Genomic and transcriptomic analyses of breast cancer primaries and matched metastases in AURORA, the Breast International Group (BIG) molecular screening initiative

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Abstract

AURORA aims to study the processes of relapse in metastatic breast cancer (MBC) by performing multi-omics profiling on paired primary tumors and early-course metastases. Among 381 patients (primary tumor and metastasis pairs: 252 TGS, 152 RNA-Seq, 67 SNP Arrays), we found a driver role for *GATA1* and *MEN1* somatic mutations. Metastases were enriched in *ESR1, PTEN, CDH1, PIK3CA* and *RB1*

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mutations; *MDM4, MYC* amplifications; *ARID1A* deletions. An increase in clonality was observed in driver

genes like *ERBB2* and *RB1*. Intrinsic subtype switching occurred in 36% of cases. Luminal A/B to HER2-

Enriched switching was associated with *TP53* and/or *PIK3CA* mutations. Metastases had lower immune

score and increased immune permissive cells. High TMB correlated to shorter time to relapse in

HR+/HER2- cancers. ESCAT tier I/II alterations were detected in 51% of patients and matched therapy

was used in 7%. Integration of multi-omics analyses in clinical practice could impact treatment strategies

in MBC.

Statement of significance

The AURORA program, through the genomic and transcriptomic analyses of matched primary and

metastatic samples from 381 patients with breast cancer, coupled with prospectively collected clinical

data, identified genomic alterations enriched in metastases and prognostic biomarkers. ESCAT tier I/II

alterations were detected in over half of the patients.

Introduction

Despite an improvement in survival rates following advances in early detection and treatments, breast

cancer (BC) remains one of the leading causes of cancer-related mortality among women (1). Metastases

are the main cause of death for patients, highlighting the need for treatment strategies to avoid

metastatic relapse and to improve the outcome of patients with *de novo* metastatic breast cancer (MBC).

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So far, progress in the treatment of MBC has mainly occurred through the conduct of empirical clinical trials in which patients are segregated by BC subtyping, namely HR+/HER2-, HER2-positive or triple-negative (TNBC). Consequently, treatment decisions are dictated by these limited subtypes, and the lines of therapy rely on minimal biological data (e.g. presence or absence of hormone receptors, HER2 status). As an example, CDK 4/6 inhibitors are now routinely added to first or second line endocrine therapies for ER+/HER2- disease, dual HER2 blockade is combined with chemotherapy as front-line treatment of HER2+ BC and PD-(L)1 checkpoint inhibitors are added to first-line chemotherapy in relapsed PD-L1+ TNBC (2). These treatment algorithms, whilst improving outcomes for some patients, have also led to marked increases in treatment costs, and contrast with growing knowledge of the molecular, genetic and immunological complexity of the disease, which can be captured by novel technologies such as targeted gene sequencing (TGS) of tissue and cell-free DNA (cfDNA).

The Breast International Group (BIG) is conducting AURORA (Aiming to Understand the Molecular Aberrations in Metastatic Breast Cancer. NCT02102165), a molecular screening program which aims to improve the understanding of MBC through the extensive profiling of paired primary tumors and metastatic samples, as well as cfDNA extracted from plasma, collected from at least 1000 patients with MBC (figure 1). The feasibility and logistics of this pan-European effort had been previously assessed in a pilot study that involved four centers in four European countries (3). Taking advantage of the large number of paired primary and metastatic samples obtained in the program, with the analyses described in this manuscript we aimed to: 1- identify molecular alterations enriched in the early phases of metastatic disease; 2- describe variations in gene expression between primary samples and their paired metastasis; 3- correlate genomic and transcriptomic markers with outcome; 4- evaluate the contribution of molecular profiling to the management of patients with MBC. The analyses were performed using genomic (TGS), transcriptomic and clinical data from the first 381 patients included in AURORA. To our

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knowledge, this is the largest study to date collecting paired samples from patients with MBC, and the largest dataset of RNA sequencing (RNA-Seq) in MBC.

Results

By February 28th 2018, 381 patients had been included in 51 centers in nine European countries (Belgium, Germany, Iceland, Italy, Luxembourg, Spain, Sweden, Switzerland, United Kingdom). Two patients were excluded for violation of the eligibility criteria leaving 379 patients for the current analysis. An additional four paired samples we excluded after a careful review of the clinical data suggested the possibility of unmatched primary tumors and metastases (e.g. due to the presence of a second primary breast or other tumor, figure S1). Based on the immunohistochemistry (IHC) status of the primary tumor, 247 (65%) patients had HR+/HER2- breast cancer, 72 (19%) had TNBC, and 60 (16%) had HER2+ breast cancer. The majority of patients were treatment-naïve for MBC at inclusion (n=274; 72%) but most had received some form of systemic therapy in the (neo)-adjuvant setting. A sizable fraction of the cohort (n=87; 23%) presented with *de novo* MBC and the metastasis site was sampled prior to any therapy in 54 cases (14% of the whole cohort). Liver was the most frequent site of metastatic biopsies (n=152; 40%) followed by lymph nodes (n=104; 27%) then skin and soft tissue (n=52; 14%). Patients and tumor characteristics are provided in supplementary table 1 and sites of metastatic biopsies are illustrated in figure S2.

The current analysis includes data from matched pairs from 242 patients with TGS, 152 patients with RNA-Seq and 67 patients with CNVs by SNP arrays. Details are available in the CONSORT diagram (figure S1) and in figure S3. Data from cfDNA is available for 99 patients as this analysis only started later after a protocol amendment.

