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Individual xenografts for personalized treatment of women with metastatic triple negative breast cancer

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Abstract

Aim: Triple negative breast cancer (TNBC) is the most severe subtype of breast cancer with poor prognosis even when treated at a localized stage. The treatment of metastatic TNBC is still challenging daily clinical practice, mainly because of the lack of targeted therapies. In the last years, a molecular sub-classification of TNBC has opened the way to personalized medicine for this type of severe cancer.

Methods: In this study, we assessed the added value of combining molecular analyses with individual xenografts to personalize the treatment of resort for five women with metastatic TNBC. While a patient was receiving one or two lines of chemotherapy, the corresponding xenograft model was tested with different drugs or drug combinations, mainly based on transcriptomic analyses of the tumor and on theoretical activated canonical pathways.

Results: On the basis of transcriptomic analyses and chemosensitivity data obtained from TNBC individual xenografts, we personalized the resort treatment for the five women in our study. In all cases, despite the fact that this resort treatment

was a third-line or a fourth-line treatment, the time to progression was longer than that observed with previous lines of chemotherapy. When we explored the 19 chemotherapy regimens given to these women and their corresponding xenograft models, there was a strong correlation between ΔSUV_{max} (maximum standard uptake value) on positron emission tomography-computed tomography and the corresponding coefficients of inhibition obtained in mice.

Conclusion: The combination of gene expression profiling and individual xenografts is a promising method and could be proposed as a personalized therapeutic resort for women with metastatic TNBCs.

Keywords: Patient-derived xenografts, triple negative metastatic breast cancer, personalized treatment

INTRODUCTION

Triple negative breast cancer (TNBC) is the most severe subtype of breast cancer, occurring in younger women and associated with poor prognosis even when treated at a localized stage. TNBCs lack any detectable expression of estrogen receptor, progesterone receptor, or human epithelial growth factor receptor 2 (HER2) amplification. Treatment of metastatic TNBC is still challenging daily clinical practice, mainly because of a lack of targeted therapies.

At the end of the 19th century, Beatson[1] opened the way to hormone therapy and personalized medicine in oncology with the first successful "surgical castration" in a young pre-menopausal woman with metastatic breast cancer. In the late 1990s, an anti-HER2 monoclonal antibody, trastuzumab, was approved for the treatment of HER2 overexpressing metastatic breast cancer[2]. To date, 48 targeted therapies have been approved for the treatment of various types of metastatic cancers[3].

High-speed whole-genome sequencing technologies have led to the identification of numerous potential molecular targets. In 2000, a first molecular classification of breast cancer individualized four subtypes: luminal A, luminal B, HER2-enriched, and basal-like (BL)[4-6], most BL subtypes corresponding to triple-negative breast cancers[7,8]. In 2011, TNBCs were classified into 6 molecular subtypes including BL1, BL2, immunomodulatory, mesenchymal-like, mesenchymal stem-like, and luminal androgen receptor (LAR)[9]. According to this classification, BL1 tumors are likely to be more sensitive to cisplatin and DNA repair inhibitors, BL2 tumors to anti-epidermal growth factor receptor (EGFR) therapies, and LAR tumors to androgen inhibitors[10-12].

Patient-derived xenografts (PDXs) are rediscovered pre-clinical pharmacological models. They enable limited available quantities of human cancer tissue to be amplified. In addition, PDX models efficiently reflect the characteristics of the original cancer, including tumor heterogeneity and metastatic potential[13-15]. As a result, there is an excellent correlation between the anti-tumor activity of a given drug in a xenograft model, and the anti-tumor effect of the same drug in the corresponding patient[16-19].

In this pilot study, we combined individual PDXs and whole-genome analyses to personalize the resort treatment for women with metastatic TNBCs.

