Circulating tumor cells in Breast cancer (BC)

CTCs are usually present at low levels in blood, thus, most approaches require an **enrichment** step including density-gradient centrifugation and/or immunomagnetic procedures before application of the detection technology. **In our laboratory**, we started the evaluation of **CTCs** in Breast Cancer (**BC**) in 2006 using the **AdnaGen technology (QIAGEN Figure 1)**. This technique combines immunomagnetic tumor cell selection and first used a "cocktail" of antibodies targeting EpCAM and MUC1 (***AdnaTest BreastCancerSelect***), followed by multiplex RT-PCR for the transcripts EpCAM, MUC1 and HER2 (***AdnaTest BreastCancerDetect***). The cDNA obtained after selection of CTCs by reverse transcription allowed the characterization of additional transcripts of interest by a separate RT-PCR e.g. for the **hormonal receptors** estrogen (**ER**), progesterone (**PR**) or the resistance marker **ERCC1** (excision repair cross-complementation group 1).

The technique was further developed using EpCAM, MUC1 and HER2 as well as EpCAM, HER2 and EGFR for selection of CTCs and subsequent detection of the the stem cell marker ALDH1 (***AdnaTest TumorStemCell***) as well as **EMT** markers using the ***AdnaTest EMT*** (multiplex RT-PCR for TWIST, Akt2, PI3K).

