, ER, PR, and HER2 status were assessed by local
94 pathologists from a biopsy taken at diagnosis before starting neoadjuvant chemotherapy and
95 prospectively recorded in the case report form. ER and PR, assessed by immunohistochemistry
96 (IHC), were reported as positive or negative according to each centre's local definition. HER2

97 negativity was defined as either absence of HER2 gene amplification by fluorescent in situ
98 hybridization or a score of 0-1+ by IHC. Pathological response after completion of neoadjuvant
99 chemotherapy was assessed by local pathologists on formalin fixed samples taken during surgical
100 resection of the residual tumour. Non pCR cases were defined as patients with residual invasive
101 disease in the primary tumour and/or in the nodes as reported by the local pathologist.

102

103 We collected and centralized in Bordeaux both frozen pre-chemotherapy samples and FFPE post-
104 chemotherapy samples taken during surgery. We have summarised in Sup Fig 1 how the 42
105 patients were selected from the initial population of 1856 patients, of whom 499 with ER and/or
106 PgR positive and HER2-negative tumours were theoretically eligible for this substudy. We initially
107 considered performing NGS on pre-chemotherapy FFPE trucut biopsies but a pilot study showed
108 that NGS was not feasible with these samples. Post-chemotherapy samples were taken from
109 surgical specimens and formalin fixed. H&E sections were examined to estimate tumour cellularity.
110 All of these samples were centrally reviewed by a board-certified pathologist (GMG) to estimate
111 the tumour cell content and to mark regions in the FFPE samples for DNA extraction. The samples
112 qualified if more than 50% of the cells in the biopsy sample were invasive cancer cells.

113

114 **DNA extraction and sequencing**

115 In Bordeaux (Bergonié Institute), DNA was purified on Qiagen columns after proteinase K digestion
116 of the pre-chemotherapy frozen samples. For post-chemotherapy FFPE samples, one or two 1 mm
117 diameter cores of 0.5 to 1 mm thickness, depending on the material available, were taken and
118 DNA was extracted with the GeneRead DNA FFPE kit (ref 180134 Qiagen, Hilden, Germany).
119 DNA concentration was measured by Qubit and tubes containing 250-300ng of DNA at >30ng/ul
120 were sent to the Wellcome Sanger Institute (WSI). An electronic manifest linking barcodes to
121 EORTC 10994 Sample IDs was sent to WSI with the tubes.

122 In Cambridge (WSI), samples were further anonymised with replacement of the EORTC
123 Sample ID with a Sanger ID. 100 SNPs were used to check that pre and post-treatment samples
124 were derived from the same patient. Genomic DNA was quality controlled and 200 ng was used for
125 library preparation. DNA was sheared to an average fragment size of 150 bp using the E210
126 Covaris plate system (Covaris, Inc. Woburn, MA). The fragmentation settings used were 200
127 cycles per burst at intensity 4 for 120 seconds. Fragmented DNA was subjected to Illumina DNA
128 sequencing library preparation using a Bravo automated liquid handling platform.

129 Sequencing libraries were amplified using the bridge-amplification process by Illumina
130 HiSeq pair read cluster generation kits (TruSeq PE Cluster Kit v2.5, Illumina) and were hybridized
131 to custom RNA baits for the Agilent SureSelect protocol. Paired-end, 75 bp sequence reads were
132 generated using Illumina HiSeq 2000 with the target of 1Gb sequence per sample. The sequenced
133 reads were aligned to the reference human genome (NCBI build 37) and deposited in bam format

134 at the European Genome-Phenome Archive (<https://www.ebi.ac.uk/ega/>, cram files
135 EGAD00001003334, study accession number EGAS00001001223). Additionally, bam files for 93
136 normal samples sequenced with the two panels used in this study were deposited under the
137 accession numbers EGAS00001002124 and EGAS00001001808 (panels V3 and V4,
138 respectively).