METHODS

Patients with metastatic TNBC and gene expression profiling

Five women with metastatic TNBC participated in this study. For each patient, five tumor samples were obtained during an imagery-guided biopsy, at the time of metastatic disease, before any medical treatment. Informed written consent was obtained from the patients. The Clinical Research Board Ethics Committee (Comité de Protection des Personnes) approved this study (CPP Ile-de-France N°13218). Among these five tumor samples, (1) two were formaldehyde-fixed and paraffin-embedded for histological analyses; (2) two

Table 1. Patients' characteristics at the time of transcriptomic analyses and of xenografting

Patients	Age (year)	TNM	Metastases	Site of biopsy	Classification		Canonical pathways	Engraf-tments
					PAM50[20]	Lehmann 2011[9]		
Patient 1	45	T2N2M1	Lung, brain, lymph nodes	Breast	Basal	BL2	EGF pathway	PDXB1
Patient 2	66	T1N0M1	Lung, liver, bone, lymph nodes	Lymph node	Basal	Stm	IGF/mTOR pathway	PDXB2
Patient 3	30	T4cN2M1	Lung, lymph node	Breast	Basal	BL1	Cell cycle/ BRCA	PDXB3
Patient 4	40	T2N1M1	Lung, liver	Liver	Luminal B	AR	AR pathway	No
Patient 5	65	T4dN1M1	Lung, liver, bone, lymph nodes	Breast	Basal	BL2	EGF pathway	PDXB5

TNM refers to international Tumor-Node-Metastasis classification; PAM50: gene set of 50 genes used to classify breast cancer sub-types; BL1: basal-like 1; BL2: basal-like 2; Stm: stem-like; AR: androgen receptor; PDXB: patient-derived xenograft of breast cancer; BRCA: breast cancer; EGF: epidermal growth factor; IGF: insulin-like growth factor; mTOR: mammalian target of rapamycin

were immediately snap-frozen in liquid nitrogen and stored in the Hôpital-Saint-Louis tumor bank for molecular analysis; and (3) one was set aside in culture medium for xenografting.

Total RNA was extracted from the frozen tumor sample, and transcriptomic analyses were performed using a MiltenyiBiotec Microarray. A linear T7-based amplification step was performed from 0.5 μg of all RNA samples. To produce Cy3-labeled cRNA, the RNA samples were amplified and labeled using the Agilent-quick-labeling kit. The yields of cRNA and the dye-incorporation rate were measured on an ND-1000 spectrophotometer (NanoDrop, LabTech, France). Hybridization was performed according to the Agilent 60-mer oligo-microarray processing protocol: 1.65 μg Cy3-labeled cRNA was hybridized overnight at 65 ℃ on Agilent-Whole-Human-Genome-Oligo-Microarrays 4x44K, and fluorescence signals were detected using Agilent's Microarray-Scanner. Agilent-FE-Software determined feature intensities and quantile normalization was performed with the Agi4x44PreProcess R package. Subsequent analyses were carried out with R3.01 software (Foundation for Statistical Computing, Vienna, Austria) and based on $\log_2$ single-intensity expression data. The classification was obtained by correlating gene expression profiles with the centroids for each of the 6 TNBC subtypes described by Lehmann *et al.*[9], and with Parker *et al.*[20] centroids for the PAM50 classification.

Patient-derived breast cancer xenografts and treatments
After imaging-guided tumor biopsies had been performed, one sample was transported in RPMI-1640 culture medium and subcutaneously grafted in 6-week-old NMRI-nude mice, under xylasin (10 mg/kg)/ketamin (100 mg/kg) anesthesia.

Four PDX models were successfully obtained, and studied [patient-derived xenograft of breast cancer 1 (PDXB1) to PDXB3 and PDXB5] [Table 1].

For each xenograft model, after successful engraftment, a clinical score was recorded daily for the mice and tumor growth was measured in two perpendicular diameters with a caliper. Tumor volumes were calculated as $V = L \times l^2 \div 2$, L being the larger diameter (length), l the smaller (width). After mouse euthanasia, the tumor was resected, cut into small pieces of 1 mm^3, and grafted again in 30 other nude mice. When tumors reached a volume of 300 mm^3 (n = 4 mice per treatment-group), after 2 to 4 weeks, the mice were treated for one month with different regimens of chemotherapy [Table 2]. A daily clinical score was recorded and tumor growth measured weekly until its weight reached the ethically recommended limit of less than 10% of mouse weight (Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes; Official Journal of the European Union L 276/33).

