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RESEARCH ARTICLE

Zhang et al: Immune modulation by gut microbiota

Role of gut microbiota and immune response in breast cancer progression

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ABSTRACT

Breast cancer is one of the most prevalent cancers among women and is associated with high mortality rates. Emerging evidence suggests a link between gut microbiota and the development of various tumors, particularly those involving immune-mediated mechanisms. However, the potential relationship between gut microbiota and breast cancer—and whether this relationship is mediated by immune cells—remains unclear. This Mendelian randomization (MR) study utilized summary statistics from genome-wide association studies of 412 gut microbiota, 731 immune cell traits, and breast cancer (including its subtypes). Two-sample MR analyses were conducted to assess potential causal relationships between gut microbiota and breast cancer. To further validate the findings, Bayesian weighted MR was applied. Robustness was ensured through sensitivity, specificity, and pleiotropy analyses. A reverse MR analysis was also performed to assess the potential for reverse causality. Finally, mediation analysis was employed to investigate whether immune cells mediate the pathway from gut microbiota to breast cancer. The MR analysis identified 15 gut microbiota and related metabolic pathways significantly associated with breast cancer, with nine showing positive associations and six showing negative associations. The reverse MR analysis did not support a causal effect of breast cancer on gut microbiota. Mediation analysis revealed that DP ($CD4^+CD8^+$) % leukocyte mediated the pathway between gut microbiota (PWY-6263: superpathway of menaquinol-8 biosynthesis II) and breast cancer. These findings suggest a causal relationship between gut microbiota and breast cancer, with a small portion of this effect mediated by immune cells. This study underscores the potential role of gut microbiota and immune modulation in the pathogenesis of breast cancer.

Keywords: Gut microbiota; immune cells; breast cancer; mendelian randomization; MR; causal inference.

INTRODUCTION

Breast cancer is one of the most diagnosed cancers in women and ranks as the second leading cause of cancer-related death among women (1, 2). Approximately one in eight women in the United States has been diagnosed with breast cancer (3). The risk of breast cancer increases with age, rising by about 0.5% per year (4). Previous studies(5) have suggested that the high incidence of breast cancer in some developed countries may be related to lifestyle and genetic factors such as BRCA1 and BRCA2 mutations(6), as well as diet and obesity(7). Treatment for breast cancer typically involves surgery, chemotherapy, radiotherapy, targeted therapy, and endocrine therapy. Although significant progress has been made in the early detection and treatment of breast cancer, treatments for advanced breast cancer remain limited and often come with high rates of adverse side effects(8). Despite advancements, the exact mechanisms underlying breast cancer development are not fully understood. Hence, further studies are needed to better understand the causes and prevent the occurrence of breast cancer. The gut microbiota is located in the human gut and establishes itself since birth and evolves in tandem with the body's growth and development. It lives in symbiosis with the host and is considered a crucial factor in maintaining host health. Gut microbiota interact with the host to form a complex ecosystem. On the one hand, a healthy gut can shape the microbiota and prevent the colonization of harmful bacteria through symbiotic relationships. On the other hand, the gut microbiota regulates both local and systemic immune function in the host. Over long-term evolution, the microbial flora and its host have developed a close symbiotic relationship through mutual adaptation and selection. The influence of the gut microbiota on local and systemic immunity has attracted widespread attention. Musso(9), Maynard et al. (10), Frosali et al.(11), and Fujisaka et al.(12) suggested that the gut microbiota was involved in and regulated metabolic and immune activities, playing a crucial role in maintaining microbial balance. Most diseases mediated by altered gut microbes are associated with impaired immune responses(13, 14). Increasing evidence suggests a potential association between gut microbiota and cancer risk. If this association is causal, targeting the gut microbiota could become a new strategy for cancer screening and prevention(15).

Mendelian randomization (MR) is a data analysis technique used in epidemiological studies to assess causal relationships. It uses genetic variants strongly associated with exposure factors as instrumental variables to assess the causal effects of these factors on outcomes. MR can circumvent the influence of residual confounders on the accuracy of association results, making the evidence for causal relationships more robust compared with observational studies or

randomized controlled trials(16). Based on the interaction between the gut microbiota and tumors, we hypothesized that a specific gut microbiota increased the risk of breast cancer. Then, a two-sample MR study was conducted to investigate the potential causal relationship between breast cancer and 412 gut microbiota, revealing relevant immune cells through mediation analysis. This study aimed to further elucidate the mechanism by which the gut microbiota contributes to the occurrence and development of breast cancer, providing a new scientific basis for personalized breast cancer treatment.

In summary, this study aimed to explore the role of gut microbiota, immune cells, and breast cancer pathogenesis. Using advanced genetic techniques and conducting comprehensive analyses of immune cell signatures, the study elucidated potential therapeutic targets and provided a deeper understanding of the complex etiology of breast cancer. It also reduced morbidity, recurrence, and mortality by intervening in the gut microbiota and immune cells to prevent breast cancer.

MATERIALS AND METHODS

Study design

The bidirectional two-sample MR analysis study design was used to investigate the causal relationship between 412 gut microbiota (exposure factors) and the risk of breast neoplasia (outcome). The first step is to assess the causal effect of gut microbiota on breast cancer and its subtypes using a two-sample Mendelian randomization analysis and to screen for gut microbiota with highly associated risk. The second step is to evaluate the causal effect of the filtered gut microbiota on the filtered immune cells. The third step is to determinate the relationship between the screened immune cells and breast cancer. Finally, mediation analysis was implemented to examine the potential pathway mediated by immune cells from gut microbiota to breast cancer. The study design is illustrated in Figure 1. Genetic variation was used as a risk factor, with single-nucleotide polymorphisms (SNPs) from the FinnGen datasets serving as instrumental variables (IVs). To ensure the validity of IVs, we considered three assumptions: (i) independence: SNPs independent of any confounding factors; (ii) correlation: SNPs strongly associated with exposure; and (iii) exclusivity: SNPs with no relationship with the exposure.

The ultimate source of the obtained data is all European populations. The relevant data are sourced from various published genome-wide association study (GWAS) databases. Since the original research has obtained informed consent, this aspect of the study does not necessitate approval from an ethics committee.

Genome-wide association study data sources for breast cancer

The GWAS data sources for breast cancer were obtained from the FinnGen R11 GWAS databases, which combine the Finnish Health Registry's digital health records with genetic data from the Finnish Biobank (https://www.finngen.fi/en). We obtained GWAS summary statistics for total breast cancer from FinnGen (20,586 cases and 201,494 controls) databases as the initial outcomes. Furthermore, GWAS data for HER2- breast cancer (8,469 cases and 201,226 controls) and HER2+ breast cancer (12,081 cases and 201,226 controls) were obtained from FinnGen to further explore the relationship between gut microbiota and breast cancer pathological subtypes. The UK Biobank database was excluded from the selection of outcome data due to sample overlap between exposure and results.

Gut microbiota data sources for GWAS

SNPs related to the composition of the gut microbiota in this study were obtained from the NHGRI-EBI GWAS Catalog (https://www.ebi.ac.uk/gwas/) under the study accession numbers GCST90027446–GCST90027857. Accession numbers for each specific taxon and pathway can be found in Table S13 or at https://dutchmicrobiomeproject.molgeniscloud.org (17). The GWAS data were obtained from Dutch microbiota, which included 207 microbial taxa and 205 functional pathways representing microbial composition and function in 7738 participants.