The first objective of AURORA is to study the genomic alterations driving metastatic relapse and progression of MBC. Driver genes potentially enriched in MBC when compared to primary tumors were

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identified in large studies by comparing genomic data from retrospectively collected samples with genomic data generated mostly from primary tumors. Compared to these cohorts, AURORA has a large number of primary/metastasis pairs collected for the majority of patients before any treatment for MBC or after only one line of therapy for some. This should allow the investigation of molecular alterations involved in metastatic relapse without the potential impact of therapy in the metastatic setting. Figure 2 illustrates, for the cohort of paired samples with TGS (n=242), IHC subtypes, intrinsic subtypes (based on PAM50 derived from RNA-Seq data), treatment history, tumor mutational burden (TMB), somatic single nucleotide variants (SNVs), short indels and copy number variations (CNVs). To identify the most common cancer genes in the AURORA cohort we applied the dN/dS algorithm that compares the normalized ratio of non-synonymous to synonymous mutations, to quantify selection in cancer genomes. Using this approach a total of 21 cancer genes were found to be under positive selection cohort-wise (in primary tumors [figure S4A] and/or metastases [figure S4B]). Fourteen (67%) of these genes overlapped with those previously identified by the Martincorena dN/dS analysis of the TCGA breast cohort (n=702) (4) and 7 additional genes (*ERBB3, ESR1, FBXW7, GATA1, KRAS, MEN1, NF1*) were identified as significantly mutated (figure S4C). There is evidence supporting these genes being drivers and the differences we observe between the two cohorts likely reflect the unique composition of AURORA. The dN/dS analyses also provided evidence that somatic mutations in *GATA1* (q=0.02 for missense mutations in the primary tumors) and *MEN1* (q=0.01 for metastatic samples) are under positive selection. These are known cancer genes but have not been identified through the analysis of large series of primary and/or MBC to date. *GATA1* encodes a transcription factor and has been associated with the induction of epithelial-mesenchymal transition in breast cancer (5). *MEN1* is a tumor suppressor gene associated with multiple endocrine neoplasia 1 syndrome. *MEN1* germline mutations are linked to an increase in breast cancer risk (6). The most prevalent point mutations in primary and/or metastases were found in *TP53*

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followed by *PIK3CA*, *ESR1*, *CDH1*, and *GATA3*. The most prevalent CN gains were in *MYC*, *CCND1*, *FGFR1*, *KAT6A, MDM4* and *ERBB2*; the most prevalent losses were in *TP53* and *RB1*.

We then compared the prevalence of molecular alterations from primary and metastatic samples found in our dataset with those enriched in the cancer genome atlas (TCGA) BC cohort (7) that used whole-exome sequencing (WES) and the METABRIC cohort (8) that used TGS. We wanted to assess if the driver alterations putatively responsible for metastatic relapse were comparable in other series. We first identified the point mutations enriched in patients that relapsed in TCGA and METABRIC by comparing the genomic landscape patients without a relapse to those with a relapse (figure S5A-B). TP53 mutations were more prevalent in AURORA while PIK3CA mutations were less prevalent (figure S5C). These differences are possibly explained by the characteristics of AURORA that only included patients with a confirmed metastatic relapse and complete clinico-pathological annotation.

The analyses of paired primary-metastasis samples in AURORA allow to directly examine changes emerging within each cancer over the course of its evolution in order to study the molecular alterations enriched in early MBC. The median TMB was higher in the metastatic samples when compared to the paired primary samples ($p<10^{-9}$; figure 2, figure S6A). This finding is consistent with the fact that somatic mutation accumulation is a continuous process. The difference did not reach statistical significance for TNBC (figure S6B), probably reflecting the fact that TNBC relapses more rapidly, so there is less time to acquire enough additional mutations within the scope of the small TGS panel to see a difference in most cases. We also looked at median TMB in the *de novo* and not *de novo* cases (figure S6C-D). The median TMB in not *de novo* cases was higher when compared with *de novo* cases and the difference was statistically significant (p=0.046).

In the full population of patients with paired TGS data (n=242), 88% of point mutations in the driver genes were shared between the primary sample and the metastasis (86% in HR+/HER2-, 93% in TNBC

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and 87% in HER2+). At least one driver point mutation acquired in the metastases was identified in 10% of cases (10% in HR+/HER2-, 12% in HER2+ and 6% in TNBC, figure S7A-D). At least one acquired CNV detected by ASCAT (n=67) was found in 31% of cases (30% in HR+/HER2-, 43% in HER2+ and 27% in TNBC, figure S7A-D). Point mutations in the following genes were enriched in the metastatic samples: *ESR1*, *PTEN*, *CDH1, PIK3CA* and *RB1* (figure 3A-D). In matched pairs of primary and metastatic samples (n=67) analyzed by SNP arrays, CN gains (including amplifications, figure 3E-L) of *MDM4*, *MYC*, *NSD3*, *FGFR1*, *AXIN1*, *TSC2*, *FLT4*, *NTRK1* and *N4BP2*; and deletions (figure 3M-P) of *ARHGEF10L*, *CASP9*, *RB1*, *ARID1A* and *PBRM1* were more frequent in the metastatic samples. The same analyses were performed for the subset of patients with *de novo* MBC. Similar alterations were found between the primaries of *de novo* and not *de novo* patients. We did not observe in the metastasis of *de novo* patients the enrichment of alterations that we observed for non-*de novo* patients, in particular *ESR1* mutations. However, the small number of patients in the *de novo* group does not allow drawing firm conclusions (figures S8A-P, S9A-P, S10A-P, S11A-P)

We then studied the evolution of the clonal composition from the primary tumors to their paired metastases (n=242). We found an increase in the median cancer cell fraction (CCF) for point mutations between the primary samples and their paired metastases, suggesting an overall increase in clonality in metastatic versus primary samples (figure 4A). The increase was statistically significant for the HR+/HER2- and HER2+ subtypes but not TNBC (figure 4B). Genes with the most significant increase were *ESR1*, *SMAD4*, *RB1* and *LRP1B* (figure 4C). An increase in clonality was seen in genes with potential clinical impact such as *ESR1* in HR+/HER2- MBC (endocrine resistance), *RB1* in HR+/HER2- and HER2+ MBC (resistance to CDK4/6 inhibitors) and *ERBB2* in all subtypes (endocrine resistance, resistance to anti-HER2 therapies, sensitivity to certain anti-HER2 tyrosine kinase inhibitors) (figure 4D-F). The increase in median CCF was consistent by metastatic biopsy site when considering the most frequent biopsy sites

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(figure 4G). Figure S12 highlights the changes between primary and paired metastasis in the CCF of driver and other genes.