139

140 **Bioinformatic Analysis**

141 The breast cancer panels used for DNA capture are described in supplementary data files 1 & 2.
142 They target all coding exons of 366 (first batch) and 280 (second batch) cancer genes that are
143 recurrently mutated, amplified or deleted plus regions showing recurrent copy number variation,
144 and recurrently rearranged introns of fusion genes. The coverage of the first panel is 2028799 bp,
145 that of the second is 2055503 bp, and the overlap is 1089744 bp. Sequencing reads were aligned
146 to the NCBI build 37 human genome using the Burrows-Wheeler Aligner (BWA-aln version 0.5.9,¹⁶
147 to create BAM files with Smith-Waterman correction [<http://bio-bwa.sourceforge.net>]. PCR
148 duplicates were marked with biobambam version 2.0.65 [<https://github.com/gt1/biobambam2>]
149 ¹⁷. The tumour samples were compared to 93 FFPE-treated normal samples sequenced with the
150 two panels to an equivalent depth. The sample mean sequencing depth was 574x in batch 1 and
151 787x in batch 2. The mean coverage with 100+ reads was 82% in batch1 and 97% in batch 2.
152 Coverage for individual samples is shown in Sup Fig 2. Variant calling was performed using the
153 Pindel version 4.2 [<https://github.com/genome/pindel>] and CaVEMan version 1.5.3
154 [<https://github.com/cancerit/CaVEMan>] algorithms as described ¹⁸. A simple tandem repeat filter
155 was applied first to remove variants observed less than five times or in less than 10% of the reads.
156 Variants present in the normal samples were excluded if the difference in VAF was <0.2. Variants
157 were only considered if observed in both forward and reverse strands. The calls were limited to the
158 regions specified in the panel bed files (sup data 3 & 4). The probes generate sequence up to 50
159 bp on either side of each exon in order to detect splice site mutations. Synonymous mutations in
160 coding regions and variants in UTRs, enhancers and copy number regions are not scored as
161 coding variants. Together with biological selection, for example, counterselection of inactivating
162 mutations in oncogenes, this explains why only 10% of variants are coding mutations. The CNVKit
163 algorithm version 0.9.7b1 was used for copy number segmentation [<https://github.com/etal/cnvkit>].
164 Unfortunately, the algorithm was unable to fully correct for the lower quality of the FFPE data,
165 leading to batch effects that are visible in the copy number heatmap . To avoid overinterpreting
166 copy number changes we only scored genes as amplified if the affected segment was <15 Mb. For
167 the reported CCND1 amplicons, the median and maximum lengths of the amplified segments were
168 3.3 and 12.8 Mb; for FGFR1 they were 3.5 and 14.6 Mb. To enrich for high-confidence somatic
169 variants, further filtering was performed to remove known constitutional polymorphisms using
170 human variation databases (Ensembl GRCh37, 1000 genomes release 2.2.2 and ESP6500, cut-off

171 VAF ≤ 0.001); and to remove alterations seen recurrently in the 93 normal DNA samples
172 sequenced with the same protocol. The Variant Allele Fraction was corrected for normal tissue
173 contamination with vafCorrect version 5.4.0 [<https://github.com/cancerit/vafCorrect>]. Enrichments
174 are defined as variants detected in pre-chemotherapy samples and increased in post-
175 chemotherapy samples. Gains are defined as variants not detected in pre-chemotherapy samples
176 but detected in post-chemotherapy samples. To distinguish between de novo mutations and clonal
177 expansion, coding variants giving VAF = 0 in one sample from a patient were called in both
178 samples without filtering: of the 20 cases where the variant was present in the bam files but
179 removed by the filters, 8 were present once, 3 present twice, 2 present 3 times, 1 present 4 times,
180 2 present 5 times, 2 present 8 times and 2 present 10 times (Sup Table 2). Code availability: Code
181 is available on request to AC or RI.

