Liquid Biopsy in Breast Cancer (BC)

Liquid biopsy, comprising of circulating immune cells, circulating tumor cells (**CTCs**) and extracellular vesicles (**EVs**), as well as proteins and nucleic acids in a blood sample of an oncologic patient, is of high potential since non-invasive blood analysis is discussed to be an ideal 'surrogate tissue' to identify and monitor prognostic and predictive factors that will help in selecting the optimal therapeutic strategy for each individual patient.

Comprehensive liquid biopsy analysis – own work in the field

We were the first to show the feasibility of **parallel** analysis of **EV mRNA, CTC mRNA, CTC gDNA** and **cfDNA** from a total of only 18 ml blood in matched samples of 35 hormone receptor **(HR)+/HER2- met BC patients** in the follow up of the disease **(Figure 1)**. Thus, we successfully established a protocol for the isolation and molecular characterization of four liquid biopsy analytes from **a minimized blood volume** on the **transcriptomic** and **genomic level.**

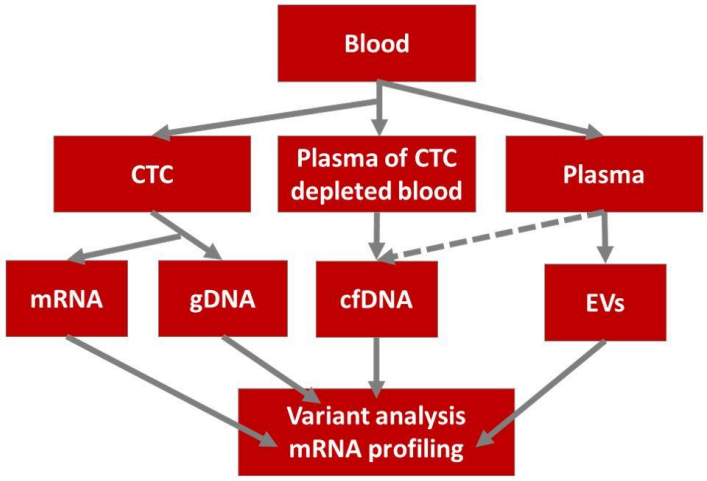


Figure 1: Experimental study design of the previous multimodal study. The indicated multimodal analysis of CTC mRNA, CTC gDNA, cfDNA and EV mRNA was performed using a minimal blood volume of only 18 ml from 35 HR+/HER2- MBC patients receiving different therapies (***Keup et al., 2016-2021***).

The **direct comparison of matched CTC and EV mRNA profiles (Figure 2 A; Keup et al., 2018)** and **matched CTC gDNA and cfDNA variants (Figure 2 B; Keup et al., 2020**) as well as the integrative statistical analyses of the multimodal approach with CTCmRNA, EVmRNA, cfDNA and CTCgDNA (**Keup et al., 2021**) clearly demonstrated an **additive value of each analyte.**

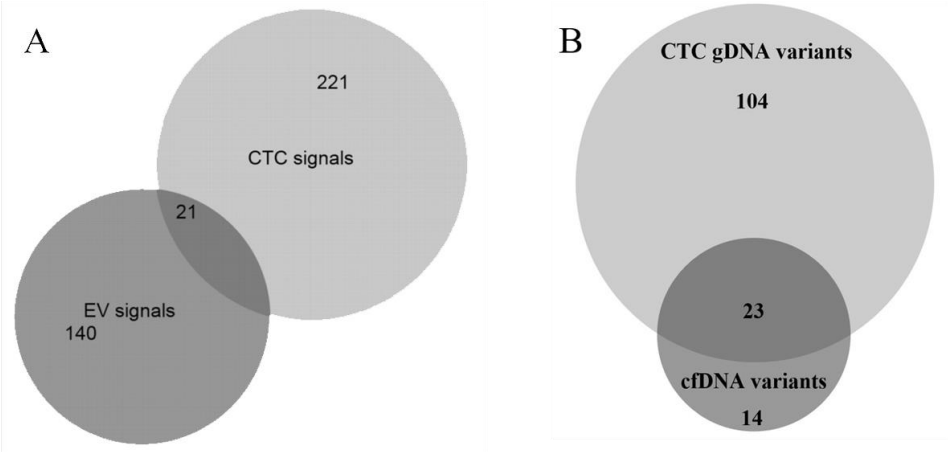


Figure 2: Venn diagrams showing the additive value of the liquid biopsy analytes (*Keup et al., 2018; 2020*).