As homologous recombination deficiency (HRD)-related genes (*BRCA1/2* and others) have therapeutic importance in MBC(9), we applied SigMA to estimate the mutational signature associated with HRD and the change between primary tumors and their paired metastasis. The SigMA computational tool allows to detect the mutational signature associated with HRD (10) using TGS (see supplementary methods). Three different callers were used (mva, mva_strict and ml). Only samples with enough mutations as per SigMA were selected: 251 samples in total from 181 patients. There were paired samples from 70 (38.7%) patients, 95 patients with metastatic samples only and 16 patients with primary tumors only. Tumor samples harboring *BRCA1* or *BRCA2* mutations (only pathogenic and likely pathogenic variants were considered) had a higher likelihood of being flagged by SigMA and the difference was statistically significant for both germline and somatic mutations (figure S13A). There was a trend towards a higher prevalence of the HRD signature in TNBC that was statistically significant with the ml caller on metastatic samples (figure S13B). The HRD signature was enriched in metastatic samples compared to primary tumors for all subtypes combined, this difference was statistically significant with the ml caller (figure S13C).

We then aimed to describe variations in gene expression between primary samples and their paired metastasis. RNA-Seq data were available for 152 pairs of primary tumors and metastases. First, we studied the correlation between mutations in selected driver genes (*ESR1* and *ERBB2*) and their level of expression. *ESR1* mutations in the metastatic samples were associated with a higher level of expression of *ESR1* mRNA (p<0.001) and a higher probability of a Luminal B subtyping (figure S14 A-E). Similarly, HR+/HER2- tumors (primary and metastasis) harboring *ERBB2* activating mutations had a higher expression of *ERBB2* mRNA (p=0.039 for primary tumors and p=0.0042 for metastases) albeit lower than

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HER2+ tumors, and were more likely to be classified as HER2-Enriched (HER2-E, p = 0.014 for the primary tumors and p = 0.0042 for the metastases; figure S14 F-J).

Previous reports have shown that the IHC and intrinsic subtypes do not completely overlap (11), bringing potential clinical implications. Furthermore, the intrinsic subtype can change between the primary tumor and the metastasis (12). Beyond IHC subtype switching (figure 5A), we wanted to study the molecular determinants of intrinsic subtype switching by assessing mutations and clonal evolution associated with this phenomenon. The prevalence of the intrinsic subtypes of the primary tumors in our cohort (n=211) as determined using RNA-seq is as follows: 22% Luminal A, 38% Luminal B, 11% HER2-E, 25% Basal, 4% Normal. Intrinsic subtype switching in the patients with paired RNA-seq data (n=152) was seen in 55 (36%) cases. Almost all Luminal A primaries switched in metastatic samples (90% of cases) and there was subtype switching from Normal, Luminal (A or B) and Basal to HER2-E in n=18 cases (figure 5B, figure S15). We focused on the 14 cases switching from Luminal A or B to HER2-E because of the prevalence in our cohort and potential clinical implications. For the cases with available TGS data (n=13), higher prevalence of *TP53* and/or *PIK3CA* mutations was found in these tumors compared to Luminal A or B cases not switching, both in the metastatic and primary samples (figure S16). In order to assess whether subtype switching was related to tumor heterogeneity and change in clonality under therapy pressure, we compared the median CCF between Luminal A or B primary tumors and their paired metastases switching to the HER2-E subtype. No decrease in median CCF could be identified in our cohort (figure S17), which is in line with previous reports (13).

The analyses of RNA-Seq data from matched pairs using uniform manifold approximation and projection (UMAP) for dimension reduction show that Basal tumors cluster by pairs in contrast to the Luminal and HER2-E subtypes, owing to closer gene expression profiles in the primaries and metastases of TNBC (figure S18A-L). The gene expression differences were significantly larger in HR+/HER2- MBC when compared to the other subtypes. Gene expression differences were also larger between primary samples

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and their paired metastasis when compared with paired samples from cases of de novo MBC. Statistical significance was however only reached in HR+/HER2- MBC. This finding remained true in the HR+/HER2- subtype after comparison with de novo MBC pairs that had metastatic tissue collection after one line of therapy for metastatic disease (figure 5 C-D). A longer time to relapse was associated with HR+/HER2- MBC and larger gene expression differences (figure S19A-B). These findings may indicate that adjuvant endocrine therapy exerts the major influence on these gene expression differences that were seen between primary tumor and paired metastasis, suggesting an adaptive transcriptional reprograming associated with endocrine resistance. Another explanation is a longer time before relapse, allowing the accumulation of more mutations.

Immune biomarkers such as tumor-infiltrating lymphocytes (TILs) are prognostic biomarkers in early breast cancer and are being investigated as predictive biomarkers (14). Immune checkpoint inhibitors have been approved in combination with chemotherapy in metastatic PDL1+ TNBC and are investigated in other subtypes such as Luminal B. We investigated immune signatures in paired primary tumors and metastases by site, as well as the difference in the immune composition between paired primary tumors and metastases by site. Expression of the immune module score (15) was lower in metastatic samples but not in lymph nodes metastases (figure 5 E-I). We looked at lymph node metastases separately because of the potential bias related to cellular composition. When performing the same analysis by metastatic site, expression of the immune signature was higher in skin metastases compared to other metastatic sites and lower in liver metastases (figure S20). Using CIBERSORT (16) we studied the immune cell composition in primary tumors and their paired metastasis. Immune cell populations increasing in metastatic samples were: Mast cell activated, Myeloid dendritic cell resting, NK cell activated, T cell regulatory, Macrophage M1 and macrophage M2 (figure S21A). Immune cell populations decreasing in metastatic samples were: T cell CD4+ memory activated and T follicular helper (figure S21A). Correlation

between the expression of the immune score and the immune cell composition by site of metastasis is seen in figure S21B.