182 **Results**

183 Demographics, tumour characteristics and treatment of the 42 eligible patients included in this
184 study are given in Sup Table 1. DNA was extracted from frozen biopsies before treatment and from
185 formalin-fixed samples after treatment. It was sequenced in two batches: an initial batch of 17
186 tumours with single pre-treatment and post-treatment samples; then a second batch including 18
187 tumours with single pre-treatment and post-treatment samples and 7 tumours with two pre-
188 treatment samples and one post-treatment sample. Thus 91 samples (49 pre-neoadjuvant
189 chemotherapy and 42 post-neoadjuvant chemotherapy) from a total of 42 tumours had evaluable
190 NGS and CNA data.

191

192 **Mutational landscape**

193 The total number of SNVs and indels in each sample is shown in Sup Fig 3; the median number of
194 variants was 86, with five samples having over 200 variants. As expected, the vast majority of
195 changes in all samples were C>T and G>A substitutions (Sup Fig 4). All three samples from
196 patient PD30315 had a mutation rate much higher than the samples from the other patients. This
197 tumour has an S314A mutation in POLE (DNA polymerase epsilon) that may explain the high
198 mutation rate. It lies in the exonuclease (proofreading) domain and is flanked by known mutations
199 (E311D and D316G, <http://mutationaligner.org/domains/PF03104?gene=POLE>). It is rare in the
200 EXAC SNP database (8×10^{-5}), and multiple computational predictions score it as pathogenic
201 (DANN, FATHMM, LRT, MutationAssessor and MutationTaster;
202 varsome.com/variant/hg19/rs770403791).

203

204 **Coding variants**

205 The median number of coding variants (SNVs and indels) per sample was 8, with 2 samples
206 having over 20 coding variants and 12 samples containing less than 5 coding variants (Sup Fig 5).
207 The coding variants in individual genes in each tumour are listed in Sup Table 2. Driver mutations
208 were defined as previously characterised oncogenic mutations^{19,20} or mutations that truncate a
209 tumour suppressor protein. The number of tumours with driver mutations in the pre-chemotherapy
210 or post-chemotherapy samples is shown in Fig 1. Seventeen genes had driver mutations in more
211 than one tumour. The commonest genes with driver mutations were PIK3CA (16/42 tumours =
212 38%), GATA3 (12/42 tumours = 29%), CDH1 (7/42 tumours = 17%), TP53 (7/42 tumours = 17%)
213 and CBF3 (5/42 tumours = 12%).

214

215 **Clonal expansion and contraction after neoadjuvant chemotherapy**

216 The primary objective was to discover mechanisms of resistance to NAC by identifying genetic
217 changes in subclones showing clonal expansion after treatment. To identify subclones showing
218 clonal expansion we compared the VAF in pre- and post-treatment samples (Fig 2a). There were

219 31 putative de novo mutations that appeared after treatment. This was balanced by loss of 30
220 mutations through clonal extinction. These changes affected 46 genes, including 28 driver
221 mutations, in 30 patients. By far the commonest change was PIK3CA mutation but there were
222 changes in both directions: 3 tumours lost mutations, 2 tumours gained them and one tumour had
223 one change in each direction. The next commonest changes were in KMT2C (1 loss, 2 gains) and
224 ARID1A (2 losses, one gain). Examination of the bam files revealed that in 75% of these cases,
225 the variant was actually present at a very low depth when it was called as absent (in two thirds of
226 these cases it was present in one, two or three reads). While it is difficult to draw definitive
227 conclusions from such small numbers of reads, it is likely that in many cases where a new
228 mutation is detected we are seeing expansion of a pre-existing clone rather than a de novo
229 mutation.