One of our previous studies revealed that the overexpression prevalence of the **same transcript** (***mTOR***) significantly differed between overall responders and overall non-responders in both, **CTCs and EVs**, but to **contrary clinical outcomes (Figure 3)**. Moreover, a **combinatorial analysis** of overexpression signals of transcripts within the **same protein family** and **in CTCs and EVs** increased the significant association with therapy failure, revealing the **synergistic potential** of both analytes **(Figure 4; Keup et al., 2018)**.

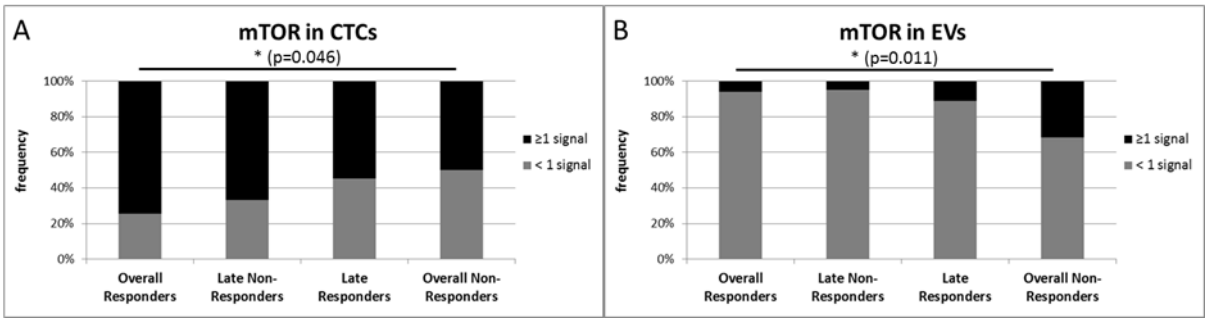


Figure 3: Overexpression frequency of mTOR in CTCs (A) or EVs (B) of met BC patients in correlation with patient groups separated based on their response characteristics across the therapy course. mTOR signals in CTCs were prominent in all groups, but the signal proportion decreased with increasing therapy failure and the results for Overall Responders and Overall Non-Responders were significantly different (p=0.046). In contrast, mTOR signals in EVs were most prominent in Overall Non-Responders (B) with a significant difference to Overall Responders (p=0.011) (***Keup et al., 2018***).