Immune cells data sources for GWAS

The publicly available accession numbers, ranging from GCST90001391 to GCST90002121, contained an extensive array of 731 immunophenotypes(18). These included data on the maturation phase of B cells, CDCs, T cells, monocytes, T-cell/B-cell/NK-cell assay, myeloid cells, and Treg cells(19). The GWAS data contained four different types of 32 morphological parameters (MPs), 118 absolute cell counts (ACs), 192 relative cell counts, and 389 median fluorescence intensities, collected from 3757 European individuals with no overlapping cohorts(18).

Selection of IVs

Significant SNPs of gut microbiota and immune cells with $P < 1 \times 10^{-5}$ and with linkage disequilibrium were excluded ($r^2 = 0.001$, kb = 10,000)(20). We clustered all genetic variants using a threshold of R2 < 0.001 within a clustering distance of 10000 kb. Subsequently, SNPs were filtered using the F-statistic method. The F-statistic was calculated for each IV, and SNPs with $F > 10$ were reserved for subsequent studies(21). If the corresponding F-statistic is <10, IVs are considered as weak IVs and then excluded.

Two-sample MR analysis

This study used two-sample MR analysis to assess the causal effects of gut microbiota and breast cancer. Causal relationships were inferred using the Inverse Variance Weighted (IVW), MR-Egger, weighted median, simple mode, and weighted mode methods. Among these, the IVW served as the primary method, which weights the inverse variance of the causal effects of different genetic variants on a trait and then combines the weighted effect estimates, while the other methods were used as supplementary approaches.

Statistical analysis

Sensitivity analysis was performed to assess the reliability and stability of the conclusions including heterogeneity analysis, horizontal pleiotropy analysis and “leave-one-out” test. Cochran's Q statistic methods were used to test for heterogeneity. Horizontal pleiotropy was assessed using the MR-Egger intercept, MR-PRESSO test. We conducted a "leave-one-out" analysis to determine the potential bias of individual SNPs on the MR analysis by sequentially removing one SNP at a time and re-estimating the effect.

Reverse MR analysis

To investigate whether breast cancer has a causal effect on the identified significant gut microbiota, we conducted a reverse MR analysis using SNPs associated with breast cancer as instrumental variables (IVs) and identified gut microbiota as the outcome. The reverse MR analysis was employed to rule out potential interactions between exposure and outcome.

Mediation analysis

Mediation analysis was used to explore the potential mechanisms of the pathways from exposure to outcomes through mediation. Firstly, β1 was obtained through the two-sample MR analysis which were used to evaluate the causal relationship between the gut microbiota and immune cells. Secondly, two-sample analysis methods were determined to evaluate the causal relationship between the screened immune cells and breast cancer to obtain β2. The mediation effect was calculated by multiplying β1 by β2. To evaluate the potential mediating role of immune cells in the pathway linking gut microbiota and breast cancer, we conducted multiple MR analyses. All analyses were performed using the R software (http://www.Rproject.org, v.4.3.3) with the "TwoSampleMR" package in this study. Figures were produced using the "ggplot2" R packages. A P-value of less than 0.05 is considered indicative of a statistically significant association between the exposure and the outcome.

RESULTS

Causal relationship of gut microbiota with breast cancer

Using the two-sample MR analysis and IVW method, this study found 15 gut microbiota and related pathways significantly associated with breast cancer (including 10 functional pathways and 5 microbial taxa). As shown in Table 1, 6 functional pathways and 3 microbial taxa were linked positively to total breast cancer. Figure 2 showed the forest plot of the positive bacterial flora of breast cancer and its subtypes. Breast cancer is a complex disease with various molecular and phenotypical backgrounds leading to different clinical outcomes. Although standard breast cancer molecular classifications often rely on ER/PR/HER2 status, the GWAS data do not providing corresponding subtypes. Therefore, we conducted subgroup analysis based on HER2 expression. Causal association ware further examined between the gut microbiota and HER2+ and HER2− breast cancer separately using two-sample MR analysis. Table 2 shows that eight functional pathways and 11 microbial taxa were associated with HER2+ breast cancer, including 4 functional pathways and 6 microbial taxa linked positively to HER2+ breast cancer. Table 3 shows the potential causal relationship between the 16 gut microbiota and HER2− breast cancer, including 5 functional pathways and 3 microbial taxa linked positively to HER2- breast cancer.

Heterogeneity, pleiotropy, sensitivity, reverse analysis, and BWMR analysis

We performed heterogeneity, sensitivity, and pleiotropy analyses to ensure the robustness of our MR causal effect estimates. The results of the IVW test and MR-Egger regression showed no heterogeneity in the causal relationship between the gut microbiota and breast cancer, as described in the Q statistics ($P > 0.05$). In addition, no significant difference was found between the intercepts derived from the MR-Egger regression analysis and zero, indicating no signs of horizontal pleiotropy (all intercept P values > 0.05). The MR-PRESSO test did not show any signs of horizontal pleiotropy in the examined causal relationship ($P > 0.05$) (Tables 4–6). No significant has been showed in Heterogeneity and pleiotropy in total breast cancer. In addition, the leave-one-out analysis showed that no single SNP significantly affected the causally related signals. The funnel plot also suggested the reliability of the causal effects of the identified associations. In addition, no supporting evidence was found for the causal effects of breast cancer on the gut microbiota in the reverse MR analysis. We performed BWMR analysis to further validate the correlation between gut microbiota and breast cancer, revealing that the aforementioned gut microbiota were significant (Tables 7–9). The relevant results are visualized in Figures 2-4. These results suggested a strong causal relationship between the identified gut microbiota and the corresponding breast cancer risk, further supporting the reliability of our findings.

Mediator analysis

We performed a two-sample MR analysis and IVW method as the primary analytical approach on 731 immune cells to filter relevant immune cells associated with gut microbiota. The selected immune cells were then separately analyzed in relation to total breast cancer, HER2+ breast cancer, and HER2− breast cancer. We found that several significant immune cells mediated the effect of gut microbiota on breast cancer. We revealed that PWY-6263: superpathway of menaquinol-8 biosynthesis II was positively linked to total breast cancer among the functional pathways of microbiota. Additionally, DP (CD4+CD8+) %leukocyte was identified as a positively mediated immune cell. *Lachnospiraceae noname* as a microbiota taxon was also positively linked to total breast cancer. Moreover, IgD− CD27− %B cell was identified to play an inhibitory mediator role. In HER2+ breast cancer, we observed a positive association with PWY-6263: superpathway of menaquinol-8 biosynthesis II. Additionally, DP (CD4+CD8+) %leukocyte was identified as a positive mediator, whereas IgD− CD27− %B cell showed a negative mediation. *Pseudoflavonifractor capillosus* exhibited a positive

relationship with breast cancer, whereas CD25hi CD45RA+ CD4 not Treg %T-cell acted as an inhibitor-mediated immune cell. In HER2− breast cancer, we observed a positive association with PWY0-1298: superpathway degradation of pyrimidine deoxyribonucleosides, and BAFF-R on CD20− was identified as an inhibitor-mediated immune cell. All relevant beta values are shown in Table 10. None of the other relevant gut microbiota was associated with mediated immune cells.

