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Active Molecular Network Discovery Links Lifestyle Variables to Breast Cancer in the Long Island Breast Cancer Study Project

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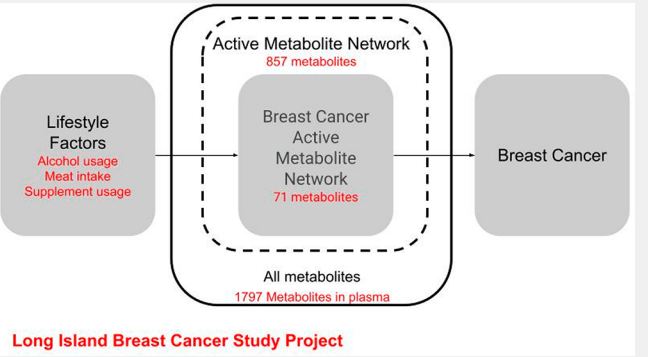
ABSTRACT: A healthy lifestyle has been associated with decreased risk of developing breast cancer. Using untargeted metabolomics profiling, which provides unbiased information regarding lifestyle choices such as diet and exercise, we aim to identify the molecular mechanisms connecting lifestyle and breast cancer through network analysis. A total of 100 postmenopausal women, 50 with breast cancer and 50 cancer-free controls, were selected from the Long Island Breast Cancer Study Project (LIBCSP). We measured untargeted plasma metabolomics using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). Using the "enet" package, we retained highly correlated metabolites representing active molecular network (AMN) clusters for analysis. LASSO was used to examine associations between cancer status and AMN metabolites and covariates such as BMI, age, and reproductive factors. LASSO was then repeated to examine associations between AMN metabolites and 10 lifestyle-related variables including smoking, physical activity, alcohol consumption, meat consumption, fruit and vegetable consumption, and supplemental vitamin use. Results were displayed as a network to uncover biological pathways linking lifestyle factors to breast cancer status. After filtering, 851 "active" metabolites out of 1797 metabolomics were retained in 197 correlation AMN clusters. Using LASSO, breast cancer status was associated with 71 "active" metabolites. Several of these metabolites were associated with lifestyle variables including meat consumption, alcohol consumption, and supplemental β-carotene, B12, and folate use. Those metabolites could potentially serve as molecular-level biological intermediaries connecting healthy lifestyle factors to breast cancer, even though direct associations between breast cancer and the investigated lifestyles at the phenotype level are not evident. In particular, DiHODE, a metabolite linked with inflammation, was associated with breast cancer status and connected to β-carotene supplement usage through an AMN. We found several plasma metabolites associated with lifestyle factors and breast cancer status. Future studies investigating the mechanistic role of inflammation in linking supplement usage to breast cancer status are warranted.



KEYWORDS: *Metabolomics, exposome, machine learning, network analysis, public health*

INTRODUCTION

Lifestyle factors can influence breast cancer risk.[1] We have previously investigated the role of a healthy lifestyle on the development of breast cancer by creating a healthy lifestyle index (HLI) using information on body mass index, physical activity, intake of plant and animal foods, alcohol consumption, breastfeeding, and smoking.[2] This analysis and other derived healthy lifestyle indices,[3] demonstrated that a healthier lifestyle was associated with decreased risk of developing breast cancer.[2] However, the biological response to lifestyle factor exposures associated with breast cancer remains unknown.

Untargeted metabolomics can be used to describe the overall molecular level changes that reflect the confluence of genetic disposition, environment, diet, and health conditions to capture an individual's susceptibility to breast cancer. Indeed, metabolomics studies have been used to identify biomarkers of nutrition, diet, and lifestyle habits,[4] some of which were directly associated with breast cancer risk.[5,6] In addition, metabolomics identified altered endogenous metabolite levels and biological pathways in breast cancer patients compared to controls.[7] However, these approaches have not yet linked the endogenous metabolites and pathways that moderate lifestyle exposures and breast cancer. In particular, exposure to dietary and lifestyle components is bidirectional where changes in physiology as a result of exposure can impact how dietary substances are metabolized.[8] This results in complex relation-

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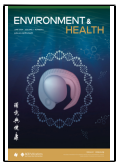

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Environ. Health 2024, 2, 401−410

ships that are difficult to uncover through traditional univariate analyses.

Previously, we developed a metabolomics data analysis workflow to identify the metabolite profiles that are associated with exposure.[9] This workflow is based on the hypothesis that some key metabolites moderate the influence of exposure to health outcomes. Those metabolites called "gatekeepers" act as sentinel nodes that link biological pathways (i.e, correlated metabolites) to exposures or health effects (Figure 1). The