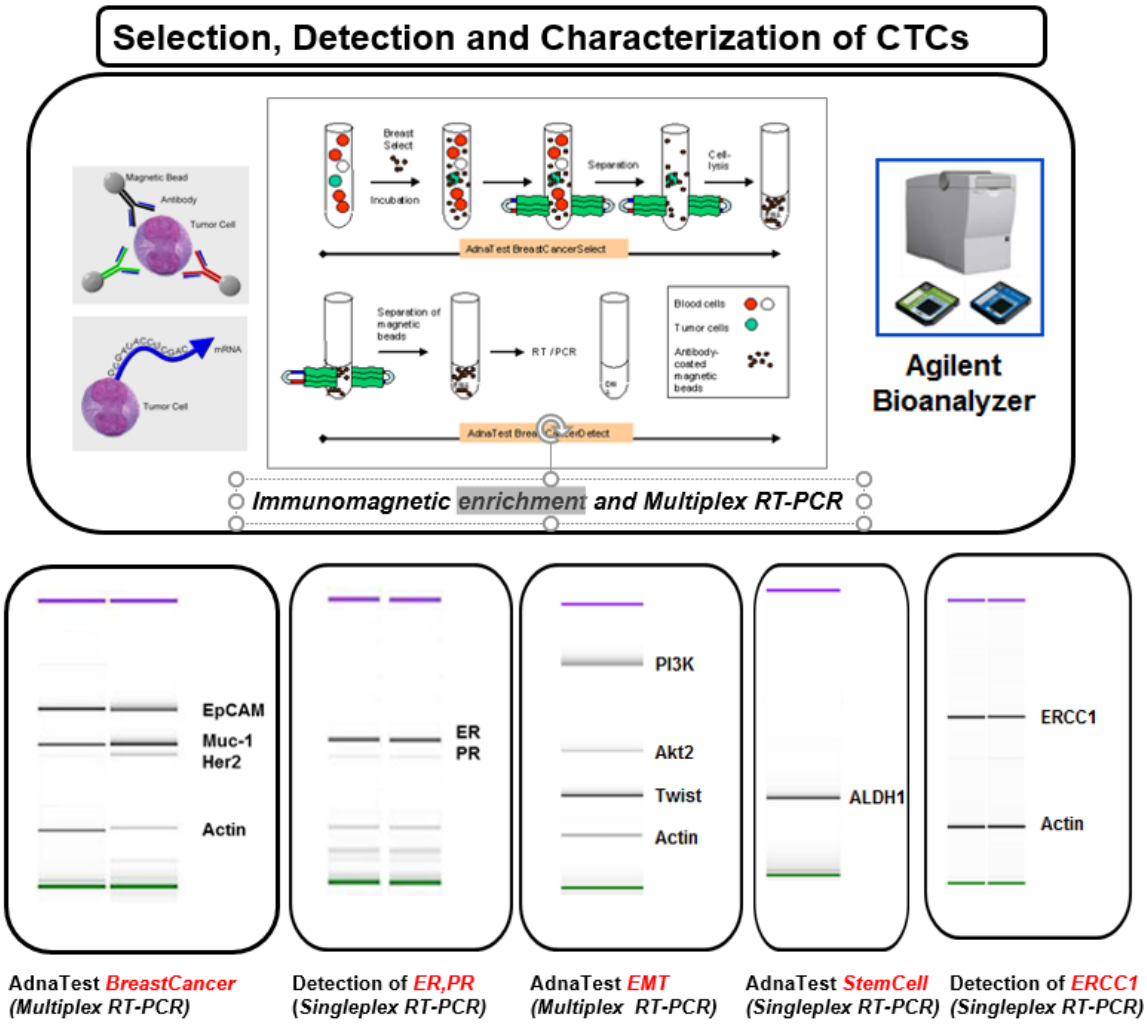


Figure 1: Selection and detection of CTCs

Main results for primary BC: Evaluating blood in 431 patients, we found a **discordant HER-2/ER/PR receptor expression** on **CTCs** as compared to the **primary tumor** and we were able to show that most of the **CTCs were "triple-negative"** (**Figure 2**), one characteristic of stem cell like cells (**Fehm, Kasimir-Bauer et al., 2009**).

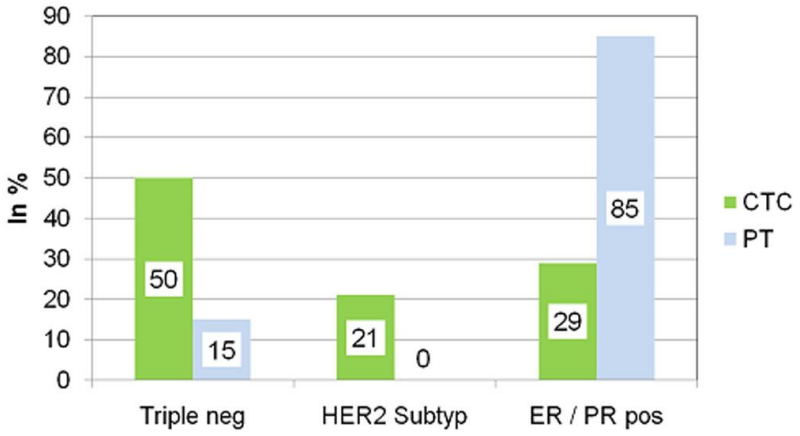


Figure 2: Expression of CTCs and corresponding primary tumors based on receptor status. Patients were stratified into three different groups based on ER, PR and HER2 status of their primary tumor (PT, blue) and their CTCs (green): ER-positive and/or PR-positive, triple-negative (ER-negative/PR-negative/HER2-negative), and HER2-positive (but ER-negative/PR-negative) (***Fehm, Kasimir-Bauer et al., 2009***).

In another 502 patients, we demonstrated **as one of the first groups** that **a subset of CTCs** shows **EMT** and **stem cell characteristics** and that the currently used detection methods for CTCs are not efficient to identify these CTC subtypes (**Figure 3; Kasimir-Bauer et al., 2012**).

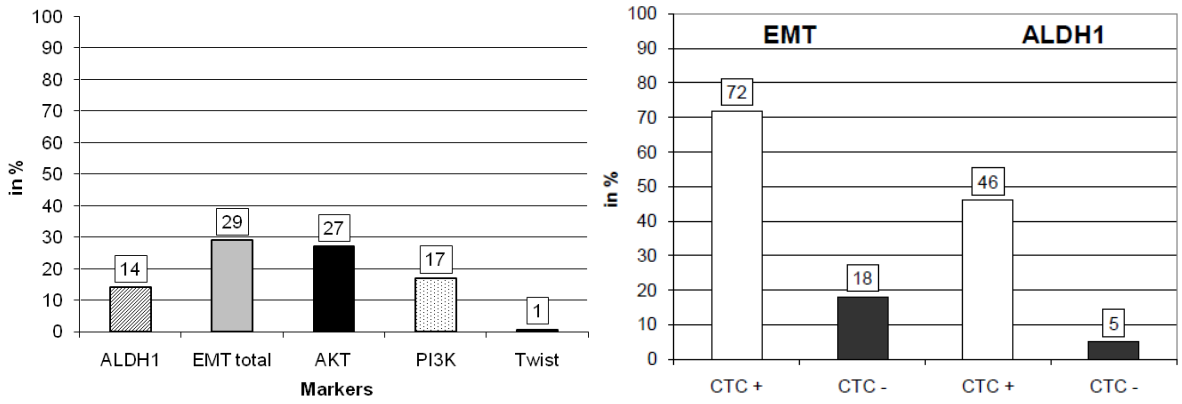
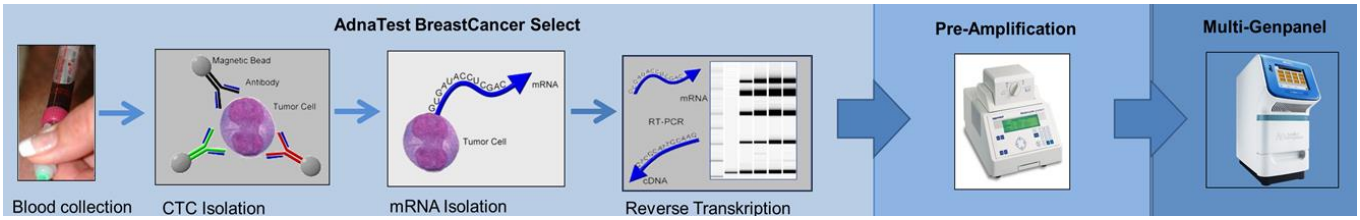


