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COMMENTARY

The potential of combined mutation sequencing of plasma circulating cell-free DNA and matched white blood cells for treatment response prediction

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Circulating cell-free DNA (ccfDNA) in the plasma of cancer patients constitutes a potential source of tumor-derived DNA. Sensitive mutation detection assays on ccfDNA extracted from plasma could be used to detect circulating tumor DNA (ctDNA). This poses opportunities to apply ctDNA as an easily accessible biomarker for cancer screening, predictive testing, and monitoring of disease and treatment responses (Heitzer *et al*., 2019; Lampignano *et al*., 2019; Pantel and Alix-Panabières, 2019). However, blood-based molecular tumor profiling has been approached with caution since the origin of the detected variants is uncertain. CcfDNA consists of mostly degraded DNA fragments shedded from various tissues through apoptosis, necrosis, exocytose, or active secretion, of which over 90% derives from the hematological lineage (Abbosh, Birkbak, and Swanton, 2018; Thierry *et al*., 2016; Xia *et al*., 2017). High-sensitive mutation detection methods using ccfDNA from cancer patients demonstrated a low overall yield of total DNA and that the ctDNA fraction accounts only for a very small proportion of the total ccfDNA of less than 0.1–1%. This fraction varies significantly according stage of disease, response to treatment, tumor burden, and tumor characteristics such as tumor grade, vascularization, cell death, and proliferation rates (Heitzer *et al*., 2019). Since the ctDNA fraction is extremely low in many cancers, ctDNA detection methods are required to be highly sensitive and highly specific (Elazezy and Joosse, 2018; Merker *et al*., 2018). Recent developments in high-sensitive, more sophisticated sequencing methodologies to detect tumor-derived mutations in ctDNA enabled to identify variants that are present at very low levels in a background of 'normal' ccfDNA using, for example, combinations of integrated digital error suppression (like unique-molecular-identifier), appropriate variant calling, multigene analysis, and in-depth sequencing (Abbosh *et al*., 2018; Heitzer *et al*., 2019; Razavi *et al*., 2019).

In this issue, Kruger and colleagues determined the presence of hotspot mutations and ctDNA load using a high-sensitive sequencing 10-gene panel approach to describe treatment outcome in estrogen receptor (ER)-positive, HER2-negative metastatic breast cancer (MBC) patients treated with everolimus and exemestane (EVE/EXE) (Kruger *et al*., 2020). In this study, 76% of the included MBC patients were considered ctDNA positive with a high prevalence of ESR1, PIK3CA, and TP53 variants. A shorter progression-free survival (PFS) was found in patients with three or more mutations ($P = 0.003$) or with 54 or more mutant ctDNA copies ($P = 0.002$). A recent study on a comparable cohort showed similar associations between high quantities of ctDNA and a diminished survival (Suppan *et al*., 2019). The study of Kruger and colleagues is one of the first to demonstrate the potential of ctDNA mutation testing using pretreatment plasma to select patients with ER-positive/HER2-negative MBC eligible for EVE/EXE with prolonged PFS and that high-sensitive

Abbreviations
CHIP, clonal hematopoiesis of indeterminate potential; ctDNA, circulating tumor DNA; ccfDNA, circulating cell-free DNA; ER, estrogen receptor; MBC, metastatic breast cancer; PFS, progression-free survival; WBC, white blood cells.

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sequencing of ccfDNA might support predicting treatment response in MBC.