BWMR analysis of biotin biosynthesis II and breast cancer

Considering the important role of biotin biosynthesis II in breast cancer, we further used BWMR to ensure the robustness of our MR causal effect estimates. The correlation between biotin biosynthesis II and breast cancer is shown in Figure 2-4. Among them, breast cancer (P values=0.03), HER2+ breast cancer (P values=0.04), HER2− breast cancer (P values=0.05). These conclusions further prove the causal relationship between biotin biosynthesis II and different subtypes of breast cancer.

Genes associated with biotin biosynthesis II in breast cancer

We further studied the genes related to biotin biosynthesis II in breast cancer. As shown in Figure 5, 30 related genes were identified. Among them, RPA2 (OR=1.137, 95%Cl=1.015-1.259), ATG13 (OR=1.292, 95%Cl=1.060-1.524), SCAMP5 (OR=1.229, 95%Cl=1.070-1.389) were positively correlated with breast cancer. MSH2 (OR=0.889, 95%Cl= 0.7855-0.993), ALMS1P (OR=0.834, 95%Cl= 0.6877-0.981), C1QTNF9 (OR=0.784, 95%Cl=0.615-0.953) were negatively correlated with breast cancer.

DISCUSSION

In our study, we found 15 gut microbiota were significantly associated with total breast cancer, 6 functional pathways and 3 microbiota taxa had a promoting effect on total breast cancer. Previous studies found a potential association of gut microbiota with carcinoma(22-24). Changes in the immune environment may lead to changes in the human gut microbiota, thereby promoting the occurrence of diseases. A large number of recent studies focused on the complex relationship between the changes in the gut microbiota and immune environment and disease. The microbiota and the immune system have a complex relationship, and their balanced interaction is crucial for maintaining health. The disruption of this balance can lead to disease development(25). 412 gut microbiota, including 205 functional pathways and 207 microbiota taxa were evaluated in this study, and 15 gut microbiota were found had significant relationship

with total breast cancer. Among these gut microbiota, FAO-PWY: fatty acid beta-oxidation I, PWY-4984: urea cycle, PWY-5005: biotin biosynthesis II, PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation, *Roseburia*, and *Bacteroides intestinalis* had protective effects on total breast cancer, whereas the other relevant gut microbiota had disadvantageous effects.

Further, we found that, among the relevant gut microbiota, 7 functional pathways (DAPLYSINESYN-PWY: L-lysine biosynthesis I, FAO-PWY: fatty acid beta-oxidation I, PWY-4984: urea cycle, PWY-5005: biotin biosynthesis II, PWY-6263: superpathway of menaquinol-8 biosynthesis II, and PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation), and four microbiota taxa (*Pseudoflavonifractor*, *Lachnospiraceae noname*, *Roseburia*, and *Oscilibacter* unclassified) participated in the link between total breast cancer and HER2+ breast cancer. Furthermore, three functional pathways (METHGLYUT-PWY: superpathway of methylglyoxal degradation, PWWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation, and PWY-5005: biotin biosynthesis II) participated in total breast cancer and HER2− breast cancer. We found that PWY-5005: biotin biosynthesis II had a protective effect on total breast cancer and HER2- breast cancer.

Previous studies found an association of gut microbiota with various diseases, including cancer. Keshet et al.(26) found that deregulation of the urea cycle (UC) metabolic pathway may inhibit cancer progression, as it is the main metabolic pathway for converting excess nitrogen into disposable urea. We found that the functional pathway of PWY-4984: UC inhibited the occurrence of breast cancer. Maiti and Paira(27) found that biotin served as an essential cofactor used by all domains of life. Also, several novel biotin-targeted Au(I) complexes proved to be efficacious as radiosensitizers with tumor-targeting capacity and acceptable safety. Hence, our study found that the gut microbiota pathway PWY-500: biotin biosynthesis II had a protective effect on total breast cancer and HER2- breast cancer. Gong et al.(28) found that the DAPLYSINESYN-PWY pathway led to obesity, which was related to breast cancer(29). PWWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation, may involve the catabolism of pyrimidine nucleotides, including deoxycytidine, deoxyuridine, and deoxythymidine nucleotides. In cells, pyrimidine deoxyribonucleosides can be degraded through various enzymatic reactions, generating important metabolic intermediates(30). PWY-6263: superpathway of menaquinol-8 biosynthesis II, may involve the catabolism of menaquinol 8(MK-8). MK-8 is a subtype of vitamin K2 and is essential for spore formation and cytochrome production in certain Gram-positive bacteria(31). The association between

vitamin K and cancer can be considered from the perspective of chemoprevention, either as a therapeutic strategy alone or as an adjunct to chemotherapy. Studies have found that vitamin K2 may exert anticancer effects by inducing autophagy and inhibiting cancer cell invasion(32, 33). This study indicated the functional pathway of PWY-6263: superpathway of menaquinol-8 biosynthesis II had the protective effect on total breast cancer and HER2+ breast cancer which supported previous studies.

However, some findings of our study were contrary to previous findings. Ma et al. (34) found that mitochondrial fatty acid β-oxidation (FAO) was a main source of bioenergy, leading to cancer. However our results found that fatty acid oxidation I played a protective role. It might also be because the gut functional pathways in our study were different from previous FAO research pathways, providing new ideas for possible future targets.

In our research of primary MR analysis, we found PWY-5005 have negative feedback with total breast cancer and HER-2- breastcancer, but positively correlated with HER2+ breast cancer. The inconsistent results may be due to the weak association of PWY-5005 with HER2+ subtype, or the difference in weighting assumptions between TSMR and BWMR. The validity of the results needs to be further verified in a larger cohort. PWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation may be associated with breast cancer, which was found to be positively correlated with total breast cancer and HER2- breast cancer, but negatively correlated with HER2+ breast cancer. While the results of IVW and BWMR in both PWY-5005 and PWY0-1298 were different, which maybe had a weak association specifically for HER2+ subtype.

The reverse analysis showed that breast cancer did not affect gut microbiota. The mechanisms by which gut microbiota influence breast cancer through B cells and T cells involve multiple pathways, including immune regulation, inflammation, cellular signaling, and metabolic products(35). Gut microbiota modulate the maturation and function of T cells and B cells, potentially promoting tumor growth and metastasis. At the same time, the mediation analysis found that five immune cells mediated the influence of gut microbiota on breast cancer and their corresponding subsets. Immune cells play a critical role in innate and adaptive immune responses, managing and regulating cellular immunity during immune diseases and cancer. Their well-coordinated functions provide significant clinical benefits. Dysbiosis in gut microbiota can lead to the overactivation of pro-inflammatory pathways (e.g., NF-κB) and the production of cytokines (e.g., IL-6, TNF-α), which create a pro-tumorigenic

microenvironment. Additionally, gut-derived metabolites such as short-chain fatty acids (SCFAs) and bile acids can alter immune cell function and signaling pathways (e.g., PI3K-Akt, β-catenin), affecting tumor cell proliferation and survival(36). Together, these interactions highlight the complex role of gut microbiota in breast cancer development through immune-mediated mechanisms. CD4+CD8+ double-positive (DP) T cells are a subset of the T cell population, have been identified in the blood and peripheral lymphoid tissues of various species. Due to their involvement in immune diseases, inflammation, and cancer have garnered our interest(37). In a previous study involving patients with malignant pleural effusion due to breast cancer metastasis to the thoracic cavity, a significant number of DP T cells were found, suggesting a potential correlation between DP T cells and the development and progression of breast cancer(37).