The next objective was to identify genomic markers that could predict the outcome for patients: overall survival (OS) from the diagnosis of MBC to death; and/or time to relapse (TTR) from the diagnosis of primary BC to the metastatic relapse. Median duration of follow-up was 847 days (range 2 - 2082 days). Two hundred fifty-nine patients had experienced at least one event of disease progression and 209 patients had died. Of those, 126 patients had ER+/HER2- BC, 57 had TNBC and 26 HER2+ BC. In a recent study utilizing TGS, a higher number of mutations were associated with a trend towards worse survival in HR+/HER2- and HER2+ MBC (17). We tested if high TMB as defined by the upper 90th percentile (see supplementary methods) was associated with patient outcomes. We had seen that median TMB was significantly higher in paired metastatic samples compared to primary tumors in HR+/HER2- and in HER2+ MBC but not in TNBC (figure S6B). High TMB in primary tumors was associated with a shorter TTR (figure 6A) and this was statistically significant for the HR+/HER2- (figure 6B) subtype but not for HER2+ nor TNBC (figure S22 A-B). High TMB in primary tumors was also associated with a statistically significant shorter OS and there was a trend towards shorter OS for high TMB in the metastatic samples (figure S22 C-D). This was driven by the HR+/HER2- subtype as the difference was not statistically significant in HER2+ and in TNBC (figure S22 E-F-G-H-I-J). In multivariate analysis controlling for other covariates like primary tumor size, nodal status and tumor grade (see Supplementary methods), high TMB in HR+/HER2- primary tumors remained an independent predictor of short TTR (p=0.0012) and OS (p=0.0088). The association between high TMB and TTR in HR+/HER2- BC was not found when tested in the TCGA cohort (figure S23 A). We therefore hypothesized that the TTR finding in HR+/HER2- could be driven in by the number of driver mutations in the primary tumor. High TMB was indeed associated with a significantly higher number of drivers in HR+/HER2- primary tumors in our dataset (figure 6C) and in TCGA (figure S23 B). A higher number of drivers in HR+/HER2- primary tumors was found to be associated with a shorter

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TTR (figure 6 D) but TMB remained and independent predictor in the multivariate model when adjusting for the number of driver mutations (p<0.001). We then looked at the association between mutations and outcome overall and by subtype, and two genes were associated with worse outcome: TP53 and LRP1B (figure S24 A-B-C-D). Mutations in *LRP1B* were correlated with worse OS (p=0.0028, FDR=0.037, figure S25A). This was true for shared and acquired mutations (p=0.0017, figure S25B). *LRP1B* is a putative tumor suppressor and a member of the low-density lipoprotein (LDL) receptor family.

Lastly, we investigated the clinical utility of molecular profiling of MBC since patients were enrolled prospectively and results reported on a sample-by-sample basis. We classified the molecular alterations identified by TGS using the ESMO Scale of Clinical Actionability for molecular Targets (ESCAT) (18). Many but not all drivers selected for the BC ESCAT tiers classification are detected and reported to the treating physicians in AURORA. We found that at least one Tier I or II alteration was identified for 51% of patients or 36% if we exclude *ERRB2* amplification that is a standard of care biomarker (table 1). The choice of therapy remained at the discretion of the treating physician. Since these genomic results are generated in a research environment, the treating physician should have ensured that the results are confirmed using conventional/approved genetic tests, prior to the introduction of any clinical action. We queried the clinical data of this cohort of 379 patients to estimate the rate of therapy matched to a genomic alteration. We have identified 102 patients (27%) treated with targeted therapies. Of those, we have excluded patients treated with a targeted therapy without a proven genomic biomarker and patients treated with a targeted therapy while we did not identify the matching genomic alteration in AURORA. Therapy matched to a genomic alteration was prescribed for 25 patients (7%, supplementary table 2).

Data from the cfDNA analyses of the baseline plasma sample were available for 99 patients. Thirty-nine patients had no mutations detected in cfDNA. At least one mutation was detected in 60% of cases (figure S26A). Out of 77 genomic alterations detected in one or both tissue samples, 31 (40%) were not found in cfDNA (figure S26B). The variants found in a tissue sample and not detected in cfDNA had a statistically

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significant lower variant allele frequency (VAF) in the tissue samples (primary and/or metastatic sample,

Mann-Whitney U test P-value<0.001 for both primary and metastatic samples, figure S26C). Tier I or II

mutations as per ESCAT (*PIK3CA, ESR1*, *AKT1*, *ERBB2*) that were identified in one or both tissue samples

were not detected in cfDNA in 11 cases (11%; figure S26D).

Discussion

Recent studies aimed to describe the genomic landscape of MBC (19–21) and identify potentially

acquired driver genes, largely by inter-dataset comparisons with series of largely primary tumors

(7,8,22). The design of AURORA based on the prospective collection of paired primary and metastatic

samples in patients that are treatment-naïve for MBC or after just on line of therapy, is built to allow the

unbiased characterization of molecular alterations causing metastatic relapse or acquired early in the

course of metastatic disease. The majority of driver point mutations were shared (88%) and only a

minority of patients (10%) had at least one mutation private to the metastatic sample. This is in contrast

with other studies that collected paired samples and that reported a higher rate of acquired genomic

alterations ranging from 45% (23) to 73% (24). Fully concordant alterations between the primary and

metastatic samples were only found in 18% of cases in another study (17). An explanation could be the

clinical setting as patients in AURORA are enrolled early in the course of MBC while acquired driver

alterations occurred late in the metastatic process (23). It was recently reported that mutations private

to metastasis are less associated with the metastatic spread and are accumulating under the pressure of

therapy (25). The heterogeneity of NGS techniques (WGS, WES, TGS) could also contribute to this

difference.

Molecular alterations found to be enriched in AURORA metastatic samples could contribute to new drug

development for MBC. Several are involved in cancer epigenetics (*MYC* amplification, *NSD3*

amplification, *PBRM1* deletion and *ARID1A* deletion) and this field is emerging as a contributor to

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endocrine resistance (21,26). Agents targeting cancer epigenetics (bromodomain and extra-terminal domain inhibitors, histone deacetylase inhibitors and EZH2 inhibitors) are currently in development in combination with endocrine therapy. *AXIN1* (Wnt pathway) and *NSD3* (27) are involved in stemness, another process that promotes therapy resistance. Agents targeting stem cells are now in clinical development (gamma-secretase inhibitors, LGR5 inhibitors) and a study testing AL101 (gamma-secretase inhibitor) has just started recruitment in metastatic TNBC (NCT04461600). Enriched alterations also include biomarkers associated with innate or acquired resistance to therapies available in the clinic such as *PTEN* loss (28), *RB1* loss-of-function alterations (29) and *FGFR1* amplification (30) for CDK4/6 inhibitors, *PBRM1* loss-of-function alterations (31,32) and *MDM4* amplification (33) for immune checkpoint inhibitors. The higher prevalence of the HRD signature in metastatic disease may widen the target population beyond germline *BRCA1* and *BRCA2* mutation carriers that could benefit from treatment with PARP inhibitors. This reinforces the case of other biomarkers such as those investigated in the TBCRC 048 trial (9).