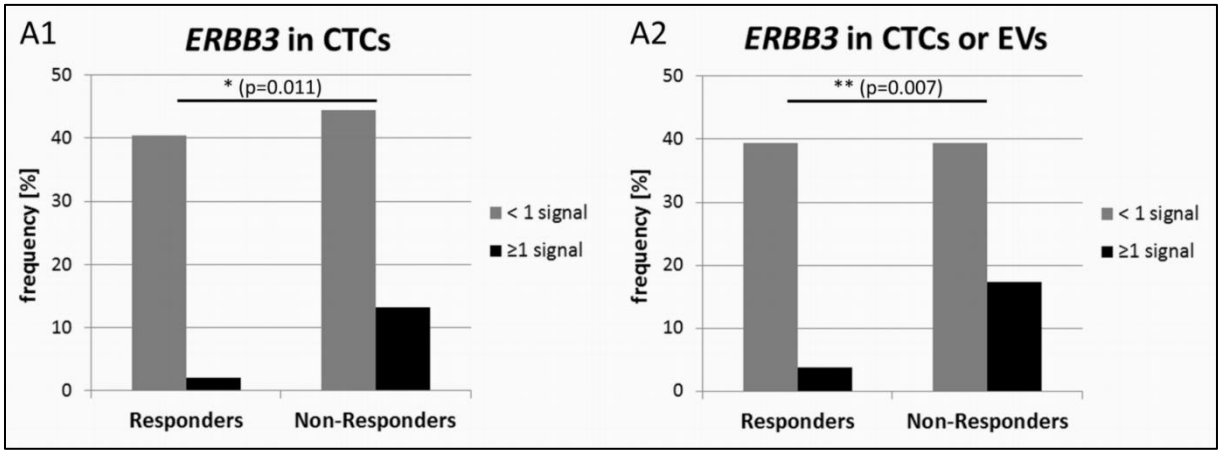


Figure 5: Overexpression frequency of ERBB transcripts in CTCs and/or EVs of met BC patients in correlation with therapy response. RNA profiles of EVs and CTCs of different time points were compared, revealing a significant higher proportion of ERBB3 signals in CTCs of Non-Responders (A1). Combined analysis of ERBB3 in the two blood components resulted in a greater difference between the two response groups (***Keup et al., 2018***).

Targeted deep sequencing of cfDNA displayed **high inter-patient variability of cfDNA concentration**. The integration of **unique molecular indices** (UMIs) was demonstrated to guarantee **specificity of called variants (Keup et al., 2019)**. Comparison of cfDNA variants isolated from whole blood and matched CTC-depleted blood presented no significant differences, enabling a **reliable combined CTC and cfDNA analysis from only 10 ml of blood (Figure 6; Keup et al., 2019a)**.

The **presence** of the specific ***ESR1* Y539S** and ***PIK3CA* E545K variants** was significantly correlated with a **decreased time** between first diagnosis of **metastasis** and **death**. The **dynamics** of *ESR1* and *PIK3CA* variant allele frequencies across treatment were reported to harbor great potential as a **sensitive monitoring approach (Figures 7 and 8; Keup et al., 2019; 2021).**

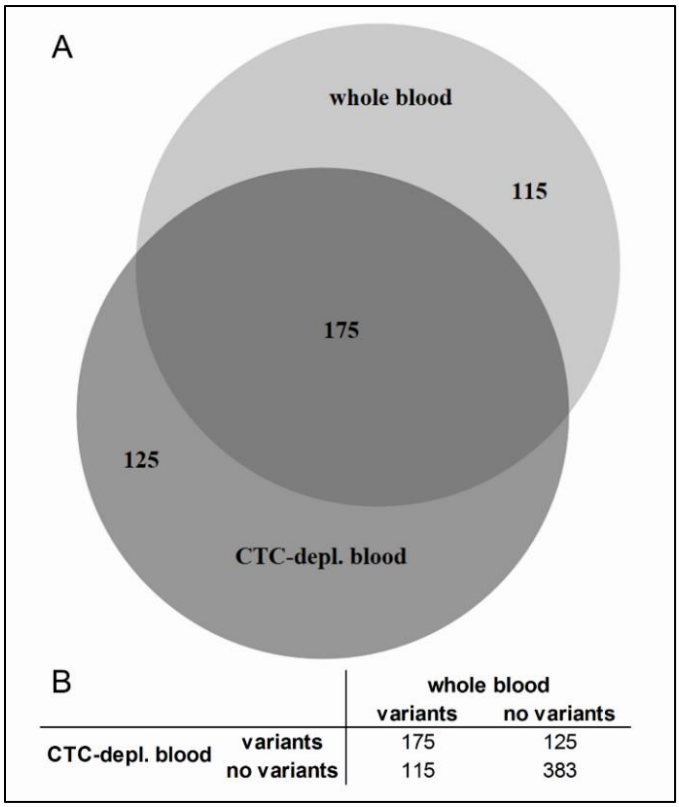


Figure 6: Venn diagram showing cfDNA variants in CTC-depleted blood and whole blood *(Keup et al., 2019).*