Assessment of tumor response in patients
For each line of chemotherapy, the patient response under treatment was characterized. Metabolic response was assessed according to positron emission tomography (PET) response criteria in solid tumors criteria[21].

Table 2. Administration of drugs in xenografted mice

Drug	Dose	Mode of administration	Frequency of administration
Epirubicin	1 mg/kg	Intra-peritoneal	Once a week
Cyclophosphamide	30 mg/kg	Intra-peritoneal	Twice a week
Bevacizumab	5 mg/kg	Intra-peritoneal	Three times a week
Paclitaxel	20 mg/kg	Intra-peritoneal	Twice a week
Docetaxel	15 mg/kg	Intra-peritoneal	Twice a week
Cetuximab	10 mg/kg	Intra-peritoneal	Twice a week
Cisplatin	3 mg/kg	Intra-peritoneal	Once a week
Oxaliplatin	1 mg/kg	Intra-peritoneal	Twice a week
Gemcitabine	400 mg/kg	Intra-peritoneal	Once a week
Capecitabine	400 mg/kg	Gavage	Once a week
Everolimus	5 mg/kg	Gavage	Twice a week
Sunitinib	40 mg/kg	Gavage	Once a week

Briefly, partial metabolic response (PMR) is defined as a reduction in maximum standard uptake value (SUV_{max}) of at least 30%, with no new lesions. Complete metabolic response (CMR) corresponds to the disappearance of all lesions in the blood-pool background. Progressive metabolic disease (PMD) is defined by an increase in SUV_{max} greater than 30%, or the appearance of new fluoro-deoxyglucose (FDG)-avid lesions. Stable metabolic disease applies when the criteria for the other categories (CMR, PMR or PMD) are not met.

[18]FDG (5 MBq/kg; not exceeding 500 MBq) was injected intravenously 60 min before data were acquired on a Philips gemini XL PET/computed tomography (CT) scanner. CT data were acquired first (120 kV; 100 mAs; no contrast-enhancement). PET 3D data were acquired with 2 min per bed position, and images were reconstructed using a 3D row-action maximum likelihood algorithm.

PET/CT images were interpreted by a nuclear medicine physician blinded to the patient's record. [18]FDG uptake was expressed as the SUV. A 3D region of interest (ROI) was drawn around the lesions and SUV_{max} (maximum SUV value within the ROI) was measured. SUV_{max} of the lesions with the highest uptake were recorded and used for the study analysis (five target lesions were assessed). The SUV_{max} of the liver was also recorded as a control value. The change in SUV_{max} at each evaluation was expressed as ΔSUV_{max} (%) = 100 × (cycle n SUV_{max} - cycle (n - 1) SUV_{max})/cycle (n - 1) SUV_{max}. The appearance of new lesions was also recorded.

For each line of chemotherapy, time-to-progression (TTP) was defined as the time between the initiation of treatment and the diagnosis of disease progression.

Assessment of tumor response in xenografts

To assess tumor response in xenografts, ultrasonography was performed twice a week on treated and untreated mice using an AplioXT ultrasonograph (Toshiba, Japan). Tumor growth was measured in two diameters, and tumor volumes were calculated as $V = L \times l^2 \div 2$. For each drug or drug combination, a growth curve was established.

The coefficient of inhibition for a drug or a drug combination was calculated as (a'- a)/a, a being the slope of the curve before the start of treatment (day 0), and a' the slope of the curve between day 0 and day 28 of treatment [Figure 1].