Figure 3: Expression of EMT-markers and ALDH1 in CTC+ and CTC- patients. Left image: At least one of the EMT markers was expressed in 29% and ALDH1 was present in 14% of the samples, respectively. **Right image:** In the CTC+ group, 66 of 92 patients (72%) were positive for at least one of the EMT markers and 42 of 92 patients (46%) were positive for ALDH1, respectively. In the CTC- group, the percentages were 18% (63 of 354 patients) and 5% (19/353 patients). (***Kasimir-Bauer et al., 2012***).

In the following years, we continued to analyze CTCs and correlated our findings with clinical characteristics of the patients. In general, **CTCs** were detected **in about of 22%** our **primary BC patients** and their presence significantly correlated with a **reduced PFS** (p=0.0227). Furthermore, their negative prognostic relevance was predominantly related to **G2** tumors (p=0.044), the **lobular** (p=0.024) and the **triple-negative subtype** (**TNBC**; p=0.005), **HR-negative** patients (p=0.001), **postmenopausal** women (p = 0.013) and patients who had received **radiation** therapy (p = 0.018) (**Kasimir-Bauer et al., 2016**). We also improved our selection using magnetic beads coupled to EpCAM, HER2 and EGFR and expanded our marker panel (**Figure 4**) to characterize CTCs more comprehensively.



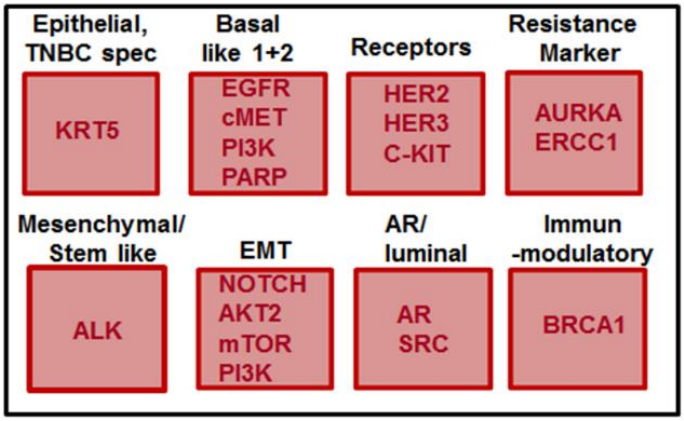


Figure 4: Selection and characterization of CTCs using a Multimarker panel.

Stratifying **BC patients** according to their **clinical subtypes**, gene expression profiles for every BC subgroup before and after therapy are documented in detail in **Figure 5**. In general, *PIK3CA, AKT2, MTOR* as well as the resistance markers *AURKA* and *ERCC1* were predominantly expressed in all BC subtypes, the latter two genes especially after therapy. In patients with **TNBC**, **all the different genes** were **overexpressed** before treatment (except for *PARP1),* probably representing the most **heterogeneous CTC population**. In addition, a variety of **genes** were **uniquely** overexpressed in this BC subgroup and particularly, ***ERBB2+ and ERBB3* + CTCs** were found at both time points in about **20%** of the **patients**.