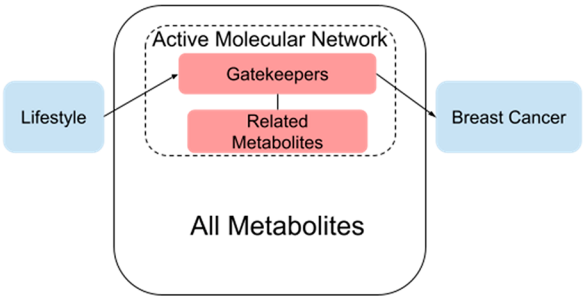

Figure 1. Active molecular network analysis to mediate the influences from lifestyle and breast cancer with the help of machine learning. Gatekeepers are key metabolites that link single- or multiple-exposure biomarkers or health outcomes with correlated clusters of related endogenous metabolites.

relationships among those gatekeepers and correlated metabolites can be used to construct a network based on statistical models and/or known biochemical reaction information.[10] Such metabolite networks [Active Molecular Networks (AMN)] cover only part of the metabolome but provide crucial information linking external stimulus and health conditions. Unlike a meet-in-the-middle approach which assigns direct associations between exposures and metabolites[11] and then metabolites and health outcomes,[12] the AMN includes more distant chemical relationships and pathways which can capture the synergistic, combined, and interactive effects of lifestyle factors on the metabolome.[13]

Here, we performed untargeted analysis using liquid chromatography-high-resolution mass spectrometry (LC-HRMS) on plasma samples from 100 postmenopausal women who participated in the Long Island Breast Cancer Study Project (LIBCSP). We then applied AMN discovery to generate hypotheses on biological mechanisms linking lifestyle factors to breast cancer with the help of machine learning.

METHODS

Population

We utilized plasma samples archived from the LIBCSP, a population-based case-control study of women residing in Nassau and Suffolk Counties on Long Island, New York, with newly diagnosed first primary in situ or invasive breast cancer recruited between 1996 and 1997. The parent study included 1508 women diagnosed with breast cancer and 1556 women without breast cancer from the same two counties, frequency matched by a 5 year age group as described in previous studies.[14] All participating institutions obtained Institutional Review Board approval, and written informed consent was obtained prior to study participation. Information on demographic characteristics, pregnancy history, hormone usage, family history of cancer, current alcohol use, cigarette smoking, and physical activity were obtained from the main study in-person-administered questionnaire completed at enrollment. Variables with missing values of more than 10% were removed from further discussion.

Additional dietary lifestyle factors were captured from the food frequency questionnaire (FFQ) for the LIBCSP, describing intake, usual frequency, and portion sizes of ~100 foods and beverages in the 12 months before diagnosis or prior to enrollment among controls.[15] Lifestyle factors selected for this analysis included alcohol use,[16] tobacco smoking,[17] meat consumption,[18] vegetable and fruit consumption,[19] physical activity,[20] and use of supplements[21] with reported association with breast cancer. Variables with missing values of more than 10% were removed. At the time of the interview, women provided a nonfasting 40 mL blood sample for laboratory analyses. The current analysis includes 100 postmenopausal women who had never used menopausal hormone therapy.

Untargeted Analysis

Plasma samples stored at −80 °C were thawed on ice. After light vortexing, 50 μL plasma aliquots were combined with 150 μL of ice-cold methanol containing internal standards. Following incubation at −80 °C for 30 min to precipitate proteins, the samples were centrifuged and the supernatant was aliquoted and evaporated to dryness using a Savant SC250EXP SpeedVac concentrator. A pooled quality control sample ("pooled QC") was generated by combining an additional 10 μL plasma aliquot from each sample. Following the same protocol, the matrix blank (replacing the plasma with water) and

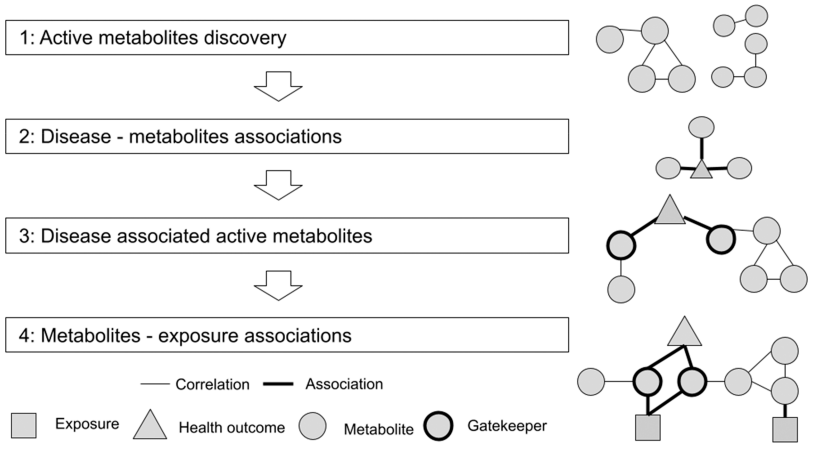

Figure 2. Active metabolite network (AMN) workflow to build the network between metabolites, exposures, and health outcomes. Step 1: Select active metabolite clusters through correlation analysis. Step 2: Determine associations between health outcome (triangle) and those metabolites in the correlated clusters (gatekeeper discovery) by machine learning. Step 3: Generate the health outcome−AMN network. Step 4: Add exposure associations to the health outcome−AMN by machine learning.