Statistical analysis

For the correlation studies, the Kendall rank correlation coefficient R^2 was calculated between patient ΔSUV_{max} for a given chemotherapy regimen and the coefficient of inhibition for the same regimen in the TNBC xenograft. A P value under 0.05 was considered to be significant.
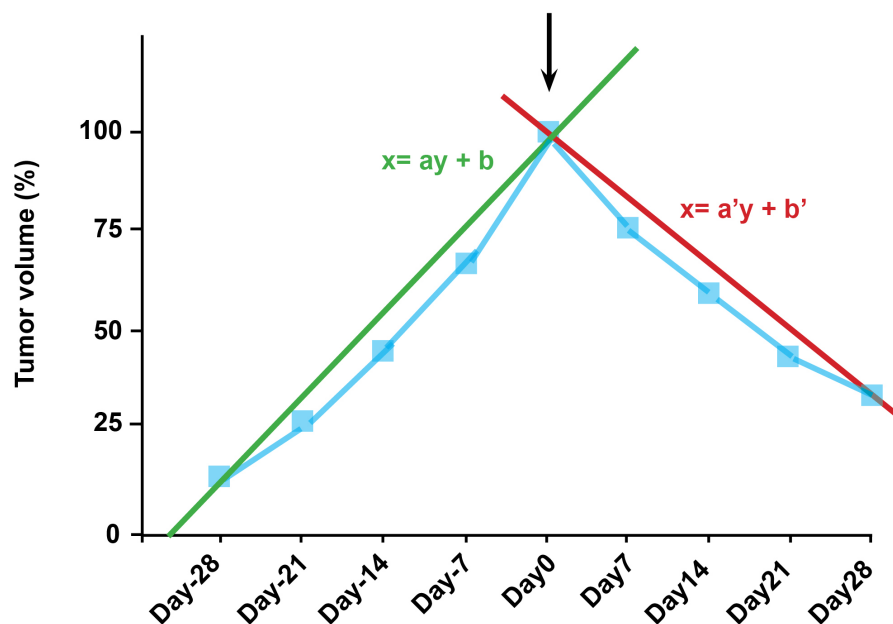
Figure 1. Treatment curve slopes. The green curve represents the tumors growth before the start of treatment (Day 0), and "a" is the slope of this curve. The red curve represents tumor growth between Day 0 and Day 28 of treatment, and a' is the slope of this curve

RESULTS

Molecular signature of metastatic TNBCs and corresponding xenografts

Five women with metastatic TNBC were included in this pilot study. Their characteristics are detailed in Table 1. With their consent, a tumor biopsy was performed at the time of metastatic disease, before any medical treatment. One biopsy sample was dedicated to transcriptomic analyses, enabling the tumors to be classified according to Lehmann's classification. Another biopsy sample was immediately processed for xenografting in nude mice. The graft was successful in 4 of the 5 patients [Table 1]. The molecular signature of each xenograft model was identical to the corresponding TNBC it derived from, and remained unchanged over successive passages for each xenograft model.

Personalizing treatment of metastatic breast cancers

While a patient was receiving one or two lines of chemotherapy, the corresponding xenograft model was tested with different drugs or drug combinations, the choice being mainly based on theoretical activated pathways identified from transcriptomic analyses [Table 1].

PDXB1, derived from the TNBC of patient 1, was classified BL2, with epidermal growth factor (EGF) pathway activation. In addition, there was no mutation of EGF pathway genes, mainly *BRAF*, *KRAS*, *NRAS* and *PIK3*. When tested with drugs or drug combinations on PDXB1, the most efficient regimen was a combination of paclitaxel and cetuximab, an anti-EGFR monoclonal antibody [Figure 2]. This regimen was then offered to the patient as a third-line resort treatment [Table 3]. Under this chemotherapy, she had almost CMR[13].