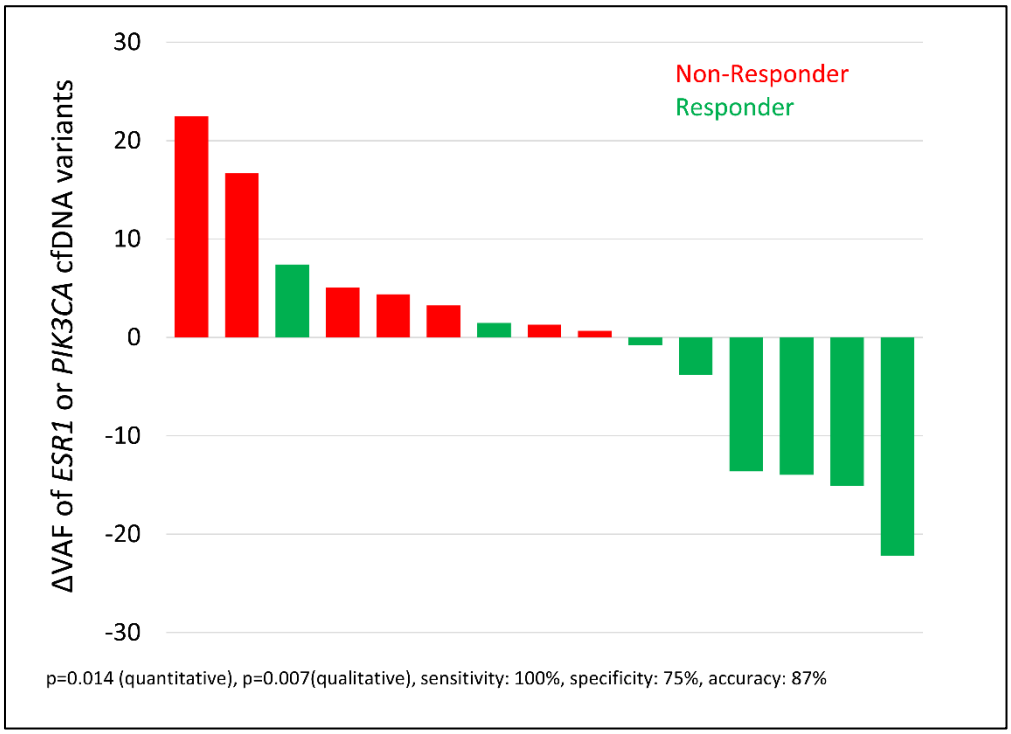


Figure 7: Waterfall plot mapping the cfDNA variant allele frequency (VAF) development of *ESR1* or *PIK3CA* variants and therapy response. Difference in variant allele frequencies (DVAF) of *ESR1* variants or *PIK3CA* variants between two consecutive staging time points correlated with therapy response at the second time point evaluated by radiologic imaging and according to RECIST ***(Keup et al., 2021).***