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Environ. Health 2024, 2, 401−410

Table 1. Metabolites in the AMN Associated with Breast Cancer and Lifestyle Factors[a]

annotation[b]	m/z	R_t (s)	potential formula	mode	lifestyle factors: continuous daily alcohol use (β)	daily meat intake (β)	supplemental B12 (β)	supplemental beta-carotene (β)	supplemental folate suppl. (β)
DiHODE	311.2223	412.3	$C_{18}H_{32}O_4$	negative		−1.5796	−0.1168	−3446.47	−122.1825
unknown	274.1836	251.9	$C_{14}H_{27}NO_2S$	positive		−4.2477		−3538.60	98.3056
unknown	325.0957	205.2	$C_{16}H_{14}N_4O_4$	negative	−10.7111	−0.7763			
unknown	460.1695	405.9	$C_{26}H_{27}N_3O_3S$	negative	−5.7003	−2.2841		−328.3488	−33.0294
unknown	514.2182	284.5	$C_{35}H_{31}NOS$	positive		−0.3512		−205.1514	−16.989
unknown	578.3014	469.2	$C_{31}H_{41}N_5O_6$	negative				−3431.26	36.8464
unknown	598.2785	61.5	$C_{25}H_{39}N_7O_{10}$	positive	3.7936	3.3787		1031.04	−12.9315
unknown	614.4827	68	$C_{27}H_{51}N_9O_7$	positive	−2.2161	22.434		4363.21	−86.7204
unknown	627.6958	549.6	$C_{44}H_{84}N$	positive	125.3808			−694.679	231.0033
unknown	724.5274	715.7	$C_{41}H_{75}NO_9$	negative	−11.6159	23.04		9358.87	−95.7523
unknown	968.7055	333.7	$C_{48}H_{85}N_7O_{13}$	positive	5.1246			−43.3207	10.3826
unknown	986.6609	141.7	$C_{60}H_{92}NO_8P$	positive		2.1691		254.8953	58.968
unknown	1076.5747	237	$C_{49}H_{85}N_{15}O_{12}$	positive	1.6495	3.1187		−1036.7028	−31.7337

[a]Associations were determined by LASSO. For categorical lifestyle variables, metabolites were retained when training accuracy was larger than 50% for predicting the lifestyle variable. For associations with continuous lifestyle variables, metabolites were retained when the best model showed nonzero coefficients with metabolites. $N = 100$. [b]All MS2 annotation results for this study are provided as GNPS links in the Supporting Information.

multiple pooled QC samples were extracted and dried. Samples, matrix blanks, and pooled QCs were stored at −80 °C until analysis. Before LC-HRMS analysis, dried extracts were reconstituted either in 100% methanol or in acetonitrile:water (8:2, v/v). Samples were analyzed using reverse-phase (RP) and hydrophilic interaction liquid chromatography (HILIC) connected to HRMS in negative and positive mode, respectively, as described elsewhere.[22] Samples were analyzed in a randomized order with pooled QCs injected routinely throughout the run.

Data Analysis

Raw LC-HRMS data were converted to open-source format and processed by R 4.2.1.[23] Features were extracted by "xcms"[24] using optimized parameters determined by the "IPO" package[25] as in the previous study.[9] Features with RSD larger than 30% in the pooled QC samples were filtered, and features with average intensity in the pooled QC samples lower than 3-fold change were compared with blank samples. Redundant features such as adducts, neutral losses, isotopologues, or common fragment ions were removed by the GlobalStd algorithm.[26] Remaining features were treated as potential metabolite features and used as precursor ion targets to collect MS/MS spectra by repeated injections.[27] The features collected from RP and HILIC modes were merged, removing those features with both a mass difference of 2.02 between positive and negative mode data and correlation coefficients larger than 0.9, as they were expected to be the same chemicals.[27] Annotation of metabolites was performed by matching to library standards analyzed under the same analytical conditions and MS/MS annotation by GNPS,[28] metlin,[29] and MS-DIAL.[30]

AMN analysis was performed by the "enet" package.[9] Analysis steps are depicted in Figure 2. First, correlation network analysis was performed among the potential metabolite features of the merged LC-HRMS data set. The correlation cutoff was determined empirically to maximize the number of correlation clusters (Figure 2, step 1). The gatekeeper workflow is modular because metabolomics is at the interface where exposure meets biology.[31] Here, instead of identifying AMN to exposures in step 2,[9] we identified the AMN to breast cancer. Association between cancer diagnosis and all active metabolite features and covariates (see Table S1) was determined using machine learning. Here, we used the Least Absolute Shrinkage and Selection Operator (LASSO) generalized linear model (Figure 2, step 2). The model was fit to feature abundances over 100 bootstrapped data sets to tune the penalized parameters (lambda), and accuracy was used to select the optimal models. The binary case-control status was used as the outcome variable with the following independent variables: logged intensities for all 1797 metabolite features and 15 covariables listed in Table 1. Next, a network connecting the selected metabolites and the metabolite clusters was generated (Figure 2, step 3). Then, for each metabolite remaining in the network, associations among the 10 lifestyle factors listed in Table S2 were individually determined using LASSO over 100 bootstrapped data sets to tune the penalized parameters, and root mean square error (RMSE) was used to select the optimal model. The lifestyle variable was used as the outcome variable with the remaining logged intensities of the metabolite features as the independent variables. For associations with categorical lifestyle variables, metabolites were retained when training accuracy was larger than 50% for predicting the lifestyle variable. For associations with continuous lifestyle variables, metabolites were retained when the best model showed nonzero coefficients with metabolites. A final network was built from the selected lifestyles and their predictive metabolite clusters (Figure 2, step 4). To help elucidate the biochemical relationships between two correlated metabolites, we used the package "pmd" to obtain reaction-level information with paired mass differences. To test any direct associations between lifestyle factors, we performed LASSO where the binary case-control status was used as the outcome variable with the lifestyle factors as independent variables.