For patient 4, the engraftment was not successful. However, since the biopsied liver metastasis was classified as LAR, we decided to personalize the anti-cancer treatment using anti-androgen drugs. The patient was first offered a treatment with bicalutamide, an anti-androgen receptor, resulting in stable disease and an 8 months period to progression. She was then offered a second-line treatment with abiraterone acetate, a CYP17A inhibitor that blocks androgen production [Figure 3], which enabled an additional 10 months of disease control [Table 3].
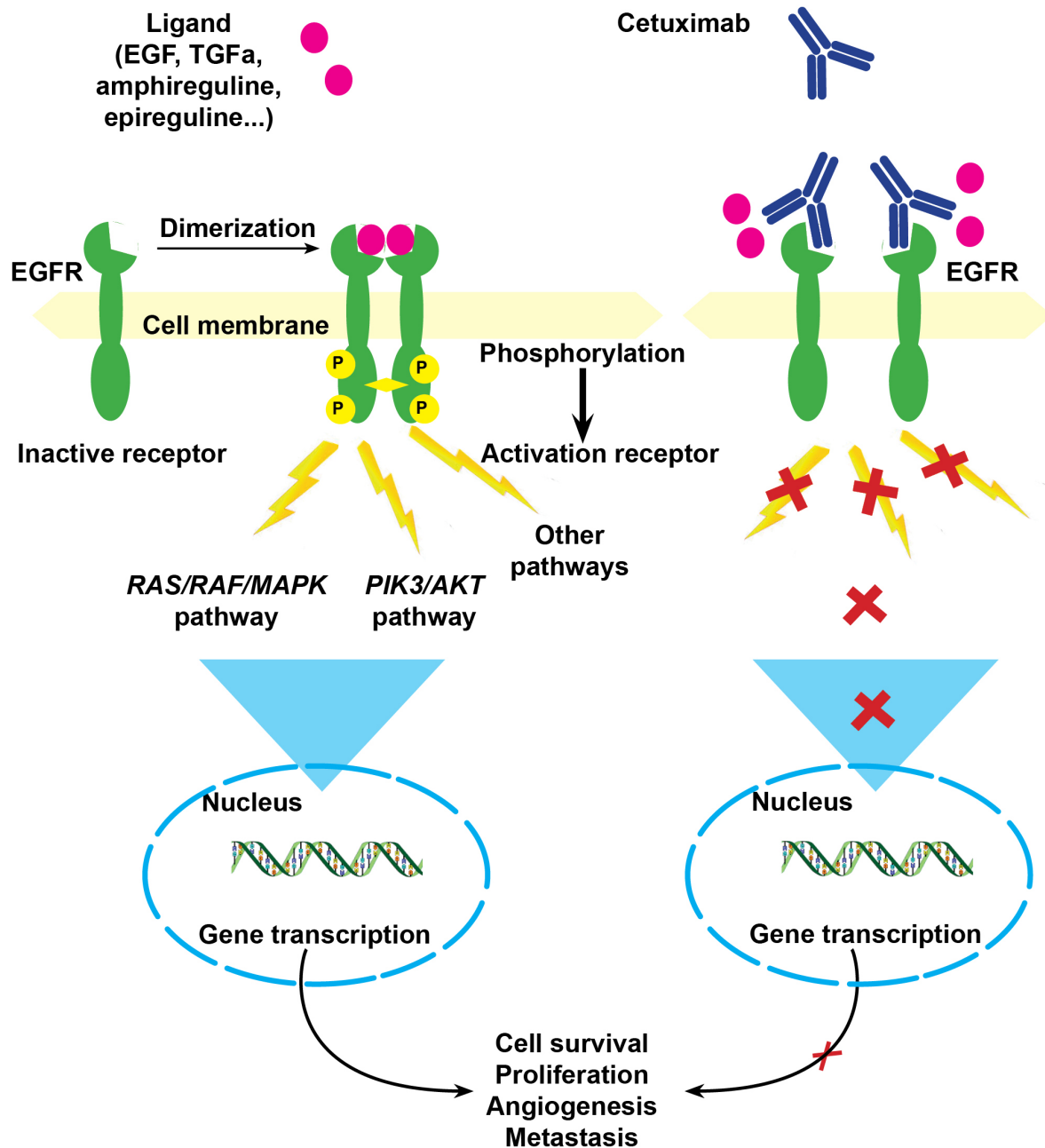
Figure 2. Epidermal growth factor pathway in triple negative breast cancers. EGFR is a tyrosine-kinase transmembrane receptor. When a ligand, mainly EGF, binds the extracellular domain, there is a dimerization of two receptors. This leads to their activation through phosphorylation of the intracellular tyrosine kinase. Cetuximab is a humanized anti-EGFR receptor, which competitively binds to the extracellular domain, blocking receptor dimerization. EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; TGFα: transforming growth factor-α; MAPK: mitogen-activated protein kinase; PIK3: phosphatidylinositol 3-kinase

Treatment efficacy in patients and in corresponding PDXs

For each of the four patients with a corresponding xenograft, we retrospectively analyzed response data for the different chemotherapy regimens they had received. In a given patient, for each regimen, we determined two parameters: the best change in SUV_{max} expressed as a percentage of SUV_{max}, and the time to progression. Each chemotherapy regimen was also tested in the corresponding TNBC xenograft, and tumor response was determined by the coefficient of inhibition. When we explored the 19 regimens given to the four patients and their corresponding xenograft models [Table 3], there was a strong correlation between ΔSUV_{max} and the corresponding coefficients of inhibition [Figure 4].