Other studies have revealed that DP T cells promote the production of interleukins, such as IL-2 and IL-4 ect, thereby facilitating tumorigenesis and tumor progression(38). In our research, DP (CD4+CD8+) %leukocyte as one immune cell plays mediate function from the pathway of PWY-6263: superpathway of menaquinol-8 biosynthesis II had the protective effect on total breast cancer and HER2+ breast cancer, further elucidating the potential mechanisms by which gut microbiota influence the initiation and progression of tumor cells.

Our MR study focused on the relationship between microbiota and the risk of breast cancer. It helped understand how changes in the intestinal microbiota led to immune disorders in breast cancer, and also helped prevent the occurrence and development of breast cancer.

However, our study still had several limitations. First, our analysis used only the European population and did not include other races, limiting disease prediction. In the future, we aim to conduct more detailed analyses and discussions encompassing all ethnicities. Second, the molecular typing of breast cancer had a strong association with the survival period, and hence breast cancer could be divided into Luminal A, Lumina B, HER2+, and triple-negative breast cancer. The Finnish database GWAS dataset was only divided into HER2+and HER2− subgroups. Therefore, this study did not further explore the causal relationship between gut microbiota and each subgroup. Third, even if we took steps to identify and eliminate outlier variables, we could not rule out the possibility of horizontal pleiotropy affecting our results, even with the application of MR-Egger and MR-PRESSO tests, some undetected pleiotropy or population stratification may persist, underscoring the necessity for further replication across diverse cohorts. Horizontal pleiotropy occurs when genetic variants affect the outcome through

pathways unrelated to the exposure of interest, violating the exclusion restriction assumption. Methods like MR-Egger regression, weighted median, and negative control outcomes can partially address this issue but have limitations. Population stratification introduces confounding due to differences in allele frequencies and phenotype distributions across subpopulations, and standard methods may not fully remove it. These challenges cannot be completely eliminated, but advanced methods and careful study design can enhance the robustness of causal inference in MR analysis. Fourth, we used summary-level statistical data in our study, which limited the depth of our analysis because we could not obtain individual-level data. Therefore, further studies are needed to quantify other mediating factors. Last but not least, the GWAS data in this study were mainly from cohorts of European ancestry, so the generalizations of the findings to other populations, such as Asian populations, should be cautious and further verified.

CONCLUSION

Our study comprehensively evaluated the relationship among gut microbiota, immune cells, and breast cancer. Our findings revealed that some gut microbiota, when considered as exposure factors, could be used as risk factors or protective factors for breast cancer. Some immune cells might mediate the effect of gut microbiota on breast cancer. These findings provided valuable novel insights into the mechanisms by which gut microbiota and immune cells affected cancer development. However, further experiments and clinical studies are needed to validate and expand our findings. Additionally, we hope that our study can provide new targets for the advancement of breast cancer treatment in the future.

Conflicts of interest: Authors declare no conflicts of interest.

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Data availability: The relevant data are sourced from various published GWAS databases.

The data used in this study are publicly available. GWAS data: https://www.finngen.fi/en https://www.ebi.ac.uk/gwas/

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TABLES AND FIGURES WITH LEGENDS

Table 1. MR result of the association of gut microbiota with total breast cancer

Exposure	snps	pval	or	or_lci95	or_uci95	Direction
DAPLYSINESYN-PWY: L-lysine biosynthesis I	9	0.043	1.117	1.004	1.243	Positive
FAO-PWY: fatty acid beta oxidation I	7	0.021	0.895	0.815	0.984	Negative
METHGLYUT-PWY: superpathway of methylglyoxal degradation	8	0.044	1.059	1.002	1.120	Positive
PRPP-PWY: superpathway of histidine purine and pyrimidine biosynthesis	13	0.0179	1.118	1.019	1.226	Positive
PWY0-1298: superpathway of	14	0.003	1.151	1.050	1.262	Positive

pyrimidine deoxyribonucleoside degradation						
PWY-4984: urea cycle	8	0.031	0.862	0.753	0.986	Negative
PWY-5005: biotin biosynthesis II	11	0.001	0.883	0.819	0.951	Negative
PWY-6263: superpathway of menaquinol-8 biosynthesis II	5	0.007	1.110	1.029	1.196	Positive
PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation	6	0.025	0.866	0.763	0.982	Negative
PWY-7446: sulfoglycolysis	10	0.034	1.048	1.003	1.095	Positive
Pseudoflavonifractor	8	0.034	1.048	1.003	1.095	Positive
Lachnospiraceae noname	5	0.034	1.087	1.006	1.174	Positive
Roseburia	14	0.005	0.887	0.816	0.964	Negative

Parabacteroides merdae	4	0.011	1.204	1.043	1.390	Positive
Bacteroides intestinalis	3	0.040	0.915	0.841	0.996	Negative

Positive: risk-increasing (OR ﹥ 1); Negative: risk-decreasing (OR ﹤ 1); pval: *p*-value; OR:Odds Ratio ; or_lci95: Odds Ratio Lower Confidence Interval at 95% ; or_uci95: Odds Ratio Upper Confidence Interval at 95% .

Table 2. MR result of the association of gut microbiota with HER2+ breast cancer

Exposure	snps	pval	or	or_lci95	or_uci95	Direction
DAPLYSINESYN-PWY: L-lysine biosynthesis I	9	0.027	1.161	1.017	1.325	Positive
FAO-PWY: fatty acid beta oxidation I	7	0.004	0.842	0.750	0.945	Negative
PWY-4984: urea cycle	8	0.021	0.835	0.717	0.973	Negative
PWY-5005: biotin biosynthesis II	13	0.0179	1.118	1.019	1.226	Positive
PWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation	11	0.048	0.911	0.831	0.999	Negative

PWY-6147: 6-hydroxymethyl dihydropterin diphosphate biosynthesis I	14	0.016	1.149	1.026	1.286	Positive
PWY-6263: superpathway of menaquinol-8 biosynthesis II	5	0.020	1.116	1.017	1.225	Positive
PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation	6	0.015	0.824	0.705	0.963	Negative
Gammaproteobacteria	4	0.038	1.212	1.011	1.453	Positive
Oscillospiraceae	6	0.036	0.837	0.708	0.989	Negative
Pseudoflavonifractor	8	0.006	1.142	1.039	1.256	Positive
Lachnospiraceae noname	5	0.020	1.230	1.033	1.464	Positive
Roseburia	14	0.009	0.857	0.763	0.962	Negative
Haemophilus	5	0.032	0.898	0.813	0.991	Negative

Rothia mucilaginosa	4	0.039	0.904	0.821	0.995	Negative
Parabacteroides merdae	4	0.013	1.244	1.048	1.478	Positive
Pseudoflavonifractor capillosus	10	0.008	1.125	1.032	1.227	Positive
Oscillibacter unclassified	6	0.037	0.837	0.708	0.989	Negative
Bacteroides clarus	9	0.017	1.077	1.013	1.144	Positive