In addition, the analysis revealed that certain (likely) pathogenic mutations in ESR1 and SF3B1 might affect PFS and OS as well (P = 0.084 and P = 0.088). In line with previous reports, specific ESR1 mutations such as Y537S were considered as adverse prognostic biomarkers while other mutations, like in PIK3CA, do not affect PFS (Moynahan *et al*., 2017; Reinert *et al*., 2017). Besides, in a similar cohort using a larger gene panel, other specific mutations in AR, MUC16, and ERBB2 (not tested in the Kruger study) revealed that each separately had a significant association with survival in MBC (Keup *et al*., 2019). These findings imply that not just the number of observed different hotspot mutations might be associated with treatment response in MBC, but the presence or absence of certain strongly pathogenic mutations such as ESR1 or MUC16 might influence survival significantly. Therefore, future experiments of larger cohorts are needed to evaluate the contribution of these separate pathogenic mutations in combination with the total number of other mutations on clinical outcome to further improve the value of ctDNA testing as a predictive biomarker for survival.

An important drawback of the implementation of innovative high-sensitive ccfDNA sequencing approaches is the detection of variants that are not derived from the vital tumor cells. Some of these variants are the result of technical artifacts during ccfDNA sequence analysis. This was recently illustrated when comparing 4 different commercially available next-generation sequencing methodologies with considerable high discordances reflected in many false-positive and false-negative results (Stetson *et al*., 2019). Other insignificant variants appear due to inappropriate variant calling resulting from inaccurate discrimination of somatic tumor-relevant variants from SNPs, germ-line mutations, sequencing artifacts, clonal hematopoiesis of indeterminate potential (CHIP) among others. All these inappropriate variant callings may confound the interpretation of ccfDNA sequencing in particular when applied to investigate associations with tumor response and clinical outcome.

Clonal hematopoiesis of indeterminate potential is the consequence of the accumulation of somatic mutations resulting from replication errors in the rapidly dividing and mutation-prone hematopoietic progenitors (Gondek and DeZern, 2020; Razavi *et al*., 2019). These somatic mutations may provide a selective benefit to some hematopoietic stem cells and their progenitors, resulting in their disproportionate expansion. Since the majority of ccfDNA is blood cell-derived, somatic mutations associated with CHIP can thus be detected during ccfDNA sequencing analysis (Gondek and DeZern, 2020; Razavi *et al*., 2019). Indeed, Chen and coworkers detected somatic mutations in the ccfDNA in 30% of healthy aging individuals in genes related to hematological malignancies including TP53 (Chen *et al*., 2019). Razavi and associates using high-intensity sequencing with 401-gene panel reported that most somatic mutations detected in control patients without cancer (81.6%) were also identified in their matched white blood cells (WBC) (Razavi *et al*., 2019). Similarly, most mutations identified in ccfDNA samples of cancer patients (including MBC) were also found in their matched WBC (53.2%). Furthermore, the number of WBC-matched ccfDNA variants in cancer patients did not correlate with the number of tumor biopsy-matched mutations. All these specific somatic mutations are less likely to be of tumor origin and have features consistent with CHIP (Razavi *et al*., 2019).

In summary, the recent achievements in high-sensitive sequencing methodologies of pretreatment plasma ccfDNA have proven to become an useful tool to detect and map tumor-derived mutations and offer opportunities as those reported by Kruger and colleagues, to investigate the clinical value for the prediction of therapy response and clinical outcome. However, these same high-sensitive sequencing methodologies now also visualize that most variants detected in ccfDNA of cancer patients represent especially CHIP and that CHIP is more prevalent than was previously anticipated (Chen *et al*., 2019; Razavi *et al*., 2019). In particular, this high prevalence of CHIP emphasizes the importance of parallel high-sensitive sequencing of DNA derived from WBCs of the same patient for appropriate variant interpretation.

Conflict of interest

PvdL has no conflicts of interest to declare. ES received honoraria for advisory board from AstraZeneca, Roche, Pfizer, Bayer, Novartis, BMS, BioRad, Illumina, Ageno BioSciences, Janssen Cilag (Johnson&Johnson), BioCartis; speaker's fee from AstraZeneca, Roche, Pfizer, Novartis, BioRad, Illumina, BioCartis; and research support from Boehringer Ingelheim, BMS, Biocartis, Bio-Rad, Ageno BioSciences, and Roche (all outside the submitted work and all fees to UMCG).

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