Table 3. Response to treatment in patients and corresponding patient-derived xenografts

Patients	Treatment	Best response in patients/ΔSUV_{max}	Coefficient of inhibition in corresponding PDX	TTP (months)
Patient 1	Cisplatin gemcitabine	PMD/2.1	1.52	2
	Capecitabine bevacizumab	SMD/0.02	-0.88	6
	Paclitaxel cetuximab	PMR/-0.88	-1.67	10
Patient 2	Paclitaxel bevacizumab	PMD/0.31	1.57	3
	Capecitabin bevacizumab	PMD/0.41	1.2	3
	Sunitinib	PMD/2.13	3.59	2
	Cisplatin everolimus	PMR/-0.78	-1.04	6
Patient 3	SIM	PMR/-0.56	-1.61	6
	Docetaxel	PMD/0.42	1.18	2
	Cisplatin gemcitabine	SMD/0.03	-1.42	8
Patient 4	Palitaxel bevacizumab	PMR	NA	12
	Cisplatin gemcitabine	PMD	NA	2
	Bicalutamide	SMD	NA	8
	Abiraterone acetate	PMR	NA	10
Patient 5	SIM	SMD/0.07	0.61	4
	Docetaxel	PMD/2.18	1.67	4

SUV: standard uptake value; PDX: patient-derived xenograft; SIM: dose-dense epirubicin-cyclophosphamide regimen; PMR: partial metabolic response; PMD: progressive metabolic disease; SMD: stable metabolic disease; TTP: time to progression; NA: not applicable

On the basis of transcriptomic analyses and chemosensitivity data obtained from the different TNBC xenografts, we personalized resort treatment for the five women in our study. In all cases, despite the fact that this resort treatment was a third-line or a fourth-line, the TTP was longer than that observed with previous lines of chemotherapy [Table 3].

DISCUSSION

TNBC is a heterogeneous, severe type of breast cancer, requiring the development of personalized therapies. Recent advances in gene expression profiling have identified TNBC molecular sub-types that could benefit from the use of targeted therapies[9].

Typically, BL2 subtype is characterized by an activation of the EGF pathway and could benefit from anti-EGFR therapies. In a phase II study on 173 women with metastatic TNBCs, where cetuximab was associated with cisplatin, it only added 2.2 months of survival[22]. However, the patients were not selected according to EGF pathway activation, which certainly lowered the benefit observed. In our pilot study, patient 1 had an activation of the EGF pathway with no mutation in the *RAS*, *RAF* or *PIK3* genes, as observed in 40% of patients with metastatic colorectal cancer[23,24], where 60% response to anti-EGFR combined chemotherapy was observed[25]. Indeed, when patient 1 received a combination of paclitaxel and cetuximab as third-line treatment, she had almost a CMR.

On the other hand, LAR tumors account for 11% of TNBCs and are characterized by an activation of the androgen receptor pathway. In two phase-II studies on women with metastatic TNBCs selected according to androgen receptor status, bicalutamide or abiraterone acetate led to 6 months disease stabilization in less than 20% of patients[26,27]. Again, these disappointing results were probably due to an inadequate selection of patients. Indeed, TNBC tumors classified as LAR usually correspond to apocrine molecular tumors[28], and are more accurately identified using gross cystic disease fluid protein-15 marker by immunohistochemistry[29]. In our pilot study, the tumor of patient 4 was classified as LAR subtype, and this young woman drew durable benefit from anti-androgen therapies, with a total of 18 months of liver metastasis stabilization.