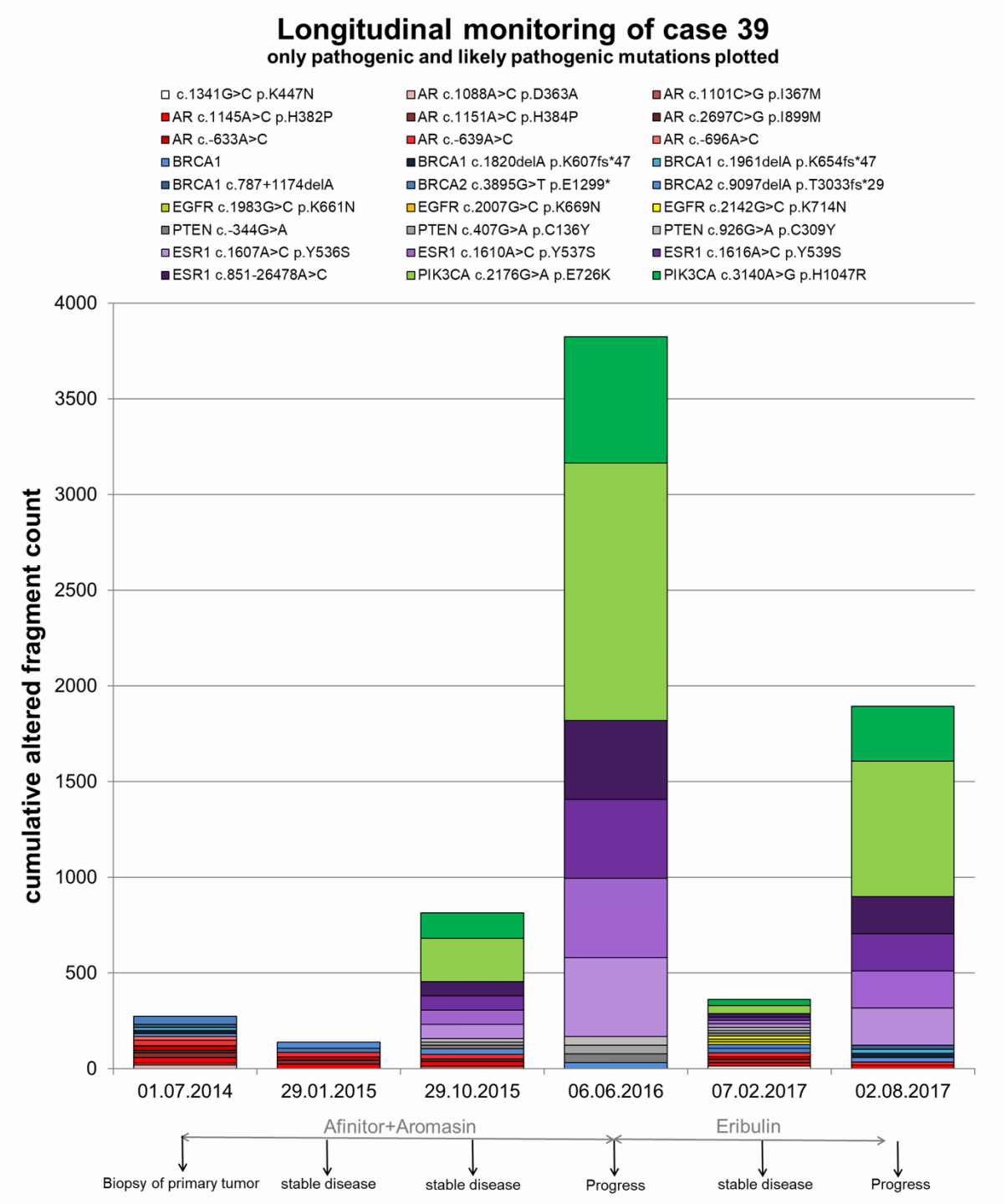


Figure. 8: Longitudinal monitoring of cfDNA variants with pathogenic/likely pathogenic impact in one HR+ HER2–patient. Cumulative altered fragment counts of all detected pathogenic and likely pathogenic variants are illustrated. Variants located in the same gene are shown in the same color but different shade. Applied therapies as well as staging results (by visual staging and RECIST) are depicted (***Keup et al., 2019***).

We developed a **method for CTC gDNA isolation** from the **remaining mRNA-depleted CTC lysates** for analysis of the **genomic** and **transcriptomic complexity in CTCs** from the same blood sample. The challenges regarding the low amounts of input CTC gDNA were solved for library preparation and sequencing. *PIK3CA* and *ESR1* variants were less common in CTC gDNA than in cfDNA, while *ERBB2* variants were only detected in CTC gDNA. The percentage of patients with no detectable cfDNA variants or CTC gDNA variants was 17%/11%, but combined analysis identified variants in 94% of all patients **(Figure 9; Keup et al., 2020)**.