230 Fisher tests were used to detect significant changes in VAF that fell short of complete gain or loss.
231 Including the complete gains and losses, 36% of coding variants changed significantly after
232 treatment (Supplementary Table 2, 152/428 variants had $p < 0.01$ after Bonferroni correction). Fig
233 2b&c illustrate different patterns of clonal behaviour. In Fig 2b (tumour PD30309) all points lie
234 close to the diagonal, indicating that there was no clonal selection: the cluster at VAF ~ 0.15
235 represents a tumour subclone whose abundance did not change; the cluster at VAF ~ 0.5
236 represents either an ancestral tumour clone or heterozygous germline variants that escaped
237 filtering. Consistent with this interpretation, the variants found at VAF ~ 0.15 are classified as driver
238 mutations in CDH1, PIK3CA, TBX3 and MAP3K1, whereas the variants at VAF ~ 0.5 are
239 heterozygous missense changes in ACAN, FGFR1, HRNR, PTPN11, RPL5, and WBP1 that are
240 probably either ancestral passenger mutations or rare germline variants. Fig 2c (tumour PD26285)
241 illustrates expansion of a malignant subclone containing CBF, CDH1 and NF1 driver mutations
242 from a VAF ~ 0.15 before treatment to a VAF ~ 0.55 after treatment. The RUNX1 mutation that goes
243 from VAF ~ 0.15 to ~ 0.3 is potentially a mutation present initially in the same parental subclone but
244 which failed to expand. Interestingly, RUNX1 is the dimerization partner of CBF. The CBF-
245 RUNX1 dimer tethers ER to enhancers; inactivating mutations in either gene could redirect ER to
246 more pernicious targets. Since the subclone containing the CBF mutation expanded faster it
247 would appear either that the CBF mutation is better able to inactivate the dimer or perhaps the
248 mutations in CDH1 and NF1 gave the CBF subclone an added advantage. The remaining
249 variants in this tumour, at VAF ~ 0.6 before and after treatment, are non-driver mutations in
250 TCF7L2 and TSC1 that could represent passenger mutations or rare germline variants. Similar
251 plots for each individual tumour showing the VAF before and after treatment are presented in Sup
252 Fig 6.

253 Next, instead of looking at all genes in individual tumours, we examined individual genes in
254 all tumours. We give an overview showing the driver oncogenes most frequently mutated before
255 and after treatment in Fig 3a; strikingly, all lie almost perfectly on the diagonal, indicating that none

256 of these genes is consistently selected for by neoadjuvant chemotherapy. Our interpretation is that
257 they are unlikely to confer resistance to chemotherapy. We can examine the behaviour of
258 individual genes by plotting the VAF for driver mutations in the gene in all tumours (Fig 3b-f). The
259 points represent individual tumours, with the size of the points scaled according to the probability
260 that the VAF changed after treatment. It is immediately obvious that for every gene there are points
261 located far from the diagonal, meaning the clonal abundance changed. Strikingly, there were
262 changes in both directions, meaning driver mutations in a particular gene became more abundant
263 in some tumours and less abundant in others. That clones containing driver oncogenic mutations
264 should become rarer is surprising, particularly for a gene like PIK3CA, but it is important to note
265 that some of the variation may be explained by incomplete correction for the amount of
266 contaminating normal tissue in the samples.

267

268 **Copy number variants**

269 Copy number changes are plotted in a heatmap in Fig 4a. Gains are shown in red, losses in blue.
270 There are frequent gains of chr1q and chr16p, indicating that the der(1;16) signature translocation
271 of luminal breast cancer was present in most samples. Large chr8q gains, commonly attributed to
272 MYC, were also present in most samples. The most commonly amplified genes were CCND1
273 (11/42 tumours = 26%) and FGFR1 (11/42 tumours = 26%) (Fig 4b). ERBB2 amplification on chr17
274 was not seen because tumours with this amplicon were excluded from the study. No fusion genes
275 were detected. Both at the level of the whole genome (Fig 4a), and when individual amplicons
276 were examined (Fig 4b), there were no consistent changes after treatment. There was also no
277 significant difference between the number of breakpoints before and after treatment in the two
278 treatment arms.