The largest dataset to date of RNA-Seq of the paired primary and metastases allowed us to study intrinsic subtype switching that is gaining clinical implications. Indeed, intrinsic subtyping, especially for the Luminal and HER2-E subtypes, is emerging as a predictor for a number of targeted therapies in different settings: Everolimus (34) and CDK4/6 inhibitors in HR+/HER2- MBC (35), CDK4/6 inhibitors (36) and anti-HER2 therapies (37) in HER2+. Treatment refinement and de-escalation based on the incorporation of the intrinsic subtype are being included in prospective clinical trials and follow single sample studies while a study has shown intrinsic subtype switching between primary tumor and paired metastasis in 31% of cases including 14.3% of luminal A or B tumors converting to HER2-E. The authors also suggested that expression of *FGFR4* could be driving the HER2-E phenotype (12). This finding was not confirmed in AURORA where we found an association of subtype switching from Luminal A/B to HER2-E with *TP53* and/or *PIK3CA* mutations. We could not demonstrate that subtype switching was

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related to tumor heterogeneity and change in clonality under therapy pressure. The UMAP analyses allowed us to postulate that adjuvant endocrine therapy may drive the differential gene expression between primary and metastatic samples in HR+/HER2- tumors. Since most patients with TNBC and HER2+ tumors have received therapy prior to the development of metastatic relapse, future analyses should study the mutational footprints of various therapeutics as in Pich *et al*. (38).

Comparing immune signatures between primary tumors and paired metastases as well as computing the differential immune cell composition in primary tumors and in different metastatic sites can contribute to the understanding of immune evasion and the planning of future trials testing immunotherapy. Our larger cohort adds to the finding of a smaller cohort (n=11) (39) by demonstrating that the immune signal is lower in metastases (excluding lymph nodes). This finding was not true for lymph node metastases where expression of the immune signatures could be influenced by the presence of immune cells. Interestingly, this may partly explain what was seen in the phase 3 clinical trial of the combination of chemotherapy and Atezolizumab in the first line setting of metastatic TNBC (40) and the phase 2 study of Pembrolizumab monotherapy in metastatic TNBC (41) that showed an increased clinical benefit with immunotherapy among patients with lymph node-only metastatic disease. The low expression of immune signatures in liver metastases mirrors the recent finding about reduced efficacy of immune checkpoint inhibitors in patients liver metastases (42). We found increases and decreases in some immune cell populations leading to a more immune permissive microenvironment in the metastatic samples, in line with studies of residual primary tumors after neoadjuvant chemotherapy (43).

While a higher TMB in MBC has been associated with a worse outcome (19), AURORA is to our knowledge the first study to demonstrate that high TMB associates with a shorter TTR after treatment for HR+/HER2- early BC. This finding remained significant after taking into consideration the number of genomic drivers, highlighting the possibility of incorporating this biomarker in the design of trials testing novel adjuvant therapies in this poor prognosis population. Correlation of outcomes with individual

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genes has associated *LRP1B* with shorter OS, particularly when the mutation occurred in the metastases. Confounders are however to be acknowledged as the *LRP1B* variants are not in known functional domains and the very large size of the gene (91 exons and a mRNA size > 16 kb) (44) makes it a probable proxy of TMB.

Molecular profiling of metastatic cancer has entered the clinic with alpelisib in HR+/HER2- MBC with *PIK3CA* mutations (45) and PARP inhibitors in MBC with germline *BRCA1/2* mutations (46). Other targets are emerging but some reputed scientific societies still do not recommend routine molecular profiling in the management of MBC (47). This is largely due to the lack of approved biomarker-driven targeted therapies in MBC other than anti-HER2 therapies. Despite the fact that 51% (193) of patients had at least one Tier I or II alteration as per ESCAT (83% of patients if we include Tier III and IV alterations currently investigated in clinical trials), only 7% of patients in this cohort had received matched therapy at the data cut-off. This can be explained by drug availability as the recruitment in AURORA started in 2014 while the first regulatory approval for a targeted therapy matched to a molecular alteration in MBC (excluding HER2+) only occurred in 2018, and access to clinical trials and new targeted agents is very heterogeneous in different countries. The results of TGS of the baseline cfDNA in AURORA expanded the landscape of actionable mutations (11% of cases with alterations not identified in a tissue samples). The clinical utility of therapy decided based on the results of cfDNA analyses has been demonstrated in settings such as *PIK3CA* mutations in HR+/HER2- MBC (48). Actionable alterations can however be missed in plasma (40% of alterations in AURORA), possible due to lower VAF in tissue samples for variants that were missed. Liquid biopsies are therefore complementary to tissue biopsies for the management of patients with advanced cancer but current sensitivity of detection is not yet ready to forego tissue biopsy of metastatic disease for full molecular understanding.

There are limitations to be recognized. AURORA has reported so far genomic results of TGS while other studies have utilized more extensive profiling such as WES and WGS. The AURORA biobank of FFPE and

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frozen samples should allow more in-depth analyses. The exclusion of variants with a VAF < 10% hinders the discovery potential of this study. This cut-off was set based on the algorithms used by the sequencing service provider and is considered to be conservative as results are reported to clinicians. Calling variants as low as 1% if the variant is seen at a higher VAF in the matched sample was used in order to describe mutations enriched in the metastases. We would still, however, miss sub-clonal alterations that might have a VAF < 10% in both samples. Also, the majority of samples being core biopsies and collected from one metastatic site, the enrichment findings could be confounded with intra-tumoral heterogeneity. Furthermore, while AURORA requires the collection of primary tumors and a paired metastasis, other samples such as a second primary tumor, pre or post-neoadjuvant therapy samples or local relapses, are not collected and could create a gap in the understanding of the metastatic process.

Beyond the findings discussed in the manuscript, the AURORA initiative is providing the basis for future research in MBC, by allowing future research on residual samples, unexploited fresh frozen metastatic samples and serial collection of plasma and serum samples currently stored in the centralized AURORA biobank. The curated clinical database as well as the central storing of pathology high-resolution scanned images, the collection of clinical data including the identification of outlier patients – exceptional responders or with highly resistant disease – that will be studied in-depth, could allow to generate hypotheses for novel therapeutic strategies.