The molecular sub-classification of TNBCs by Lehman *et al.*[9] has opened the way to personalized medicine for metastatic TNBCs. However, molecular analyses still have limitations, and different, complementary methods need to be implemented. Individual xenografts from metastatic samples of TNBCs are an additional, innovative tool and are more physiological than genomic analyses. A major limitation of PDXs is the low
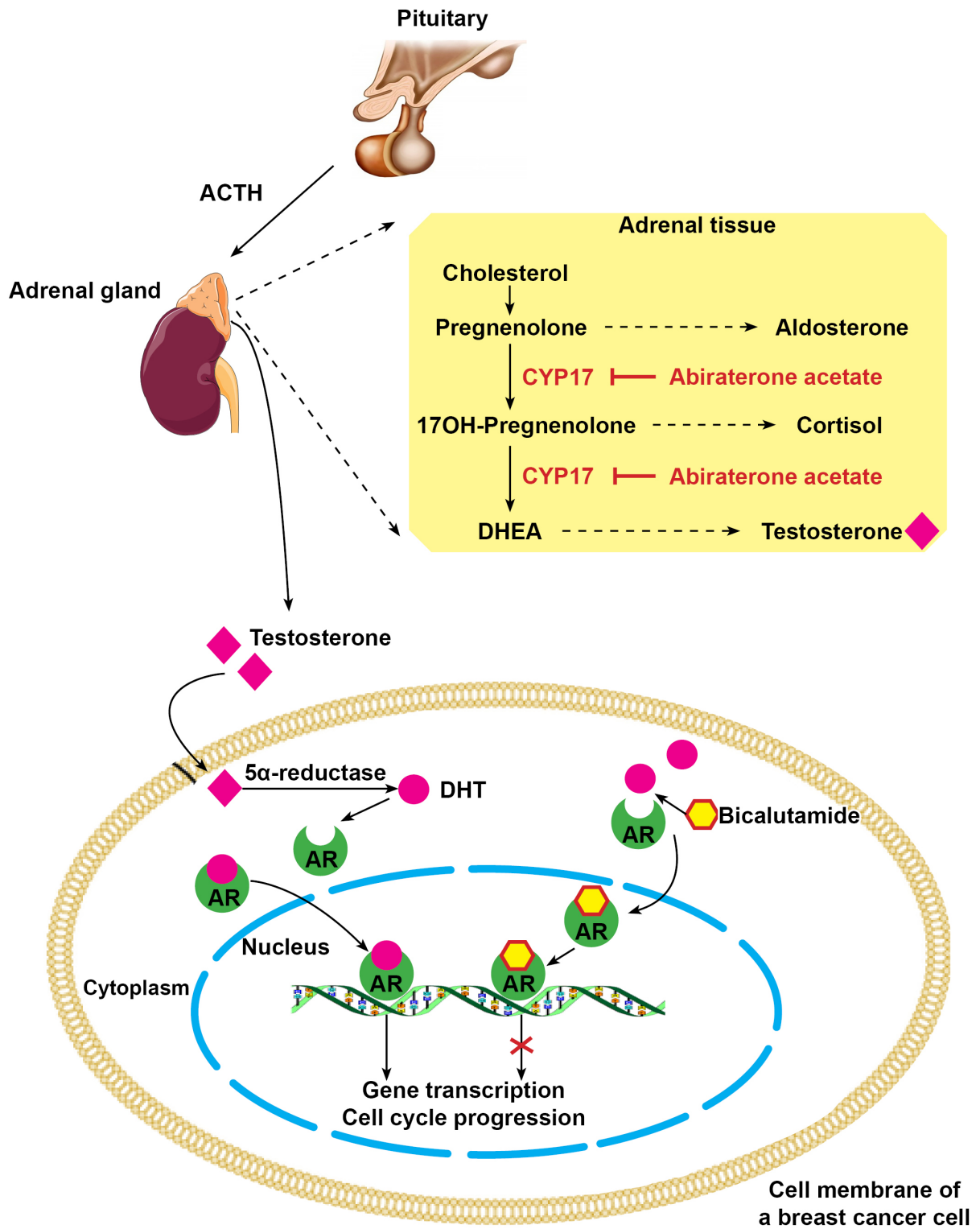
Figure 3. Androgen pathway in triple negative breast cancers. Bicalutamide acts as an inhibitor competing with DHT on cytoplasmic AR. Abiraterone acetate inhibits CYP17, thus reducing testosterone synthesis in adrenal tissue. DHT: dihydrotestosterone; AR: androgen receptors; CYP17: cytochrome P450 17 alpha-hydroxylase; ACTH: adrenocorticotropic hormone; DHEA: dehydroepiandrosterone
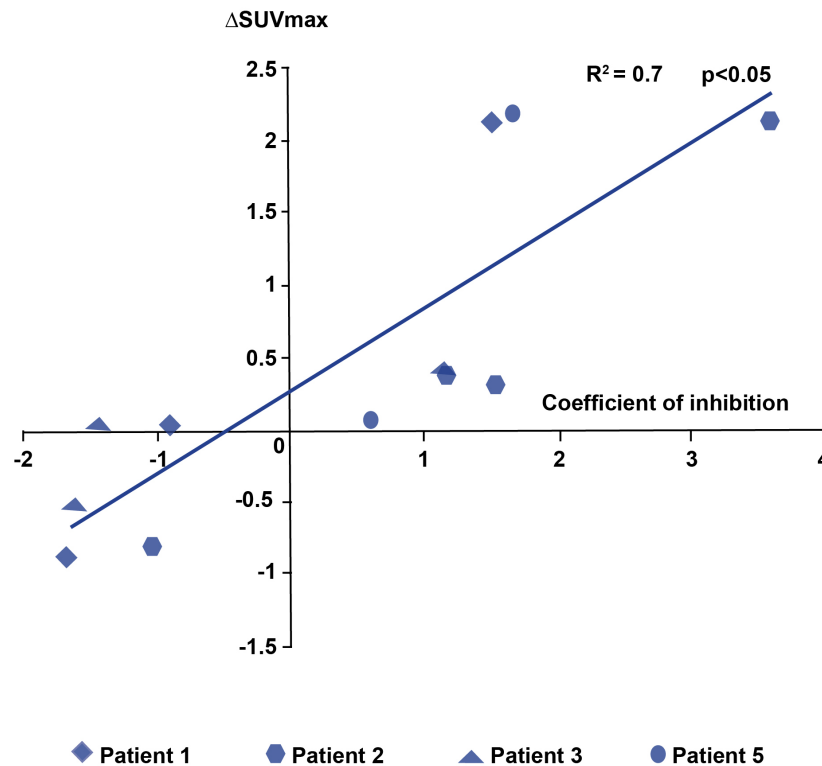
Figure 4. Correlation between inhibition coefficients and ΔSUV_{max}. For a drug or a drug combination, the inhibition coefficient is calculated as (a'-a)/a. The Kendall rank correlation coefficient is calculated between a given patient ΔSUV_{max} for one chemotherapy regimen and the inhibition coefficient for the same regimen in his corresponding TNBC xenograft. There is a significant correlation between these two parameters ($R^2 = 0.7$, $P < 0.05$). TNBC: triple negative breast cancer; SUV: standard uptake value