RESULTS AND DISCUSSION

Summary Statistics for the Participants

Summary statistics for participants included in this analysis and potential confounding variables are presented in Table S1, and lifestyle factors are given in Table S2. Differences between case and control groups were tested for each covariate using the "t.test" or "chisq.test" functions in R, specifying a two-sided alternative. There was evidence for a difference in age at menarche, which was earlier for cases than controls (nominal p value = 0.066). There was also evidence for differences in physical activity (nominal p value = 0.013) and fruits and vegetables intake (nominal p value 0.048) between the cases and the controls. There was no evidence of a statistically significant difference for any of the other covariates or lifestyle characteristics.

Active Metabolites Selection by Correlation Network Analysis

Peak picking resulted in 6615 (HILIC) and 5171 (RP) features measured in the samples. After the removal of redundant peaks, 913 (HILIC) and 890 (RP) peaks were retained as

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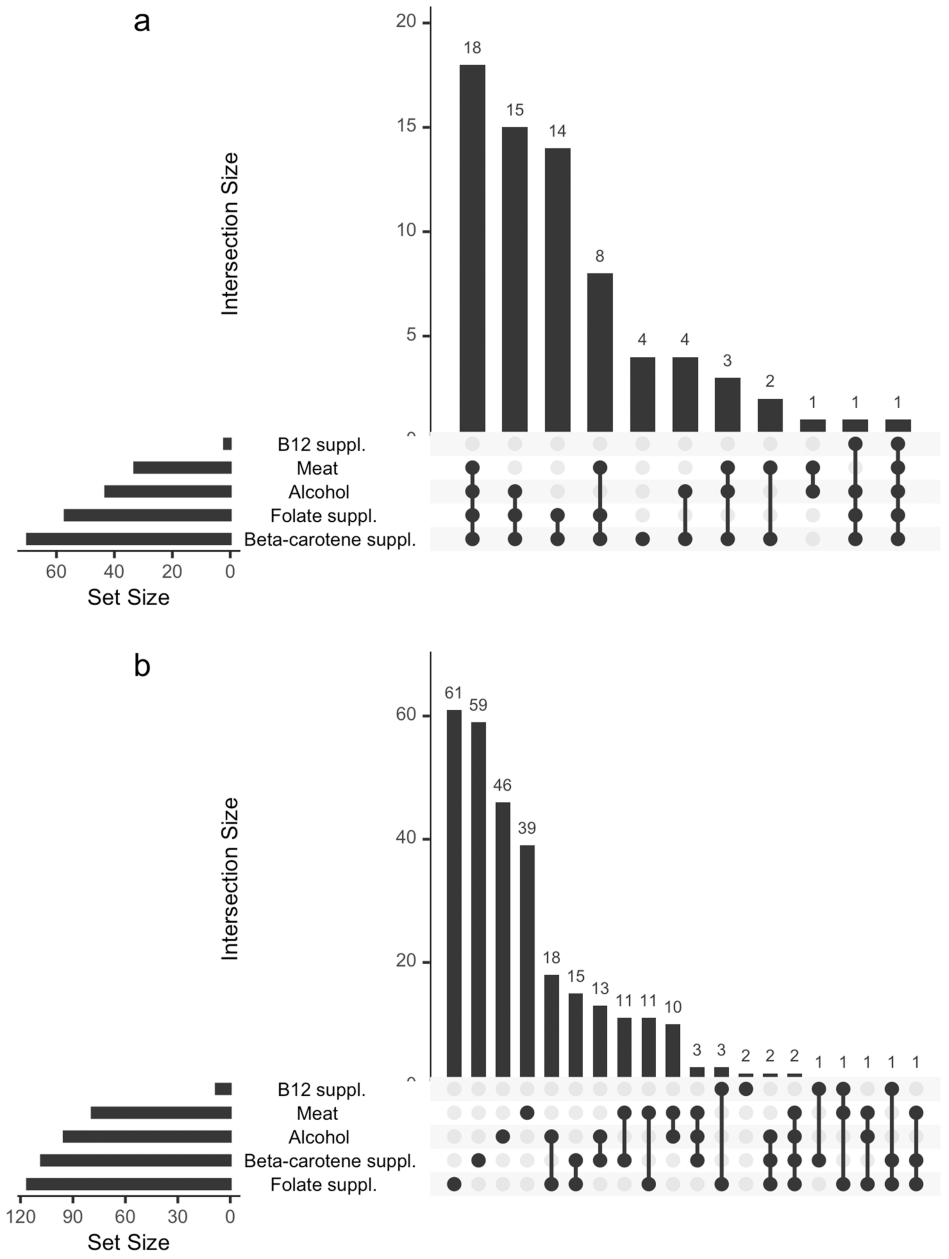