Survival analysis with regard to **PFS** and **OS** was only feasible for the group of **TNBC patients** with 10 relapses after a median follow-up time of 30 months (range 2-57 months) and eight deaths, six of them BC-specific, after a median follow-up time of 19.85 months (range 3-33 months), respectively. As shown in **Figure 6A and B**, the presence of ***EGFR+* or *ERBB2+* or *ERBB3+* CTCs** in TNBC patients **before** therapy and ***ERBB2+/ERBB3+* CTCs after therapy** significantly correlated with a **reduced PFS** (0.01 and P = 0.02). Using **COX multivariate proportional hazard analysis** with standard staging parameters like nodal stage and tumour size as well as menopausal status and grading, the **CTC 'all ERBB family status'** turned out as a significant, independent **unfavourable predictor for PFS** (**Bittner et al., 2020**).

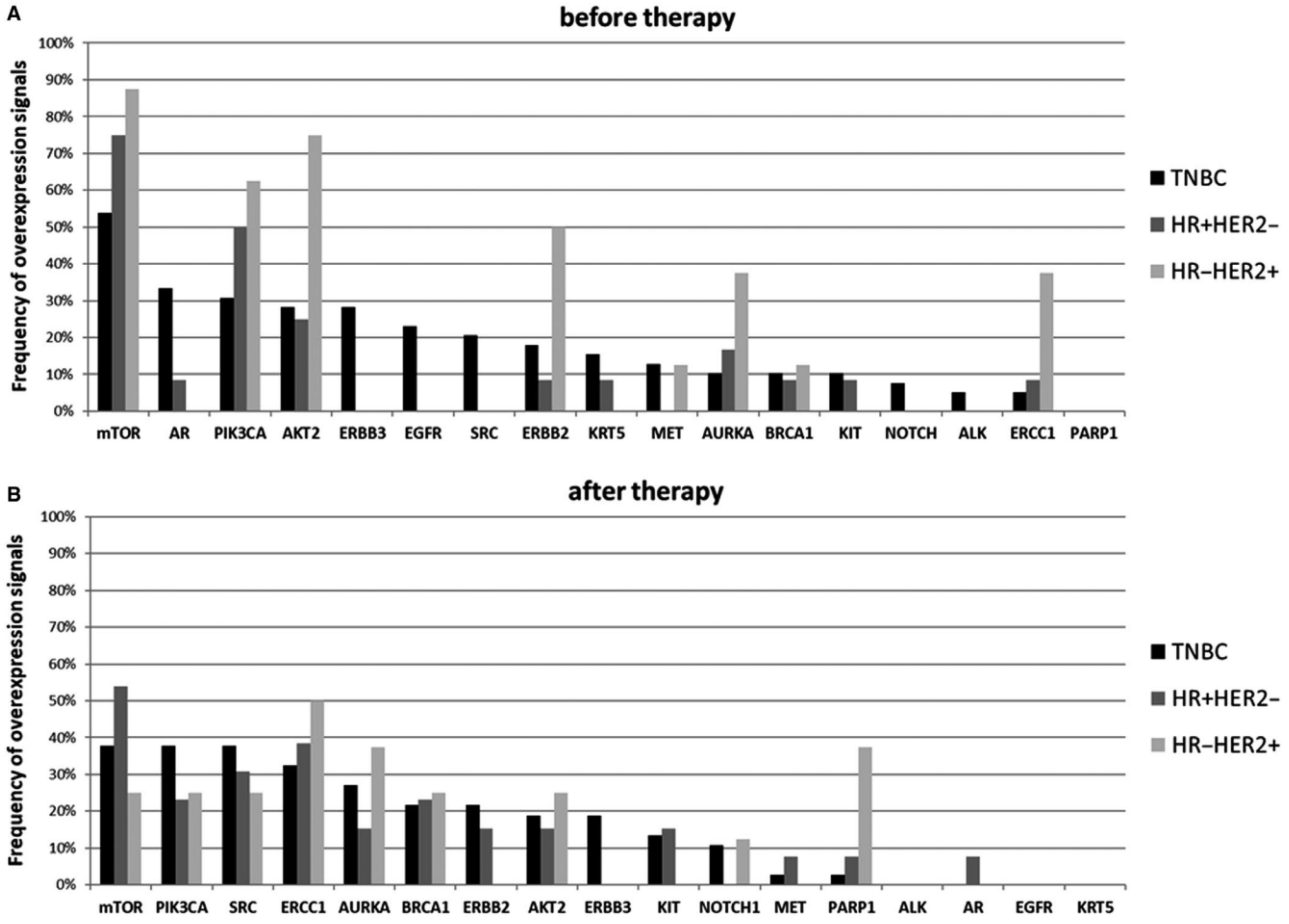


Figure 5: Gene expression profiles in the different BC subgroups. A, before therapy (BT). B, after therapy (AT). In general, *PIK3CA, AKT2, MTOR* as well as the resistance marker *AURKA* and *ERCC1* were predominantly expressed in all BC subtypes, the latter two genes especially AT. In patients with TNBC, all the different genes were overexpressed BT (except for *PARP1*), probably representing the most heterogeneous CTC population. The most frequently overexpressed genes were *MTOR* (54%), *AR* (33%), *PIK3CA* (31%) and *AKT2* (28%), respectively. A variety of genes including AR (with the exception of one positive patient in the HR+/HER2− group), *ERBB3* (28%), *EGFR* (23%), *SRC* (21%), *NOTCH* (8%) and *ALK* (5%) were uniquely overexpressed in patients with TNBC BT (***Bittner et al., 2020***).

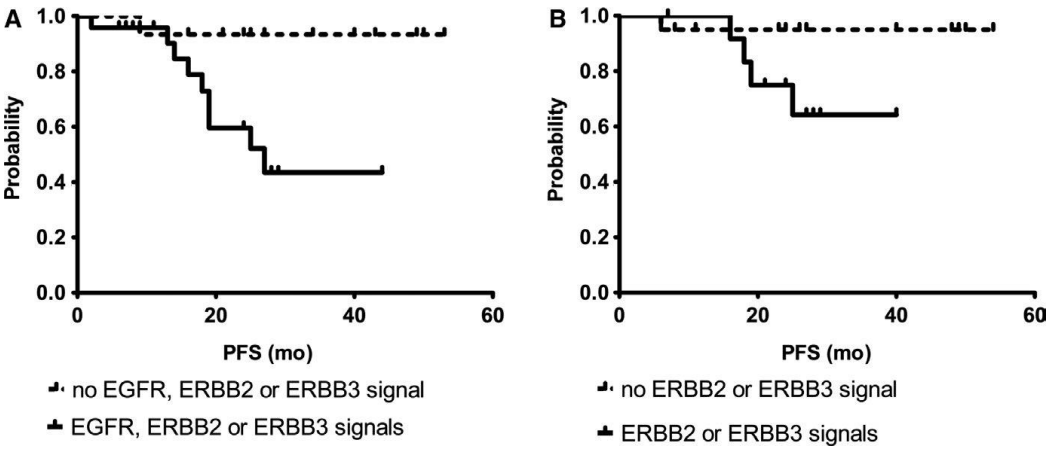