279

280 **Pathway analysis after treatment**

281 In Fig 5 we categorise the driver mutations present after chemotherapy into different functional
282 pathways. The only ESCAT²¹ tier IA target is PIK3CA, which has hotspot mutations in 14 tumours.
283 We score the POLE mutation in tumour PD30315 as tier IC, because the tumour has a high
284 mutation burden making it a candidate for immunotherapy. Three tumours had tier IIA PTEN
285 mutations, two tumours had tier IIB AKT1 T17E mutations, two tumours had tier IIIa MDM2
286 amplification, and one tumour had a tier IIIa somatic BRCA1 mutation. Together, 48% of tumours
287 (20/42) had at least one of these defects. If one adds FGFR1 and CCND1 amplicons, which do
288 not respond to current targeted therapy (tier X), 74% of tumours (31/42) had potentially interesting
289 targets. The remaining mutations in Fig 5 are tier IVA or not in the ESCAT breast cancer list,
290 although many of the latter are targets for preclinical or clinical drugs in other tumour types.

291

292 **Discussion**

293 Our working hypothesis was that clones with recurrent mutations in driver oncogenes would
294 expand after neoadjuvant chemotherapy but we have found no evidence to support it. Since there
295 were multiple changes in VAF and copy number of driver oncogenes after treatment but they show
296 no consistent pattern we conclude that there is no driver oncogene that undergoes strong positive
297 selection under chemotherapy. We confirmed the presence of targetable mutations in many driver
298 oncogenes previously reported to be mutant in breast cancer, and showed that they are not
299 eradicated by neo-adjuvant chemotherapy, but we did not identify any recurrent genetic changes in
300 clones surviving chemotherapy. We therefore conclude that classic mammary oncogenes are
301 probably not responsible for resistance to chemotherapy. This echoes the conclusion from the
302 original EORTC 10994 "p53 study" ¹⁵, that p53 status can not be used to select patients for
303 chemotherapy with taxanes. The likely explanation for the negative results in both studies is that
304 chemotherapy targets dividing cells whose rapid division is a distant consequence of the
305 oncogenic mutations that created the tumour. Crucially, the targets of chemotherapy are never
306 mutant in tumours. This contrasts with the paradigm of modern targeted therapy. Pharmacologists
307 will not be surprised by our results: they have long known the metabolic pitfalls that dictate the
308 exposure and response of tumour cells to chemotherapeutic drugs.

309

310 We have confirmed the presence of targetable mutations in many driver oncogenes previously
311 reported to be mutant in pretreatment samples from luminal ER+ breast cancers ^{10,22,23}. We have
312 shown here that the same mutations are also present in residual disease after chemotherapy. The
313 persistence of classic oncogenic mutations in functionally important pathways in so many tumours
314 after chemotherapy highlights their importance as drug targets. Targeted therapies are either
315 licensed or in development for most of these pathways. ESMO has developed a Scale for Clinical
316 Actionability of molecular Targets (ESCAT) to classify mutations according to the strength of
317 evidence supporting their use in patients with metastatic breast cancer ²¹. Tier of evidence IA
318 refers to genetic alterations for which a licensed treatment is known to be effective. The two main
319 contenders are ERBB2 amplification and PIK3CA mutation. PIK3CA was the commonest driver
320 oncogene mutated both before and after chemotherapy in this study. Tumours with ERBB2
321 amplification in the pretreatment samples were excluded from this study, and new ERBB2
322 amplicons were not detected after treatment. Tier IC includes immunotherapy for tumours with a
323 high mutation rate. This is mainly caused by mismatch repair defects but can also result from
324 defective proofreading by the replicative polymerase, DNA polymerase epsilon (POLE). Several
325 tumours had MSH2 mutations of unknown effect. One had a POLE mutation that produced a large
326 increase in the mutation burden, making that tumour an obvious candidate for immunotherapy. Tier
327 II includes gene-drug pairs which have shown activity in breast cancer but for which there is
328 currently no proof that they increase survival in prospective studies. In our data, PTEN loss (IIA)
329 and AKT1 mutation (IIB) fall into this category. Tier III covers gene-drug pairs that show activity in