Figure 3. (a) UpSet plot of pairwise associations between 71 metabolites in the BC−AMN network and lifestyle factors. (b) UpSet plot of pairwise associations between 851 metabolites in the AMN network and lifestyle factors. Associations were detected by LASSO, where metabolites were retained when the best model showed nonzero coefficients with metabolites. The set size is the total number of unique metabolites associated with each lifestyle factor. The intersection size (vertical axis) then describes the distribution of those unique metabolite associations as single and multiple exposures.

potential metabolite features. Merging of the data sets resulted in 1790 peaks selected for AMN. Among these metabolites, an empirically derived Pearson's correlation threshold of 0.84 was selected to maximize both the number of metabolites and the number of clusters to retain the most information in the samples with less random correlation and distinguish potential pathway networks as described in our previous publication.[9] This automated correlation threshold selection process resulted in 197 metabolite correlation network clusters found containing 851 metabolites, considered active metabolites (see Figure S1).

Association between AMN and Breast Cancer

In this study, breast cancer is associated with 13 metabolites connected to 13 correlation clusters containing a total of 71 active metabolites (see Figure S1). Active metabolites included those annotated as trazodone hydrochloride, lysoPC(20:4-(5Z,8Z,11Z,14Z)), DiHODE, glycodeoxycholic acid, glycocholic acid, taurodeoxycholic acid, taurocholic acid, and PE(P-18:0/18:1(9Z)) (Table 1 and Table S3). The 13 metabolites are considered gatekeepers for breast cancer, while the 71 active metabolites may be biological links from external exposures. No individual lifestyle factors were associated with breast cancer in this study according to LASSO. Therefore, several metabolomics gatekeepers linked lifestyle factors to health outcomes, even when the main effects of individual

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Environ. Health 2024, 2, 401−410

lifestyle factors on breast cancer were absent. This suggests that the molecular level changes in an active metabolite network may be more sensitive than direct exposure−health outcome associations.

Annotation of Dihydroxyoctadeca Dienoate (DiHODE)

The MS/MS spectra of the MS1 feature (m/z 311.2220 with a retention time of 6.8 min) had major fragmental ions m/z 293.166, 197.0273, and 183.0118, which matched online MS/MS databases GNPS and MS-DIAL for 13-HPODE. Since the isotope and adduct patterns agreed with a molecular formula of $C_{18}H_{32}O_4$, we purchased analytical standards for confirmation. However, the analytical standard peak for 13-HPODE eluted at 7.5 min, confirming that this compound was not 13-HPODE but a chemical of similar structure.

We then checked the retention times of chemical standards analyzed on the same method for 9-HPODE, 12,13-DiHOME, and 9,10-DiHOME. The retention times of 12,13-DiHOME (6.8 min) and 9,10-DiHOME (6.9 min) are similar to this compound, while their molecular ions are both m/z 313.2384. The retention time for 9-HPODE eluted at 7.7 min (m/z 311.228). This suggests that the unknown feature of interest (m/z 311.2220 and R_t = 6.8 min) likely contains two hydroxyl groups.

We then explored the experimental MS/MS spectra of 13-HPODE, 9-HPODE, 12,13-DiHOME, and 9,10-DiHOME. 13-HPODE, 9-HPODE, and 12,13-DiHOME share the major fragmental ions with the unknown compound, while 9,10-DiHOME did not have the fragmental ions for m/z 197.0273 and 183.0118. The mass distance between the molecular ions m/z 311.220 and m/z 183.0118 is C_9H_{20}, while the mass distance between the molecular ions m/z 311.220 and m/z 197.0273 is C_8H_{18}, suggesting a neutral loss of saturated chain hydrocarbons. We checked the structures of 13-HPODE, 9-HPODE, and 12,13-DiHOME, and they all have unsaturated chain hydrocarbons, which could be saturated by a hydrogen rearrangement. However, 9,10-DiHOME does not have this structure for an alpha or beta cleavage but has a neutral loss of $C_7H_{14}O$, which showed m/z 201.1133. Therefore, we concluded that the unknown feature of interest (m/z 311.2220 and R_t = 6.8 min) is likely a DiHOME but is not 9,10-DiHOME or 12,13-DiHOME. Unfortunately, HMDB-predicted spectra for 15,16-DiHODE could not confirm a match to our unknown—it contained the product ion m/z 311.2220 but not the same fragmental ions observed in our experiment. Therefore, we conclude from retention time and MS/MS spectra that the MS1 feature of interest at m/z 311.2220 with R_t = 6.8 min is a dihydroxyoctadeca dienoate (DiHODE) with a chain hydrocarbon of nine carbon atoms.