engraftment rate. However, for metastatic TNBCs, the engraftment probability of metastatic samples is high, up to 80%[30]. Individual PDXs could thus be useful in co-clinical trials, using individual xenografts for the development of new therapeutic agents in patients with cancer resistant to standard treatments[13,31,32].

The combination of gene expression profiling and individual PDXs is a promising method, and could be proposed as a personalized therapeutic resort for women with metastatic TNBCs.

DECLARATIONS
Acknowledgments

Authors' contributions
Collected and assembled the data: Nguyen TT, Bousquet G
Provided data analysis and interpretation: Nguyen TT, El Bouchtaoui M, Janin A, Bousquet G
Drafted the manuscript: Nguyen TT, Janin A, Bousquet G
Analyzed the transcriptomic data: Feugeas JP
Provided the analysis of PET/CT data: Vercillino L
Performed imaging-guided tumor biopsies: de Bazelaire C
Located financial support and provided administrative support: Janin A
Conceived and designed the study, provided the study material or patients: Bousquet G
Read and approved the final manuscript: Nguyen TT, El Bouchtaoui M, Feugeas JP, Vercillino L, de Bazelaire C, Janin A, Bousquet G

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The Clinical Research Board Ethics Committee (Comité de Protection des Personnes) approved this study (CPP Ile-de-France N°13218). Informed written consent was obtained from the patients.

Consent for publication

Written informed consent was obtained from the patients for publication.

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