330 other tumour types, or family members of tier I genes. Two genes fall in this category in our data
331 (BRCA1 and MDM2). The commonest changes we saw are classified as tier IVA, meaning there is
332 good preclinical data to support their exploration in clinical studies. Tier X includes perplexing
333 defects for which treatment should work but does not. It includes CCND1 and FGFR1, the genes
334 most commonly amplified in our study. It is surely only a matter of time before the failure of
335 tumours with these amplicons to respond to ostensibly good drugs is explained, leading to the
336 development of new therapeutic strategies that improve the survival of affected patients. Many of
337 the other mutations in Fig 5 were not assigned a level of evidence because they were not in the 40
338 genes ESMO chose to classify, but effective preclinical or clinical drugs exist for many of them.
339 Mutations in others, like RB1 and FAT1, provide useful information because they confer resistance
340 to licensed drugs. There is thus good reason to be optimistic about the utility of sequencing data,
341 and to foresee an increasing dependence on it in the future. Considering the high risk of relapse of
342 patients with ER+ HER2- tumours with residual disease after NAC, a personalised post NAC
343 strategy based on genomic analysis should be considered a clinical research priority.

344
345 The gene panels we tested cover all currently known mammary oncogenes. The pace of discovery
346 of new oncogenes and tumour suppressor genes in breast cancer has fallen to essentially zero
347 because of the enormous scale of the genomic studies that have already been completed^{10,22,23}.
348 This means we can confidently exclude the possibility that a common oncogenic driver was
349 missing from the present study. A whole genome sequencing approach would have allowed us to
350 detect mutations in genes that are not known breast cancer genes, but, had we done this without a
351 normal sample to exclude germline variants, the enormous number of unknown variants
352 discovered would have rendered identification of true mediators of resistance a near impossible
353 task. In cases where the mutant fraction for a particular variant changed in one direction in one
354 tumour, there were changes in the opposite direction in other tumours, suggesting that the
355 differences were not caused by clonal selection by the treatment, but only PIK3CA, TP53, GATA3,
356 CDH1 and CFB were mutant often enough, and CCND1 and FGFR1 amplified often enough, to
357 warrant this type of analysis. This is a recurring theme in genomic studies of breast cancer: many
358 genes are infrequently mutated leading to a multiplicity of tiny groups that defy statistical inference.

359
360 Many drug resistance studies based on clinical trials have produced interesting but inconclusive
361 results^{7,8,15,24,25,26}. What is missing is proof that the observed changes play a causal role in drug
362 resistance. To fill this gap requires sophisticated cell biological studies linked to clinical trials.
363 Recent progress in cell culture and xenografting techniques²⁷⁻²⁹ mean it is now possible, at least in
364 principle, to perform these studies but the technical challenges are daunting. Specifically, it means
365 establishing cultures of samples taken before treatment, introducing putative genetic changes
366 mediating resistance with viral or CRISPR technology, and showing that the genetically engineered

367 cells acquire the predicted phenotype. Given the risk of sampling artefacts, this should be done
368 with samples containing the broadest possible representation of clones, and with techniques that
369 allow tracing of individual cells in complex mixtures, for example by barcoding the starting material
370 and analysing large numbers of single cells at each step⁸.

371

372 In summary, we have performed a targeted sequencing study comparing driver mutations before
373 and after neoadjuvant chemotherapy of luminal breast cancer and found no evidence that
374 expansion of clones containing recurrent driver mutations is responsible for resistance to
375 neoadjuvant chemotherapy. There may be multiple such mechanisms in play, each contributing
376 occasionally to resistance, but new approaches based on functional analysis of material from
377 clinical trials will be required to prove it. Despite our failure to discover new chemotherapy
378 resistance mechanisms, we identified targetable defects both before and after chemotherapy in
379 most of the tumours examined. Future clinical studies should seek to increase the number of
380 licensed drugs available to target these defects

381

382 **Additional information**

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387

388 **Authorship**

389 HB, RI and DC conceived the study. DC, HB and EORTC clinical investigators provided clinical
390 samples. CP analysed the clinical data. GMG analysed the histology. JS extracted the DNA. AC
391 and RI analysed the NGS data and made the figures. RI, HB and AC wrote the manuscript. All
392 authors approved the final version of the manuscript and agreed to be accountable for all aspects
393 of the work.