Table3. MR result of the association of gut microbiota with HER2− breast cancer

Exposure	snps	pval	or	or_lci95	or_uci95	Direction
METHGLYUT-PWY: superpathway of methylglyoxal degradation	8	0.027	1.125	1.013	1.249	Positive
POLYISOPRENSYN-PWY: polyisoprenoid biosynthesis *Escherichia coli*	8	0.027	0.887	0.797	0.987	Negative
PWY0-1298: superpathway of pyrimidine	14	0.002	1.252	1.083	1.448	Positive

deoxyribonucleosides degradation						
PWY0-781: aspartate superpathway	11	0.038	1.197	1.010	1.418	Positive
PWY-5005: biotin biosynthesis II	11	0.005	0.848	0.756	0.952	Negative
PWY-6628: superpathway of L-phenylalanine biosynthesis	12	0.021	0.888	0.803	0.982	Negative
PWY-6700: queuosine biosynthesis	15	0.039	0.870	0.762	0.993	Negative
PWY-6892: thiazole biosynthesis I *Escherichia coli*	6	0.018	1.213	1.034	1.422	Positive
PWY-GLYCOLYSIS: glycolysis I from glucose-6-phosphate	15	0.030	0.870	0.767	0.987	Negative
PWY-HEME-BIOSYNTHESIS-II: heme biosynthesis I aerobic	8	0.030	0.829	0.700	0.982	Negative

TRNA-CHARGING-PWY: tRNA charging	12	0.009	1.205	1.048	1.386	Positive
Veillonellaceae	10	0.047	1.141	1.002	1.300	Positive
Butyrivibrio	10	0.021	0.925	0.865	0.988	Negative
Streptococcus thermophilus	4	0.030	0.871	0.768	0.987	Negative
Ruminococcus torques	7	0.015	1.185	1.034	1.358	Positive
Roseburia intestinalis	9	0.034	1.163	1.011	1.337	Positive

Positive: risk-increasing (OR › 1); Negative: risk-decreasing (OR ‹ 1).

Table 4. Heterogeneity, pleiotropy, and sensitivity of total breast cancer

Exposure	Outcome	Heterogeneity		Pleiotropy		
		MR Egger Q (*P* value)	IVW Q (*P*-value)	PRESSO RSSobes (*P* value)	Egger_intercept	Value of *P*
DAPLYSINESYN-PWY: L-lysine biosynthesis I	Total breast cancer	0.733	0.749	0.771	−0.013	0.435
FAO-PWY: fatty acid beta oxidation I	Total breast cancer	0.433	0.550	0.612	−0.006	0.781

METHGLYUT-PWY: superpathway of methylglyoxal degradation	Total breast cancer	0.368	0.407	0.441	−0.019	0.455
PRPP-PWY: superpathway of histidine purine and pyrimidine biosynthesis	Total breast cancer	0.979	0.938	0.944	0.026	0.199
PWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation	Total breast cancer	0.923	0.947	0.948	−0.006	0.743
PWY-4984: urea cycle	Total breast cancer	0.138	0.203	0.207	0.011	0.853
PWY-5005: biotin biosynthesis II	Total breast cancer	0.738	0.727	0.737	−0.023	0.352

PWY-6263: superpathway of menaquinol-8 biosynthesis II	Total breast cancer	0.810	0.903	0.904	0.009	0.801
PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation	Total breast cancer	0.782	0.709	0.730	0.034	0.336
PWY-7446: sulfoglycolysis	Total breast cancer	0.555	0.642	0.649	−0.008	0.738
Pseudoflavonifractor	Total breast cancer	0.705	0.793	0.800	−0.007	0.774
Lachnospiraceae noname	Total breast cancer	0.336	0.495	0.557	0.003	0.940
Roseburia	Total breast cancer	0.280	0.324	0.362	−0.011	0.568
Parabacteroides merdae	Total breast cancer	0.417	0.364	0.489	−0.066	0.354

Bacteroides intestinalis	Total breast cancer	0.204	0.410	NA	−0.019	0.801

Table 5. Heterogeneity, pleiotropy, and sensitivity of HER2+ breast cancer

Exposure	Outcome	Heterogeneity		Pleiotropy		
		MR Egger Q (*P* value)	IVW Q (*P* value)	PRESSO RSSobes (*P* value)	Egger_intercept	Value of *P*
DAPLYSINESYN-PWY: L-lysine biosynthesis I	HER2+ breast cancer	0.352	0.442	0.454	−0.007	0.735
FAO-PWY: fatty acid beta oxidation I	HER2+ breast cancer	0.784	0.864	0.870	0.008	0.778
PWY-4984: urea cycle	HER2+ breast cancer	0.225	0.315	0.359	0.009	0.901
PWY-5005: biotin biosynthesis II	HER2+ breast cancer	0.363	0.453	0.479	−0.004	0.910
PWY0-1298: superpathway	HER2+ breast cancer	0.217	0.274	0.316	0.007	0.786

of pyrimidine deoxyribonucleosides degradation						
PWY-6147: 6-hydroxymethyl dihydropterin diphosphate biosynthesis I	HER2+ breast cancer	0.804	0.827	0.842	−0.028	0.527
PWY-6263: superpathway of menaquinol-8 biosynthesis II	HER2+ breast cancer	0.605	0.589	0.603	0.038	0.372
PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation	HER2+ breast cancer	0.402	0.547	0.582	0.031	0.639
Gammaproteobacteria	HER2+ breast cancer	0.468	0.612	0.616	0.005	0.921

Oscillospiraceae	HER2+ breast cancer	0.679	0.781	0.817	0.002	0.951
Pseudoflavonifractor	HER2+ breast cancer	0.485	0.469	0.534	0.052	0.369
Lachnospiraceae noname	HER2+ breast cancer	0.104	0.129	0.162	−0.013	0.609
Roseburia	HER2+ breast cancer	0.583	0.707	0.683	−0.001	0.979
Haemophilus	HER2+ breast cancer	0.323	0.406	0.498	−0.055	0.553
Rothia mucilaginosa	HER2+ breast cancer	0.670	0.420	0.521	0.066	0.291
Parabacteroides merdae	HER2+ breast cancer	0.875	0.954	0.965	−0.017	0.825
Pseudoflavonifractor capillosus	HER2+ breast cancer	0.834	0.781	0.827	0.028	0.283
Oscillibacter unclassified	HER2+ breast cancer	0.463	0.608	0.615	0.003	0.949
Bacteroides clarus	HER2+ breast cancer	0.424	0.417	0.456	0.030	0.326

Table 6. Heterogeneity, pleiotropy, and sensitivity of HER2− breast cancer

Exposure	Outcome	Heterogeneity		Pleiotropy		
		MR Egger Q (*P* value)	IVW Q (*P* value)	PRESSO RSSobes (*P* value)	Egger_intercept	Value of *P*
METHGLYUT-PWY: superpathway of methylglyoxal degradation	HER2− breast cancer	0.104	0.154	0.188	0.013	0.799
POLYISOPRENSYN-PWY: polyisoprenoid biosynthesis E coli.	HER2− breast cancer	0.379	0.445	0.467	0.025	0.547
PWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation	HER2− breast cancer	0.337	0.414	0.436	−0.001	0.984