Association between AMN and Lifestyle Factors

We found no significant associations between individual lifestyle factors and breast cancer in our study population, a subset of the LIBCSP (data not shown). However, we performed gatekeeper discovery to link lifestyle factors with active metabolites associated with breast cancer to generate mechanistic hypotheses. As shown in Figure 3a, most of the lifestyle factors have shared sets of associated gatekeeper metabolites. Beta-carotene supplement usage had the most associated gatekeepers with 4 unique metabolites and 66 gatekeepers linked with multiple lifestyle factors followed by folate supplement usage, suggesting that they heavily influence the breast cancer metabolome. Folate supplement usage linked 57 gatekeepers with multiple lifestyle factors. Alcohol and meat consumption followed with 43 and 33 gatekeepers linked with multiple lifestyle factors, respectively. B12 supplement usage was only associated with two gatekeepers. However, one gatekeeper (m/z 614.4827 and R_t = 68 s, Table 1 and Table S3), was associated with all 5 lifestyle factors and breast cancer. Since the metabolites in this BC−AMN are biased toward those that are also associated with breast cancer, our results suggest that supplement usage, meat, and alcohol have the largest influence on the breast cancer-active metabolome compared to the other lifestyle factors of physical activity and fruit and vegetable intake.

As a sensitivity analysis, we also checked the associations between the full set of 851 AMN metabolites and the lifestyle factors (Figure 3b). Overall, there were over four times as many AMN metabolites associated with lifestyle factors than the BC−AMN (300 versus 71, respectively). Similar to that of the BC−AMN, lifestyle factors with the most influence on the AMN network included folate and beta-carotene supplement usage, followed by alcohol usage, then meat intake, then B12 supplement usage. However, for the AMN, most (207/300) of the lifestyle−metabolite associations were unique to a single lifestyle factor. There were 61 metabolites associated with only folate, 59 metabolites associated with only beta-carotene, 46 metabolites associated with only alcohol, 39 metabolites associated with only meat intake, and 2 metabolites associated with only B12 supplement usage. Therefore, these results suggest that lifestyle factors have a strong influence on the BC−AMN and metabolome in general, and the BC−AMN is most influenced by overlapping exposure influences, suggesting complex interactions and possibly shared pathways among different lifestyle factors.

Emerging metabolomics studies of dietary factors including supplement usage, meat, and alcohol intake suggest strong and overlapping effects on metabolite profiles. In a study of dietary exposures and breast cancer in 1242 participants, 113 metabolites were significantly associated with ≥1 dietary exposure while 37% of these were significantly associated with multiple dietary exposures,[5] suggesting similar chemicals found in different food groups. Indeed, gamma-tocopherol measured with metabolomics was positively correlated with processed meat intake but negatively correlated with vitamin E intake, and ergothioneine was positively correlated with both red meat intake and total alcohol intake,[5] showing the complexity of associations between specific metabolites and multiple dietary exposure.

Oxidative stress is one pathway to which several of these lifestyle and dietary factors have been linked. Oxidative stress can cause DNA damage that when unbalanced can contribute to increased risk of cancer.[32] Folate, B12, and beta-carotene are individually considered antioxidants, while processed meat and alcohol are considered pro-oxidants.[33] Folate, a nutrient in one-carbon metabolism, affects DNA methylation by regulation of *S*-adenosylmethionine levels, which are ubiquitous methyl donors. Reduced *S*-adenosylmethionine can cause DNA hypomethylation, inducing the expression of proto-oncogenes.[34] In addition, folate insufficiency can cause methylation of uracil which incorporates into DNA causing chromosome breakage and carcinogenesis.[35] Similarly, B12 is an essential cofactor in the methionine cycle as part of one-carbon metabolism, which together with folate regulate DNA synthesis and methylation reactions.[36] However, while considered antioxidants, folate or B12 intake has inconclusive associations with breast cancer in several studies.[37] Further,

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Environ. Health 2024, 2, 401−410