Figure 6: Survival correlations for CTCs expressing *EGFR/ERBB2/ERBB3*. A, PFS before therapy. **B**, PFS after therapy. Survival analysis with regard to PFS was only feasible for the group of TNBC patients with 10 relapses after a median follow-up time of 30 months (range 2-57 mo). The presence of *EGFR*+ or *ERBB2*+ or *ERBB3*+ CTCs (A) in patients with TNBC before therapy and (B) *ERBB2*+ or *ERBB3*+ CTCs after therapy significantly correlated with a reduced PFS (P = 0.01; P = 0.02) (***Bittner et al., 2020***).

Looking for **new predictive biomarkers**, **prostate cancer related markers** have been evaluated in **TNBC** for additional treatment options. Consequently, we analyzed the mRNA profiles of CTCs for the expression of the full-length androgen receptor (***AR-FL***), AR splice variant 7 (***AR-V7***), the prostate specific antigen (***PSA***) and the prostate specific antigen membrane antigen ***PSMA*** in blood samples of 41 TNBC patients **before** and 26 TNBC patients **after therapy** to elucidate their prognostic value and their potential as therapeutic targets.

Before therapy, at least **one prostate cancer related gene** was detected in 15/41 pts (**37%**). Notably, in **73%** of *AR-FL* positive cases, ***AR-V7* was co-expressed**. After therapy, CTCs of only one patient harbored prostate cancer related genes (**Figure 7**). ***AR-V7*+ and *PSMA*+ CTCs** significantly correlated with **early relapse** ($p = 0.041$; $p = 0.00039$) whereas ***PSMA*+ CTCs** also associated with a **reduced OS** ($p = 0.0059$) (**Figure 8**). This correlation was confirmed for *PSMA*+ CTCs in univariate (PFS $p = 0.002$; OS $p = 0.015$), but not multivariate analysis (**Kasimir-Bauer et al., 2020**).