PWY0-781: aspartate superpathway	HER2− breast cancer	0.132	0.185	0.204	0.002	0.952
PWY-5005: biotin biosynthesis II	HER2− breast cancer	0.778	0.472	0.506	−0.073	0.076
PWY-6628: superpathway of L-phenylalanine biosynthesis	HER2− breast cancer	0.572	0.615	0.626	0.025	0.498
PWY-6700: queuosine biosynthesis	HER2− breast cancer	0.815	0.797	0.810	−0.032	0.318
PWY-6892: thiazole biosynthesis I E coli.	HER2− breast cancer	0.521	0.525	0.562	−0.042	0.386
PWY-GLYCOLYSIS: glycolysis I from glucose-6-phosphate	HER2− breast cancer	0.967	0.924	0.924	0.043	0.190

PWY-HEME-BIOSYNTHESIS-II: heme biosynthesis I aerobic	HER2− breast cancer	0.401	0.469	0.482	0.026	0.542
TRNA-CHARGING-PWY: tRNA charging	HER2− breast cancer	0.791	0.838	0.884	−0.019	0.657
Veillonellaceae	HER2− breast cancer	0.764	0.837	0.850	0.007	0.881
Butyrivibrio	HER2− breast cancer	0.504	0.563	0.576	−0.018	0.543
Streptococcus thermophilus	HER2− breast cancer	0.613	0.793	0.837	0.016	0.837
Ruminococcus torques	HER2− breast cancer	0.855	0.923	0.939	0.000	0.997
Roseburia intestinalis	HER2− breast cancer	0.369	0.411	0.456	−0.024	0.473

Table 7. BWMR analysis of total breast cancer

Exposure	Method	or	or_lci95	or_uci95	pval

DAPLYSINESYN-PWY: L-lysine biosynthesis I	BWMR	1.136	1.032	1.250	0.009
FAO-PWY: fatty acid beta oxidation I	BWMR	0.891	0.805	0.986	0.025
METHGLYUT-PWY: superpathway of methylglyoxal degradation	BWMR	1.061	1.001	1.124	0.046
PRPP-PWY: superpathway of histidine purine and pyrimidine biosynthesis	BWMR	1.123	1.019	1.237	0.019
PWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation	BWMR	1.154	1.047	1.272	0.004
PWY-4984: urea cycle	BWMR	0.861	0.755	0.982	0.025
PWY-5005: biotin biosynthesis II	BWMR	0.878	0.811	0.951	0.001
PWY-6263: superpathway of menaquinol-8 biosynthesis II	BWMR	1.112	1.026	1.205	0.010
PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation	BWMR	0.862	0.753	0.987	0.032

PWY-7446: sulfoglycolysis	BWMR	1.051	1.003	1.101	0.038
Pseudoflavonifractor	BWMR	1.089	1.004	1.182	0.041
Lachnospiraceae noname	BWMR	1.221	1.047	1.423	0.011
Roseburia	BWMR	0.884	0.805	0.971	0.010
Parabacteroides merdae	BWMR	1.191	1.039	1.365	0.012
Bacteroides intestinalis	BWMR	0.908	0.841	0.981	0.014

Positive: risk-increasing (OR ﹥ 1) ; Negative: risk-decreasing (OR ﹤ 1).

Table 8. BWMR analysis of HER2+ breast cancer

Exposure	Method	or	or_lci95	or_uci95	pval
DAPLYSINESYN-PWY: L-lysine biosynthesis I	BWMR	1.163	1.025	1.319	0.019
FAO-PWY: fatty acid beta oxidation I	BWMR	0.839	0.741	0.949	0.005
PWY-4984: urea cycle	BWMR	0.852	0.729	0.996	0.044
PWY-5005: biotin biosynthesis II	BWMR	0.907	0.822	1.000	0.049
PWY0-1298: superpathway of pyrimidine	BWMR	1.149	1.020	1.294	0.022

deoxyribonucleosides degradation					
PWY-6147: 6-hydroxymethyl dihydropterin diphosphate biosynthesis I	BWMR	1.118	1.012	1.235	0.028
PWY-6263: superpathway of menaquinol-8 biosynthesis II	BWMR	0.818	0.691	0.969	0.020
PWY-6590: superpathway of *Clostridium acetobutylicum* acidogenic fermentation	BWMR	1.222	1.006	1.484	0.044
Gammaproteobacteria	BWMR	0.831	0.697	0.992	0.040
Oscillospiraceae	BWMR	1.147	1.036	1.271	0.008
Pseudoflavonifractor	BWMR	1.241	1.029	1.495	0.024
Lachnospiraceae noname	BWMR	0.853	0.753	0.967	0.013
Roseburia	BWMR	0.841	0.710	0.995	0.044
Haemophilus	BWMR	0.894	0.805	0.993	0.037
Rothia mucilaginosa	BWMR	0.901	0.812	0.999	0.049
Parabacteroides merdae	BWMR	1.246	1.055	1.471	0.010

Pseudoflavonifractor capillosus	BWMR	1.131	1.032	1.240	0.009
Oscillibacter unclassified	BWMR	0.832	0.697	0.993	0.041
Bacteroides clarus	BWMR	1.082	1.014	1.153	0.017

Table 9. BWMR analysis of HER2− breast cancer

Exposure	Method	or	or_lci95	or_uci95	pval
METHGLYUT-PWY: superpathway of methylglyoxal degradation	BWMR	1.126	1.013	1.251	0.027
POLYISOPRENSYN-PWY: polyisoprenoid biosynthesis *Escherichia coli*	BWMR	0.883	0.789	0.989	0.031
PWY0-1298: superpathway of pyrimidine deoxyribonucleosides degradation	BWMR	1.265	1.069	1.497	0.006
PWY0-781: aspartate superpathway	BWMR	1.206	1.010	1.440	0.038
PWY-5005: biotin biosynthesis II	BWMR	0.841	0.743	0.951	0.006
PWY-6628: superpathway of L-phenylalanine biosynthesis	BWMR	0.884	0.795	0.983	0.023
PWY-6700: queuosine biosynthesis	BWMR	0.864	0.752	0.992	0.039

PWY-6892: thiazole biosynthesis I *E. coli*	BWMR	1.221	1.027	1.452	0.024
PWY-GLYCOLYSIS: glycolysis I from glucose 6 phosphate	BWMR	0.869	0.761	0.991	0.036
PWY-HEME-BIOSYNTHESIS-II: heme biosynthesis I aerobic	BWMR	0.823	0.679	0.998	0.047
TRNA-CHARGING-PWY: tRNA charging	BWMR	1.183	1.028	1.362	0.019
Veillonellaceae	BWMR	1.140	1.000	1.298	0.049
Butyrivibrio	BWMR	0.921	0.859	0.989	0.023
Streptococcus thermophilus	BWMR	0.868	0.759	0.993	0.038
Ruminococcus torques	BWMR	1.194	1.043	1.368	0.010
Roseburia intestinalis	BWMR	1.177	1.003	1.381	0.045

Positive: risk-increasing (OR › 1); Negative: risk-decreasing (OR ‹ 1).