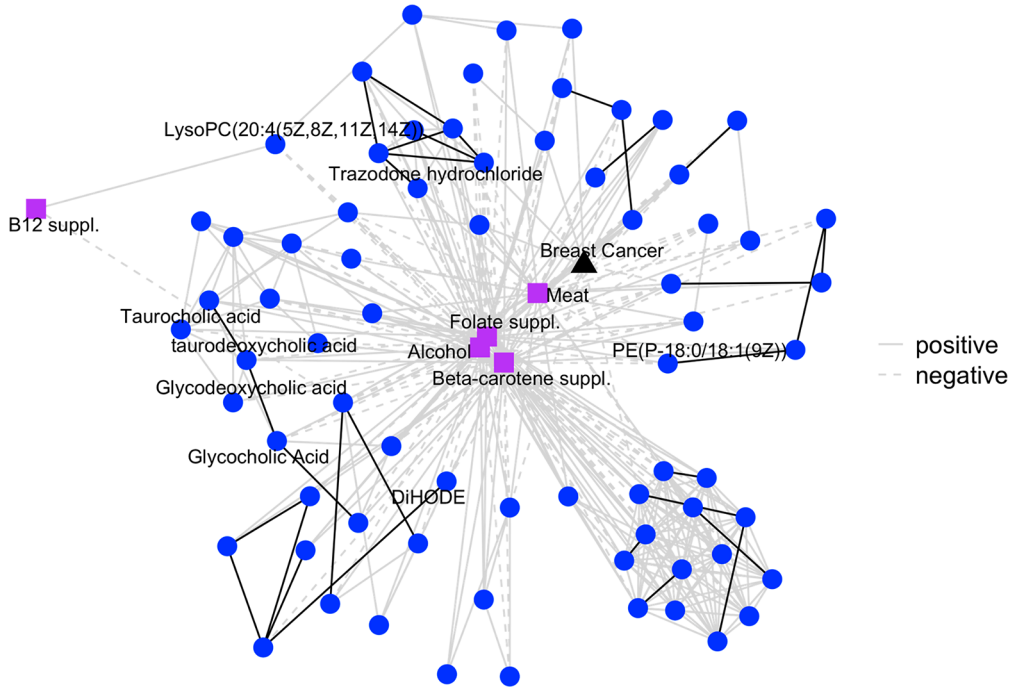

Figure 4. Association between lifestyle, breast cancer, and metabolites in the breast cancer−active metabolite network (BC−AMN). Edges between metabolites and lifestyle/breast cancer are associations determined by LASSO. Edges among metabolites were determined by correlation. Black edges indicate paired mass differences (PMDs) consistent with PMDs from common KEGG database reactions.

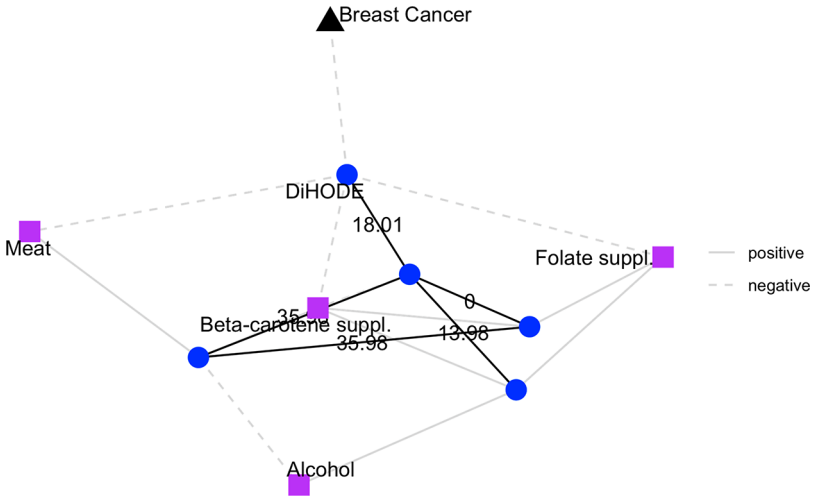

Figure 5. Association between lifestyle, breast cancer, and DiHODE involved BC−AMN metabolites. Edges between metabolites and lifestyle factor or breast cancer risk indicate associations, while edges among metabolites indicate correlation. Black edges depict paired mass differences (PMDs) that can be explained by PMDs in KEGG reactions.

beta-carotene's antioxidant actions are based on their ability to quench singlet oxygen and trap peroxyl radicals[38,39] as well as protecting lipid tissue from peroxidation in vivo.[40] However, increased risk of several cancers has been observed with beta-carotene supplementation.[41] The results of these epidemiological studies point to the complexity of not yet defined interactions between dietary components and cancer initiation and progression.

Dietary exposures have been shown to interact with each other and resultant biology linked with cancer. Mechanisms for alcohol-induced carcinogenesis suggest that one-carbon (folate) metabolism may play an important role[42] and the formation of aldehydes and ROS that promote carcinogenesis by covalently modifying DNA, proteins, and lipids, resulting in altered function.[43,44] Interestingly, alcohol consumption was shown to increase blood beta-carotene levels, likely due to interference by ethanol in its conversion to vitamin A even at moderate alcohol intake, potentially promoting carcinogenesis.[45] In addition, alcohol diminished vitamin B12 status in postmenopausal women[46] but can also potentially modify protective associations between folate and breast cancer[47] or increase the risk of breast cancer for women with higher vitamin B12 levels and either low plasma folate or increased alcohol consumption.[37] These observations further support the importance of investigating dietary exposures as interacting mixtures in association with breast cancer.

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AMN Identifies Inflammation Linking Lifestyle Factors to Breast Cancer

A network was built to link lifestyle factors, active metabolites, and breast cancer (Figure 4). Here, lifestyle factors connect between these correlated metabolite clusters to form a single network, and alcohol, folate, and beta-carotene are the most central lifestyle factors in the network with B12 the most adjacent, having the least interaction with the BC−AMN. The BC−AMN suggested that several long-distance (e.g., multi-node) connections are required to link outcome gatekeepers to lifestyle factors. In this case, the active metabolites in the cluster may play a moderating role between lifestyle and breast cancer, where metabolites linking exposure and outcome play causal biochemical roles.