Methods

The objectives, study design and eligibility criteria of AURORA have been previously described (49). The study aims to include at least 1000 patients with MBC at the diagnosis of metastatic disease or after one line of therapy. Collected samples include FFPE tissue from the primary tumor, FFPE and fresh frozen samples from a metastatic biopsy, whole blood, plasma and serum. At the central lab (OncoDNA, Gosselies, Belgium) and after pathology review and standard IHC for estrogen receptor (ER),

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progesterone receptor (PR) and HER2, TGS is performed on DNA extracted from the primary tumor, the FFPE metastatic sample (gene list in supplementary table 3), whole blood and baseline plasma sample (gene list in supplementary table 4). Results are reported on sample-by-sample basis to the investigators after annotation by a molecular advisory board (MAB). RNA-Seq and CNVs analyses using SNP arrays are performed on the available tumor samples in batches (at EUROFINS). The oncoplot included CNVs as determined by TGS and SNP arrays. Discordances were seen mainly in genes involving deletions (supplementary methods and figure S27). Liver genes were excluded for transcriptomic analyses (supplementary methods and supplementary file – liver genes). Indeed, visualization of the biopsy site of the samples using UMAP showed that liver samples were clustered together (figure S28A-V). Residual FFPE tumor samples, frozen tumor samples and plasma and serum samples collected serially (every 6 months and at every disease progression for up to 10 years) are shipped in batches and stored in the study biobank (IBBL, Dudelange, Luxembourg). In the first version of the protocol, a patient was only considered included if TGS was successful on both primary and metastatic tumor samples. The protocol was amended following a high rate of screen failures to allow inclusion of patients if one of the two tissue samples was successfully sequenced. The amendment also introduced the sample-by-sample concomitant TGS of cfDNA extracted from the baseline plasma sample. A cohort capped at 100 patients with bone-only metastases was introduced; patients were allowed to provide only material from the primary tumor and were considered included if TGS of the primary tumor was successful. The AURORA study recruited patients at 51 sites in 11 European countries. A dedicated IT platform served for the logistics, collection of data on the disease and samples, pathology images scanning and for reporting of the genomic results. The study is conducted in collaboration with Institut Jules Bordet (IJB) and Frontier Science (FS). IJB's Clinical Trials Support Unit holds the clinical database for AURORA, in which clinical data including information on previous and subsequent treatments as well as survival follow-up for up to 10 years are collected. The MAB is composed of experts in molecular biology, genetics, drug

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development, pathology and bioinformatics; it convenes remotely every two weeks and provides variant annotations and clinical implications that are included in a consolidated molecular report. The study was approved by the ethical committees of the participating institutions and is performed in accordance with relevant guidelines and procedures. Participating patients provided written informed consent.

Data availability

Instructions to access the manuscript processed data for reproducibility purposes are available at the webpage https://aurora.bigagainstbreastcancer.org/ and can be obtained upon signature of an appropriate data transfer agreement subject to applicable laws. Instructions to access processed or raw manuscript data to perform original research are also available on the webpage and investigators can contact aurora.researchproposals@bigagainstbc.org for enquiries. Access to data for research will be granted upon review of a project proposal & endorsement by the study Steering Committee, and after entering into an appropriate data access agreement subject to applicable laws.

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Table 1 legend:

We have reproduced the different molecular alterations and their level of evidence as per ESCAT (18).

The molecular alterations reported on a sample-per-samples basis in AURORA are in bold.

Table 1: actionability of alterations as per ESCAT

	Readiness of use in clinical practice	ESCAT for alterations in breast cancer	Prevalence in the AURORA population	Cumulative prevalence in the AURORA population
Tier I	Targets ready for implementation in routine clinical decisions	ERBB2 amplification (IA), germline BRCA1/2 mutations (IA), PIK3CA mutations (IA), MSI (IC), TRK fusions (IC)	143 (38%)	143 (38%)
Tier II	Investigational targets likely to define patients who benefit from a targeted drug, but additional data needed	PTEN loss (IIA), ESR1 mutations (IIA), AKT1 mutations (IIB), ERBB2 mutations (IIB)	65 (17%)	193 (51%)
Tier III	Clinical benefit previously demonstrated in other tumor type or for similar molecular targets	Somatic BRCA1/2 mutations (IIIA), MDM2 amplification (IIIA), ERBB3 mutations (IIIB)	35 (13%)	206 (54%)
Tier IV	Preclinical evidence of actionability	ARID1A/B, ATM/ATR/PALB2, CDH1, IGF1R, INPP4B loss, MAP2K4/MAP3K1, MT4, MYC, NF1, PIK3R1, RUNX1/CBFB, SF3B1, TP53 (IVA)	233 (61%)	313 (83%)
Tier V	Evidence supporting co-targeting approaches			
Tier X	Lack of evidence of actionability	FGFR1 amplification, CCND1 amplification	99 (26%)	328 (86%)

Figure legends

Figure 1: study design

Illustration of the design of the AURORA molecular screening program including the baseline and longitudinal collections of samples as well as the clinical data.

Figure 2: repertoire of somatic gene alterations

Oncoplot of the relevant genomic alterations in the set of 242 patients with available Target Gene Panel (TGS) data for primary and metastatic samples. From top to bottom, the oncoplot includes three sections: Tumor Mutational Burden (TMB), clinical data and genomic alterations. TMB section shows the bar plots of TMB in primary and metastatic samples. Dashed lines refer to the TMB threshold used to

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defined high TMB patients based on 90 percentile of the TMB distribution (corresponding to 8 for primary and 11 for metastatic samples). Clinical data section includes information about the number of treatment lines for metastatic disease, de novo metastatic disease, adjuvant and neo-adjuvant therapy and molecular subtype information in primary and metastatic samples by PAM50 and IHC. Genomic alterations are classified as shared (if present in both primary and metastatic samples), primary (private to primary sample) and metastatic (private to metastatic samples). Genomic alterations include driver mutations (single-nucleotide variants and Insertions/Deletions) in driver genes, amplifications in oncogenes and deletions in tumor suppressor genes. On the right, the bar plots summarize, for each gene, the frequency of shared, private to primary and private to metastatic events. The asterisks refer to genes showing significant difference in terms of alteration frequency in metastatic compared to primary samples (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Genes (SNVs) with significant positive selection on missense mutations and/or truncating substitutions on the dN/dS analysis are represented in the figure. We have included in the figure CNVs of genes known as breast cancer drivers.