394

395 **Ethics approval and consent to participate**

396 The EORTC 10994 clinical trial was registered with ClinicalTrials.gov number NCT00017095 and
397 approved by national and/or local ethics committees in all participating centres. Before registration,
398 all patients signed an informed consent for the trial and for mandatory p53 gene assessment on
399 tumour samples. Patients involved in this substudy gave consent for additional biological research
400 on their tumour samples. The study was performed in accordance with the Declaration of Helsinki.

401

402 **Data availability**

403 Clinical data can be accessed through the EORTC data sharing platform (for details see
404 www.eortc.org/data-sharing). NGS data can be accessed through the European Genome-
405 Phenome Archive (<https://www.ebi.ac.uk/ega/>, cram files EGAD00001003334, study accession
406 number EGAS00001001223). The NGS data is only accessible under a Managed Access
407 agreement (for details see www.ebi.ac.uk/ega/dacs).

408

409 **Competing interests**

410 The authors declare no conflict of interest.

411

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421

422

423 **EORTC 10994/BIG 1-00 Consortium (**)**

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544 apocrine breast tumours. *J Pathol* **240**, 256-261 (2016).

545

546 **Figure legends**

547

548 **Fig 1. Commonest driver mutations before and after treatment.** The mutation count is non-
549 redundant (ie, genes are only counted once if multiple mutations were identified in a tumour, or the
550 same mutation was present in multiple samples from a tumour).

551

552 **Fig 2. Variants gained and lost after neoadjuvant chemotherapy.** a, Number of coding
553 variants gained and lost in each tumour. b&c, Examples of tumours showing different patterns of
554 clonal change after treatment (b, PD30309, FEC arm; c, PD26285, T-ET arm). The size of the
555 plotting symbol reflects the Fisher p value (mutations supported by fewer reads have a smaller
556 symbol).

557

558 **Fig 3. Frequency and VAF of the most frequently mutated oncogenes and tumour**
559 **suppressor genes before and after neoadjuvant chemotherapy.** a, Percentage of tumours with
560 specific genes mutated before and after treatment. b-f, VAF for the most commonly mutated
561 drivers. There are substantial changes in both directions after treatment. The size of the plotting
562 symbol reflects the Fisher p value (mutations supported by fewer reads have a smaller symbol).

563

564 **Fig 4. Copy number variants in pre- and post-neoadjuvant chemotherapy samples.** a,
565 Heatmap showing regions gained and lost coloured red and blue, respectively. The y axis is
566 ordered by patient ID with pre- followed by post-treatment samples. b, Log₂ ratio before and after
567 treatment for the most commonly amplified genes (CCND1 and FGFR1). The dashed grey line
568 corresponds to a copy number of 4. The dotted grey line corresponds to likely gains (3 sd above a
569 copy number of 2). If two pretreatment samples were tested, the second sample is indicated by an
570 X and joined to the first sample by a horizontal line. Note that the FGFR1 sample with log ratio 0.6
571 pre/1.6 post was not scored as amplified because it was not a focal gain. FEC: 5-fluorouracil +
572 epirubicin + cyclophosphamide x6; T-ET: docetaxel x3 then docetaxel + epirubicin x3.

573

574 **Fig 5. Pathway analysis.** Driver oncogenes mutated or amplified after treatment are grouped
575 according to biological pathway. CCND1, FGFR1, AURKA, MDM2 and NCOA3 are within amplified
576 regions; all of the other genes shown contained driver mutations. Note that for amplified regions
577 the true driver may be a nearby gene rather than the one shown.