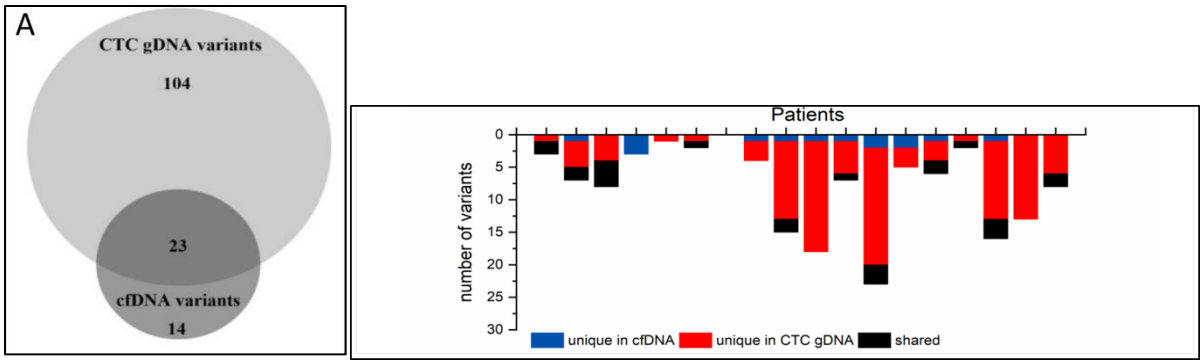
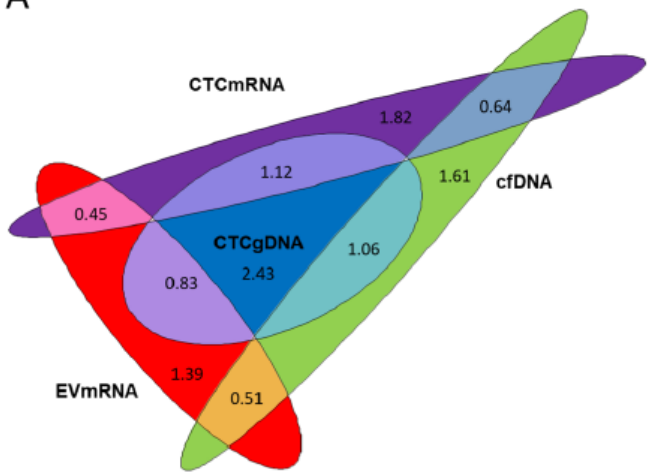


Figure 9. Direct comparison of cfDNA and CTC gDNA variants. (A) Venn diagram illustrating the concordance of detected cfDNA and CTC gDNA variants in 18 matched samples..**Right image:** Number of variants separated by patients and fractions. Colors indicate whether the variants were found in cfDNA only (blue), CTC gDNA only (red) or whether the same variant was found in both cfDNA and CTC gDNA (black) ***(Keup et al., 2020).***

The assessment of the **dynamics** of **matched CTC mRNA, EV mRNA** and **cfDNA** at **disease progression** and at the two subsequent radiologic staging time points revealed that **each of the three analytes** has its own **unique feature**, useful for **disease monitoring** (**Keup et al., 2020)**.

The **final integration** of the information from matched **CTC mRNA, EV mRNA, cfDNA** and **CTC gDNA**, assessed at disease progression **in 26 HR+/HER2- met BC** patients using **statistical approaches**, indicated the **complementary nature of the analytes** for prognosis and therapy stratification. By calculation of the mutual information, we have shown that **some of the information** within the four analytes **overlap**, while **all** of the **analytes added information** to the global multimodal data set **(Figure 10; Keup et al., 2021)**.

A



B

	cfDNA	CTCgDNA	CTCmRNA	EVmRNA
cfDNA	1.61	1.06	0.64	0.51
CTCgDNA	1.06	2.43	1.12	0.83
CTCmRNA	0.64	1.12	1.82	0.45
EVmRNA	0.51	0.83	0.45	1.39

Figure 10: Mutual information (b) illustrated by an area-proportional Euler diagram (a). Pairwise comparison revealed the greatest overlap between information contained in CTC gDNA with the information contained in the other three analytes (threshold >0.8). The mutual information values were found to be rather low. Each analyte therefore, added information that was absent in the other analytes ***(Keup et al., 2021).***

References

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