Figure 3: comparison of the truncal aberrations with those private to the metastasis.

All plots are on paired samples, each point representing the percentage of tumors with an aberration common between the primary and the metastasis versus the percentage of tumors with an aberration found only in the metastasis. Mutations are shown (A: all subtypes, B: TNBC, C: HER2+, D: HR+/HER2-) as well as CN amplifications (normalize CN > 4; E: all subtypes, F: TNBC, G: HER2+, H: HR+/HER2-), gains (normalized CN > 1.5; I: all subtypes, J: TNBC, K: HER2+, L: HR+/HER2-) and deletions (normalized CN < 1.5; M: all subtypes, N: TNBC, O: HER2+, P: HR+/HER2-). The points are colored in function of their q-values, which assess whether a given aberration is more often private to the mutation than expected by the play of chance, corrected for multiple testing by panel.

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Figure 4: cancer cell fraction (CCF) changes between primary and metastatic samples

Box plots showing the distribution of median CCF by patient in **A**: paired primary and metastatic samples and **B**: stratified by subtype (HR+/HER2-, HER2+, TNBC). Grey lines refer to paired samples. **C**: Median CCF by driver genes in metastatic (y-axis) versus primary (x-axis) samples. The size of the circles refer to each gene alteration frequency. Box plots of the distribution of CCF for driver mutations in **D**: ESR1, **E**: RB1 and **F**: ERBB2, stratified by subtype. **G**: distribution of median CCF in paired primary and metastatic samples by biopsy site. P-values are estimated by paired Wilcoxon-Mann-Whitney test.

Figure 5: RNA-Seq of paired primary tumors and metastatic samples

A-B: Subtype switching, on IHC subtypes (A) or on PAM50, estimated from RNA-Seq (B). **C:** Distribution of the distances between primaries and metastases in term of expression of the PAM50 genes, in function of the clinical subtype. **D:** Similar comparison as C, but between untreated *de novo* metastatic patients, treated *de novo* metastatic patients and patients with a later relapse. **E-I:** Difference in immune signal between primary and metastasis across PAM50 subtypes. "Meta LN" are lymph node metastases, "Meta other" are all other metastases.

Figure 6: TMB and patient outcome

A: TTR by TMB in all subtypes primary samples; **B**: TTR by TMB in HR+/HER2- primary samples; **C**: TMB and number of drivers correlation HR+/HER2-; **D**: TTR by number of drivers HR+/HER2- primary samples.
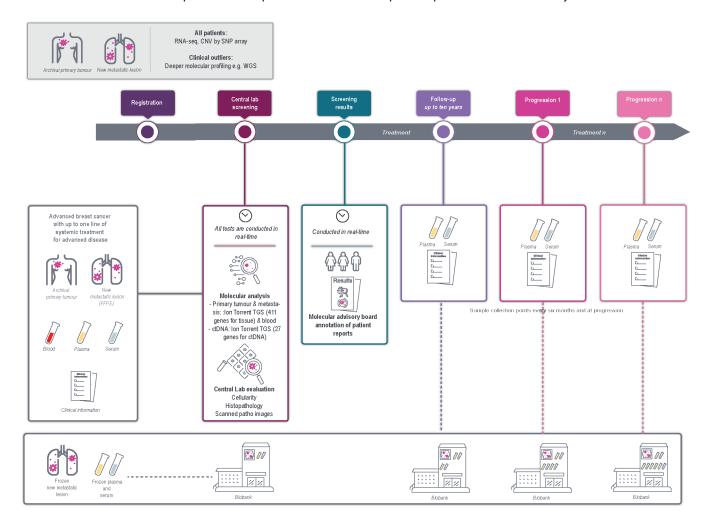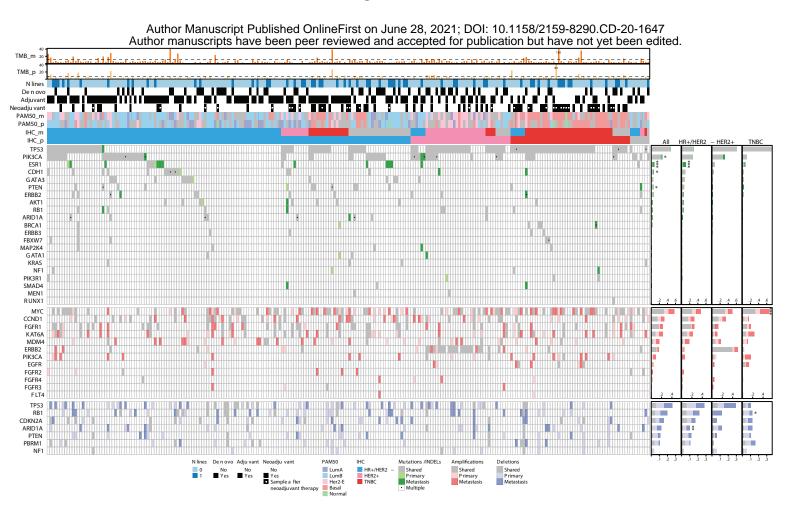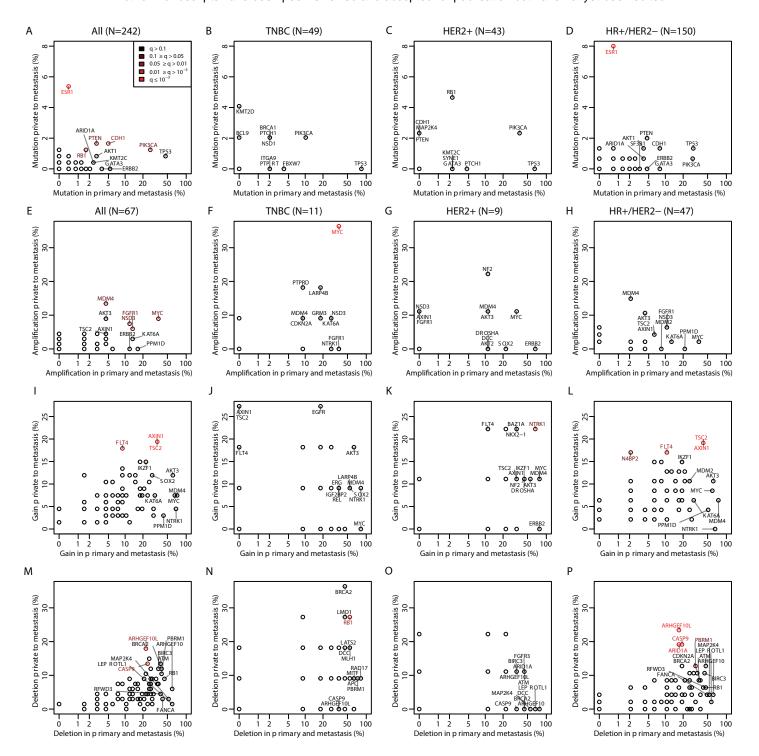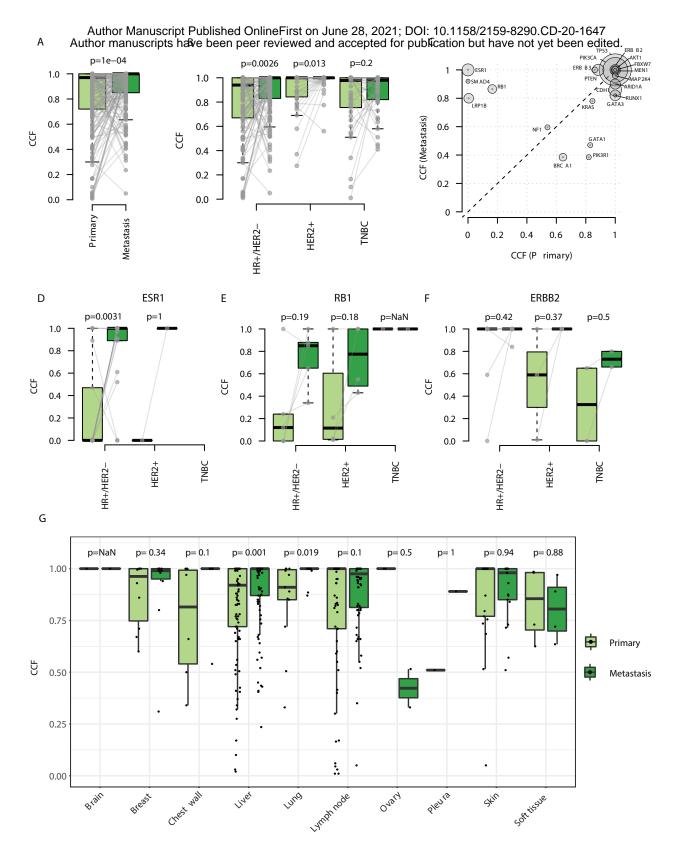
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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