Table 10. Mediation analysis

Exposure	Mediation	Total effect (Beta)	A (Beta)	B (Beta)	Indirect effect (Beta)	Indirect effect/Total effect
Total breast cancer (outcome)						

PWY-6263: superpathway of menaquinol-8 biosynthesis II	DP (CD4+CD8+) %leukocyte	0.104	0.259	0.505	0.013	0.126
Lachnospiraceae_noname	IgD− CD27− %B cell	0.192	−0.274	0.052	−0.014	NA
HER2+ breast cancer (outcome)						
PWY-6263: superpathway of menaquinol-8 biosynthesis II	DP (CD4+CD8+) %leukocyte	0.110	0.259	0.076	0.020	0.178
PWY-6263: superpathway of menaquinol-8 biosynthesis II	CD25 on activated Treg	0.110	0.214	−0.061	−0.013	NA
Pseudoflavonifractor capillosus	CD25hi CD45RA+ CD4 not Treg %T cell	0.118	−0.125	0.021	−0.003	NA
HER2− breast cancer (outcome)						
PWY0-1298: superpathway of pyrimidine	BAFF-R on CD20−	0.225	0.199	−0.076	−0.015	NA

deoxyribonucleosides degradation						

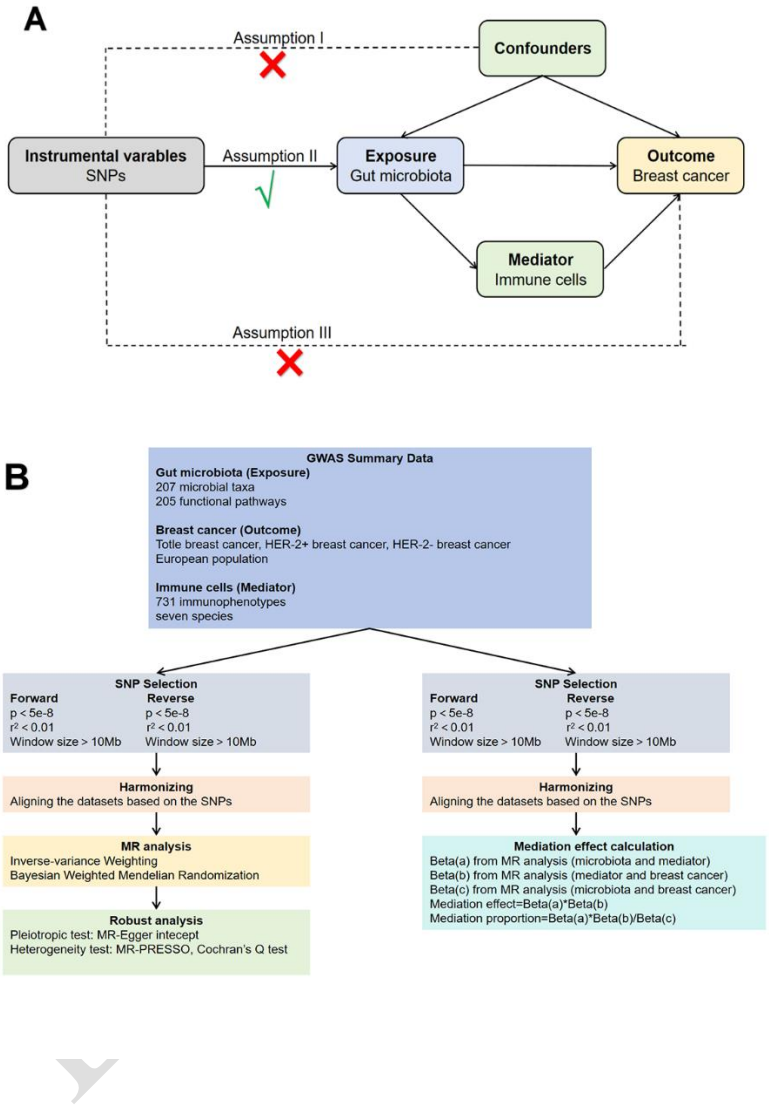


Figure 1. Forest plot of the positive bacterial flora of breast cancer and its subtypes

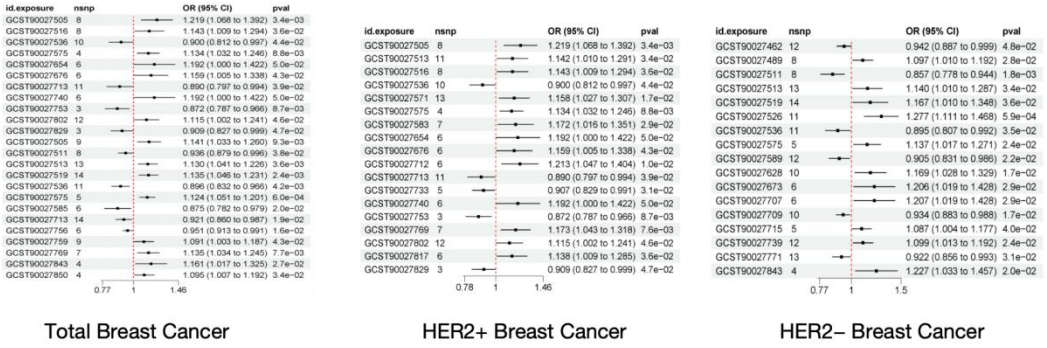


Figure 2. The forest plot of the positive bacterial flora of breast cancer and its subtypes