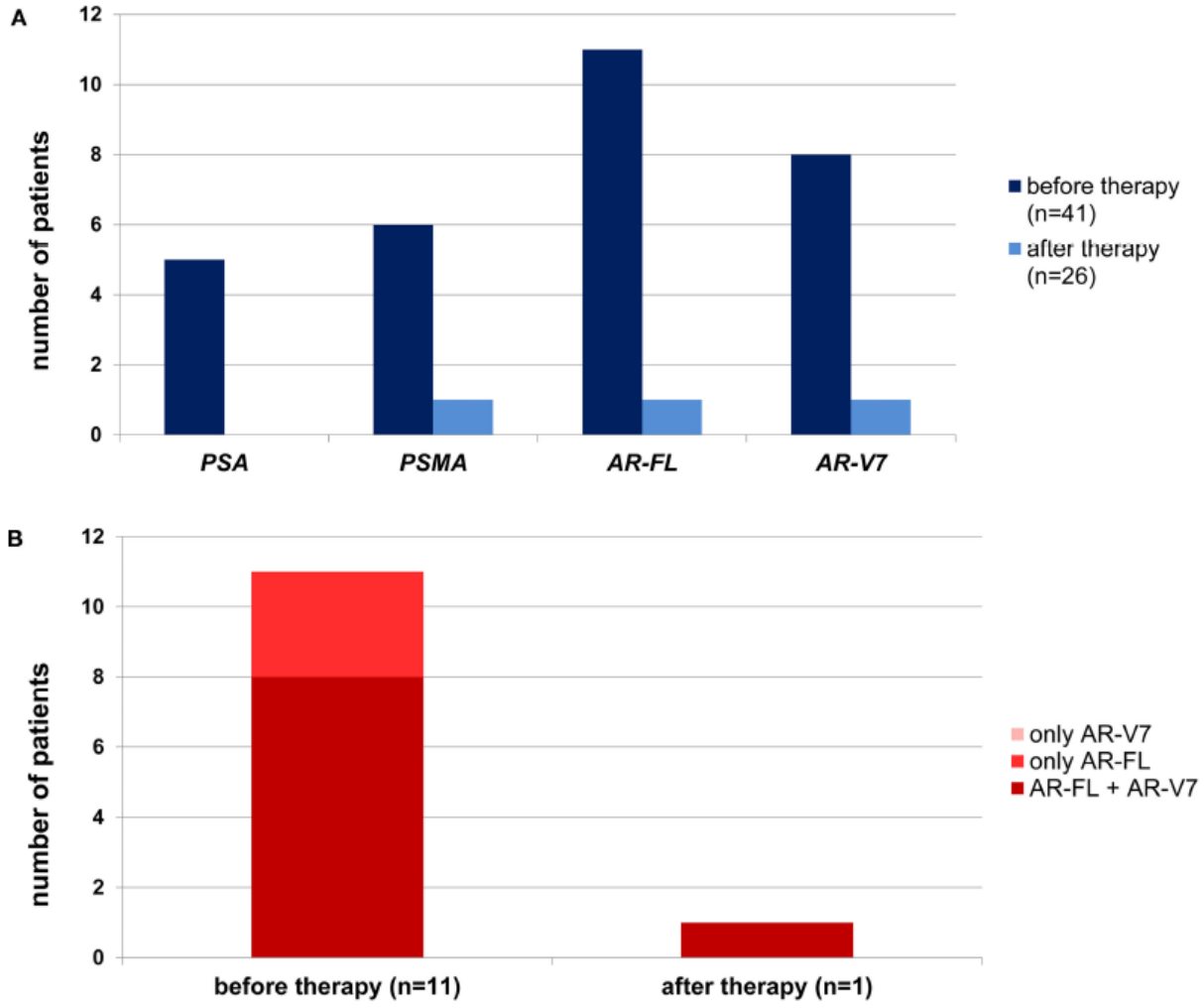


Figure 7: Prevalence of primary TNBC patients with prostate cancer related transcripts detected in CTCs. (A) In 41 TNBC patients before therapy (dark blue) and 26 TNBC patients after neoadjuvant therapy (light blue) *PSA, PSMA, AR-FL*, and *AR-V7* RNA profiles were examined. **(B)** Co-expression of *AR-FL* and *AR-V7* (dark red) was detected in the majority of *AR-FL*+ CTCs. Some patients displayed only *AR-FL*+ CTCs (red), but no patient was examined to have only *AR-V7*+ CTCs (light red) (**Bittner et al., 2020**).

References

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