CANCER DISCOVERY

AACR American Association for Cancer Research
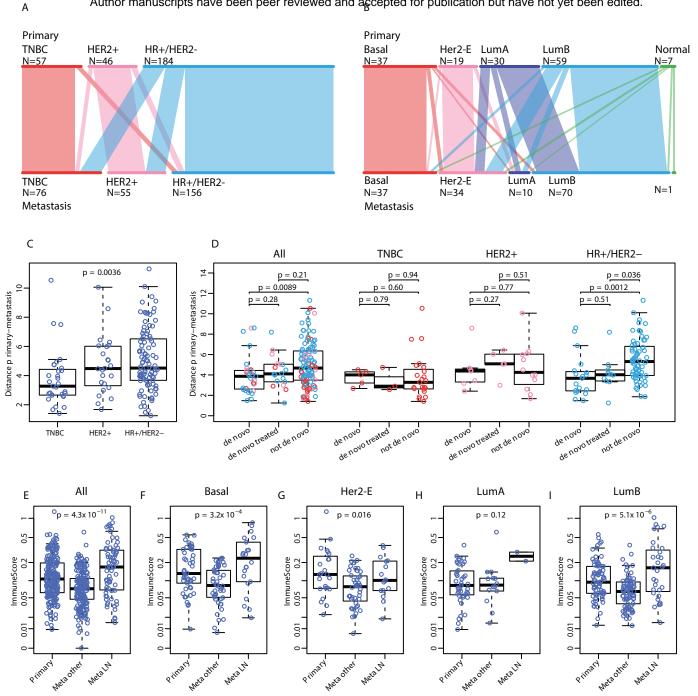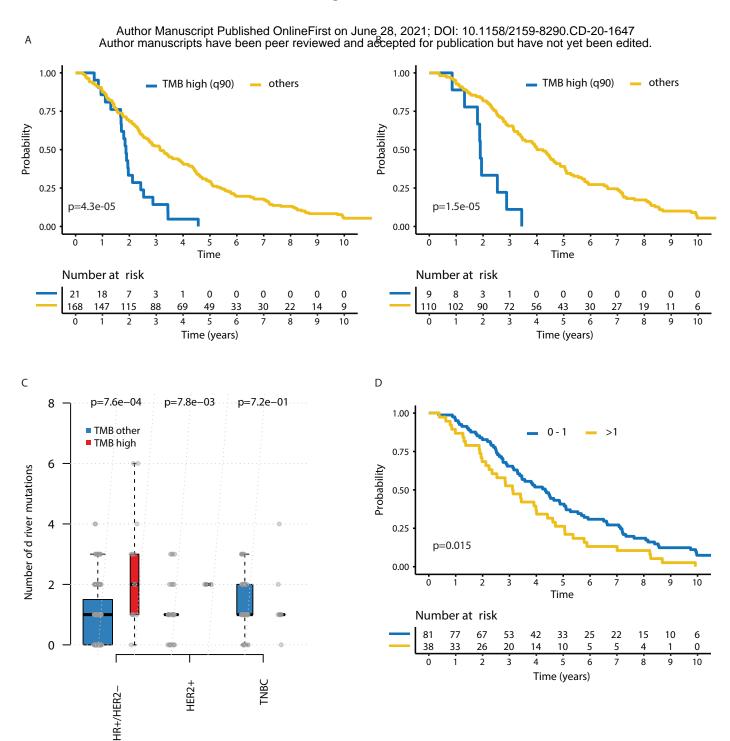
Genomic and transcriptomic analyses of breast cancer primaries and matched metastases in AURORA, the Breast International Group (BIG) molecular screening initiative

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