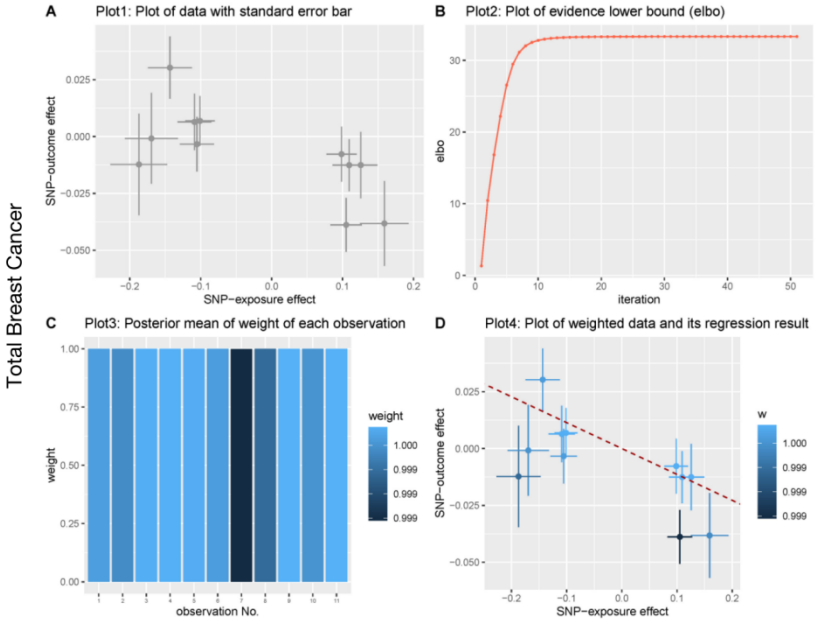


Figure 3. BWMR analysis of biotin biosynthesis II and breast cancer

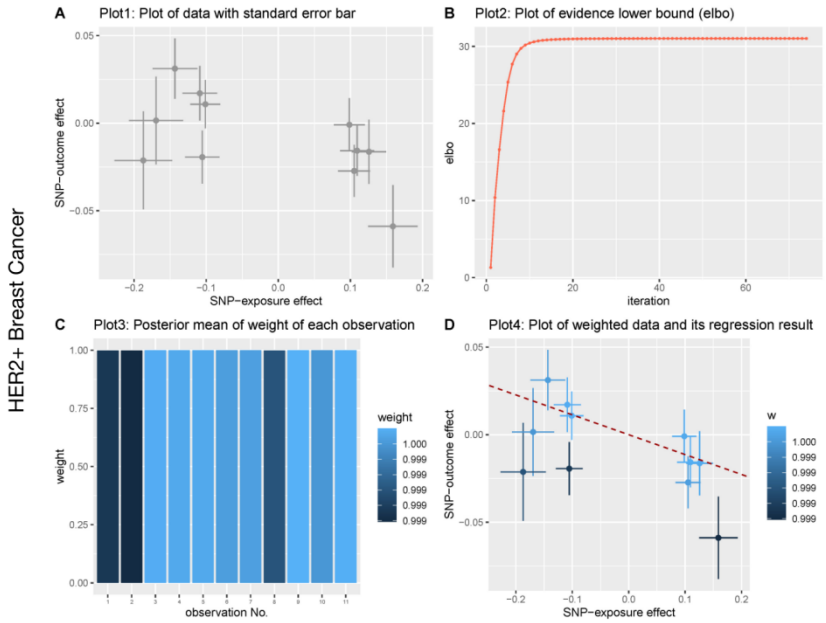


Figure 4. BWMR analysis of biotin biosynthesis II and HER2+ breast cancer

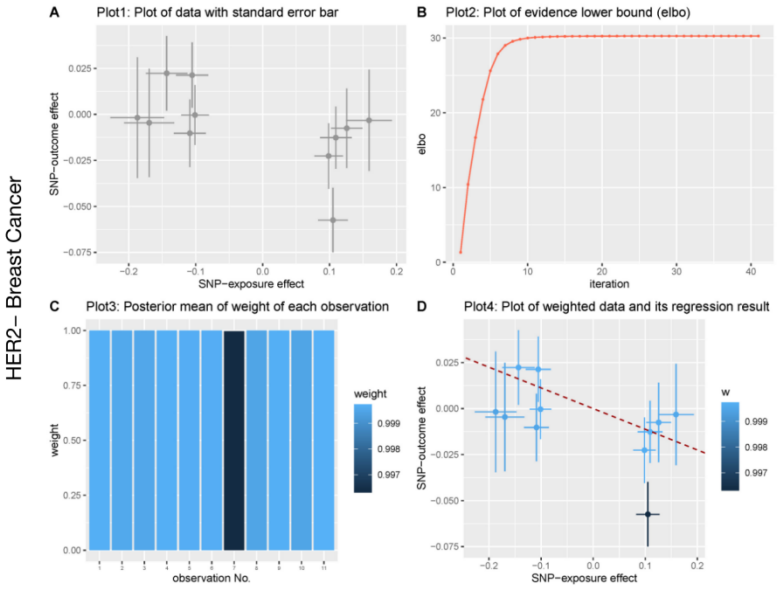
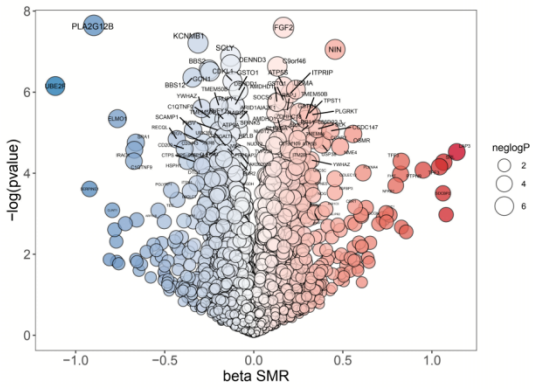


Figure 5. BWMR analysis of biotin biosynthesis II and HER2- breast cancer



Gene	tissue	topSNP	OR (95% CI)	p_SMR
RPA2	Breast_Mammary_Tissue	rs28904914	1.137 (1.015 to 1.259)	3.94e−02
MSH2	Breast_Mammary_Tissue	rs11883630	0.889 (0.785 to 0.993)	2.61e−02
ALMS1P	Breast_Mammary_Tissue	rs17349321	0.834 (0.687 to 0.981)	1.54e−02
ESPNL	Breast_Mammary_Tissue	rs60512353	1.080 (1.020 to 1.140)	1.22e−02
RP11−141M3.5	Breast_Mammary_Tissue	rs12494055	0.847 (0.695 to 1.000)	3.30e−02
LINC00960	Breast_Mammary_Tissue	rs7610215	1.062 (1.008 to 1.115)	2.80e−02
KRT18P34	Breast_Mammary_Tissue	rs9831830	0.889 (0.778 to 1.001)	3.95e−02
VPS8	Breast_Mammary_Tissue	rs73067325	0.840 (0.684 to 0.996)	2.81e−02
RP11−448G15.3	Breast_Mammary_Tissue	rs17385872	0.828 (0.652 to 1.003)	3.47e−02
CTD−2227C6.2	Breast_Mammary_Tissue	rs2974445	0.897 (0.814 to 0.981)	1.07e−02
FAM170A	Breast_Mammary_Tissue	rs2636966	1.147 (1.040 to 1.254)	1.18e−02
STK19P	Breast_Mammary_Tissue	rs2844463	0.888 (0.780 to 0.996)	3.17e−02
SNHG5	Breast_Mammary_Tissue	rs2758845	0.953 (0.905 to 1.000)	4.34e−02
RP11−43A14.1	Breast_Mammary_Tissue	rs35412259	1.087 (1.011 to 1.163)	3.19e−02
RP11−578O24.2	Breast_Mammary_Tissue	rs1441850	1.067 (1.013 to 1.121)	1.93e−02
NUDT2	Breast_Mammary_Tissue	rs10971957	1.087 (1.025 to 1.148)	8.39e−03
ATG13	Breast_Mammary_Tissue	rs61884324	1.292 (1.060 to 1.524)	3.02e−02
TAS2R64P	Breast_Mammary_Tissue	rs35622190	0.913 (0.826 to 0.999)	3.80e−02
PYROXD1	Breast_Mammary_Tissue	rs3825289	1.085 (1.011 to 1.159)	3.01e−02
C1QTNF9	Breast_Mammary_Tissue	rs1856987	0.784 (0.615 to 0.953)	4.70e−03
DCAF4	Breast_Mammary_Tissue	rs11158997	1.132 (1.027 to 1.236)	2.03e−02
SCAMP5	Breast_Mammary_Tissue	rs9672995	1.229 (1.070 to 1.389)	1.13e−02
GOLGA6L4	Breast_Mammary_Tissue	rs62021171	1.075 (1.014 to 1.136)	1.97e−02
SCAND2P	Breast_Mammary_Tissue	rs62021171	1.173 (1.036 to 1.311)	2.30e−02
DECR2	Breast_Mammary_Tissue	rs2363758	1.223 (1.063 to 1.383)	1.37e−02
CDK10	Breast_Mammary_Tissue	rs12924138	1.094 (1.012 to 1.176)	3.17e−02
SPATA2L	Breast_Mammary_Tissue	rs13330285	0.819 (0.634 to 1.005)	3.50e−02
VPS9D1	Breast_Mammary_Tissue	rs57119673	0.787 (0.582 to 0.991)	2.17e−02
FANCA	Breast_Mammary_Tissue	rs68092778	0.859 (0.721 to 0.997)	3.14e−02
SHPK	Breast_Mammary_Tissue	rs11652474	1.177 (1.022 to 1.332)	3.97e−02

Figure 6. Genes associated with biotin biosynthesis II in breast cancer