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Reply to: Validation of blood-based detection of breast cancer highlights importance for cross-population validation

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REPLYING TO B. Theeuwes et al. *Nature Communications* https://doi.org/10.1038/s41467-025-57265-z (2025)

We have read with great interest the Matters Arising manuscript by Theeuwes and colleagues, who evaluated our newly identified breast cancer (BC) diagnostic methylation sites from peripheral blood mononuclear cells (PBMCs) in the whole blood methylation dataset from five hospitals and countries in Europe. The results showed that although most of the sites in the whole blood methylation dataset were consistently differentially expressed in BC compared to healthy controls, their diagnostic efficacy was lower than our study. Therefore, they concluded that our newly discovered BC diagnostic methylation sites have limited cross-population portability. However, we respectfully disagree with this conclusion, because the components of whole blood and PBMCs are completely different, and the scientific validity of using whole blood methylation datasets to verify the methylation sites obtained in PBMCs needs further verification.

Whole blood is a complex specimen consisting of red blood cells, white blood cells, and platelets suspended in plasma. White blood cells are composed of multiple cell types, with granulocytes being the most abundant (50–70%), followed by lymphocytes (20–40%) and monocytes (1–7%). In contrast, PBMCs primarily consist of lymphocytes and monocytes. Therefore, DNA methylation changes in whole blood are significantly influenced by other cell types, such as granulocytes, in addition to the methylation status of PBMCs. Due to the heterogeneity of whole blood, some studies have investigated DNA methylation differences among its various cell components. Most studies have shown that there are differences in DNA methylation levels in the promoter regions of certain genes or other specific genomic sites when studying different blood cell components[1,2]. For example, Cruzata et al. examined the correlation between PBMCs and granulocyte DNA methylation levels in 112 women participating in the multi-ethnic BC project in New York City. The results showed that there was a significant difference in the overall DNA methylation level between granulocytes and PBMCs, and the difference still existed after stratification by age and race[3]. Adalsteinsson et al. measured the methylation levels of four genes CpG Islands in PBMCs and granulocyte of 20 individuals using pyrosequencing method. They observed a higher average methylation in granulocyte in 21 of the 29 CpG sites analyzed in total[4]. The DNA methylation between different cell types varies, which may be the reason why the results obtained by the authors in the whole blood methylation dataset are different from those obtained in our PBMCs methylation dataset.

In addition, population differences, such as genetics and environmental factors, may also explain the discrepancies between our study and that of Theeuwes et al. Previous studies have found many regions with abnormal methylation during carcinogenesis by comparing genome-wide DNA methylation analysis of tumors and non-malignant tissues, and these regions show differential DNA methylation patterns between ethnicities[5–8]. Several studies have shown that racial differences in gene-specific DNA methylation levels are even present at birth[9,10]. Besides that, some environmental factors can also change the methylation of human cancer genes, thereby exerting carcinogenic or anticancer effects. Such as air pollution, living environment, eating habits, economic status and social security, etc.[11,12]. It is noteworthy that we found that four methylation sites (cg11754974, cg16652347, cg13828440, and cg18637238), verified by multiple methods in our study, still showed significant differences in the whole blood methylation dataset used by Theeuwes and colleagues. However, in the prospectively collected IARC and PLCO cohorts, our signature failed to effectively distinguish BC from healthy controls prior to cancer diagnosis. The design of the IARC and the PLCO cohorts, which involved collecting blood samples before cancer diagnosis to study epigenetic predisposition, may not be suitable for validating our signature, which aims to detect breast cancer early after its onset.

In addition, Theeuwes and colleagues found that patients with systemic sclerosis (an autoimmune disease) showed the same directionality of methylation changes as PBMCs in BC patients in our study, so it was concluded that some of the signals in our study may come from inflammation, such as before the histological diagnosis of BC was

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triggered by compression mammography and/or core needle biopsy, rather than BC itself. Since autoimmune diseases and acute inflammation can affect PBMC methylation[13,14], we excluded individuals with concurrent autoimmune diseases during patient recruitment. Furthermore, we collected samples prior to invasive procedures (e.g., diagnostic puncture or minimally invasive surgery), with most patients undergoing only ultrasound examination. Therefore, the hypothesis proposed by Theeuwes and colleagues that the methylation signal is derived from inflammation induced by compression mammography, which is not established in our study. However, we cannot rule out that some cancer-related chronic inflammation may mediate the methylation changes of immune cells in this study. Increasing evidence suggests that the pathogenesis of breast cancer (both primary and recurrent) may be governed by various signaling pathways involved in chronic inflammation[15]. In early 1863, Rudolf Virchow postulated that cancer could be originated at the site of chronic inflammation[16]. Several risk factors associated with inflammation, which may correlate with breast cancer, include aging, obesity, heavy alcohol consumption, postmenopausal status, adiposity, physical inactivity and subclinical infections[17,18]. Chronic inflammatory response mainly carried out by immune cells (such as monocytes, macrophages, neutrophils, eosinophils, dendritic cells, mast cells and lymphocytes) in breast carcinogenesis. The genes of the four methylation sites screened and verified in our study were all related to immune receptors. For example, KLRK1 (cg18637238) mediates the antitumor functions of NK cells, as well as conventional and unconventional T cells, and the presence of KLRD1/NKG2A (cg13828440) on human tumor-specific T cells impairs IL2 receptor-dependent proliferation. Hence, it cannot be excluded that some chronic inflammation related to tumor may mediate the methylation changes of immune cells.

Finally, the minor inconsistencies between the data analyzed by the authors and our research are summarized as follows. First, the granulocyte ratio calculated by Theeuwes and colleagues is inconsistent with our study, and we speculate that this may be due to differences in analysis methods (the authors used hierarchical EpiDISH analysis, while we used EpiDISH analysis, both of which are acceptable). Second, there are differences between the methylation sites identified by the authors in TCGA BC tissues and our results, which we suspect may be due to incorrect classification of samples. In our study, the cohort included 96 normal samples and 794 tumor samples, while the study by Theeuwes and colleagues used 97 normal samples and 792 tumor samples. We checked the relevant data again and found no error in classification. In addition, the authors found that the methylation site cg14507403 exhibited a trimodal distribution, and its methylation level may be affected by single nucleotide polymorphisms (SNP) or genetic components. Considering the impact of SNP on methylation, our study has already filtered out SNP-related sites when analyzing the 850k methylation chip[19].

Due to different underlying population characteristics such as genetics, ethnicity, clinicopathological features, etc., we agree with the authors' emphasis on the need for cross-population validation and robust assessment of biomarkers across multiple clinically relevant populations. However, in general, we believe that it is not advisable to validate the results obtained from the PBMCs methylation dataset using whole blood methylation datasets.

Data availability

The Genome-wide DNA methylation data generated in this study have been deposited in the Gene Expression Omnibus database under accession code No. GSE237036.

Code availability

The codes used to generate the analysis can be accessed at http://www.bioconductor.org/packages/release/bioc/vignettes/ChAMP/inst/doc/ChAMP.html?from=singlemessage&isappinstalled=0#section-.

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Author contributions

T.W., Q.Q., P.L., J.L., L.D., and C.W. conceived the work and provided substantial contributions to the interpretation of the data. T.W. wrote the first draft of the paper. All authors have read, reviewed, and approved the final version of the manuscript. All authors are accountable for all aspects of the work.

Competing interests

The authors declare no competing interests.

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