Meanwhile, there are several important paired mass differences (PMDs) linking lifestyle factors to breast cancer. We identified a total of 23 different PMDs with reactions that included oxidation (PMD 2.02 Da), hydroxylation (PMD 15.99 Da), and dehydration (PMD 18.01 Da). These reactions are consistent with KEGG PMDs, suggesting that there are some enzymes that could link lifestyle factors to breast cancer and that the interference of those reactions might regulate or moderate such influences. For example, both oxidation and dehydration reactions of lipids are important in inflammation processes.[48,49] BC−AMN and the biochemical reactions among the active molecules are used to generate hypotheses on important biochemical reactions and causal pathways linking lifestyle factors to breast cancer.

Among the AMN molecules, DiHODE is negatively associated with breast cancer risk as an outcome gatekeeper and remotely connected to beta-carotene supplement usage, meat intake, and folate supplement usage through an active molecular network of five nodes (Figure 5). Increased DiHODE is associated with decreased breast cancer risk in this population. DiHODE is a degradation compound of epoxy-fatty acid,[50] which is positively associated with inflammation in other studies.[51] Since epoxide hydrolases can generate DiHODE from the corresponding epoxy-fatty acid,[52] this enzyme might be important in mediating the influences from lifestyle factors to cancer. DiHODE is also an oxylipin, which is influenced by a high fat diet.[53]

PMD was then used to interpret this connection. A PMD 18.01 Da between nodes DiHODE and M329.2328T384.4 suggests a dehydration process between DiHODE and an unannotated compound. These results suggest a pathway where increased beta-carotene is involved in dehydration of an unknown metabolite from lipid metabolism leading to increased DiHODE. Since the untargeted assay is broad but not comprehensive of every cellular metabolite, it is possible that compounds along the pathway linking lifestyle factors to breast cancer are missing from the analysis. Nevertheless, even in the absence of further annotation information about the unknown active metabolite and possible missing compounds, our results suggest that enzymes that participate in dehydration reactions may play an important role in the pathways linking supplemental usage to breast cancer.

Inflammation is a hallmark of cancer,[54] including breast cancer.[55] However, the role of inflammation in linking lifestyle factors to breast cancer initiation and progression remains undefined. We found that metabolite DiHODE linked beta-carotene, folate, and meat intake to breast cancer, supporting the role of dietary-induced inflammatory compounds in breast cancer. Alcohol and meat intake have been positively associated with inflammation,[56−59] while folate has been negatively associated with inflammation.[60] Similarly, even in large human population studies, associations between breast cancer and a high-inflammation diet show contradictory findings. In a prospective study of 49 258 women in Sweden, a dietary inflammatory index (DII) was positively associated with breast cancer incidence, with slightly higher risk observed in postmenopausal women.[61] Breast cancer risk in 34 700 women in the United States was positively associated with DII, with slightly higher risk in obese women.[62] In the Sister Study cohort of 43 563 participants, breast cancer risk was only associated with a high-inflammation diet for triple-negative breast cancer cases[63] or when combined with low oxidative balance diets. In addition, a high-inflammatory diet score was positively associated with breast cancer risk in the 318 686 participants in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. However, here, the association was strongest among premenopausal women compared to postmenopausal women.[64] Finally, DII was not associated with breast cancer risk in the prospective study of 122 788 postmenopausal women in the Women's Health Initiative.[65] While our metabolomics study supports a common inflammation pathway linking beta-carotene, folate, and meat intake to breast cancer, it is likely that we did not capture all of the important and/or unique metabolites linking lifestyle factors to breast cancer. These results further highlight the complexity of the role of dietary factors in inflammation pathways related to breast cancer and the need for further studies to investigate these interacting exposures on a molecular level.

CONCLUSION

We constructed a breast cancer−active molecular network (BC−AMN) to identify metabolites and pathways that link between breast cancer and lifestyle factors. In this way, we have used a dimension-reduction technique to focus on the functional metabolome of breast cancer. Using this workflow, we found that supplement usage of beta-carotene and folate, alcohol usage, and meat intake were the most influential lifestyle factors on metabolites associated with breast cancer, with B12 supplement usage also contributing but to a lesser degree. Further, these lifestyle/dietary factors likely influence the metabolome through synergistic or interactive pathways, suggested by the multiple associations observed between specific metabolites and several lifestyle factors. In particular, the metabolite DiHODE emerged as a metabolite linking beta-carotene, folate, and meat intake to breast cancer, supporting the role of dietary-induced inflammatory compounds in breast cancer.

There are several limitations to this study. This study used a cross-sectional design with a moderate sample size of 100 women. Therefore, causality cannot be fully addressed for etiologic effects given the postdiagnostic timing of the sample collection. In addition, this study focused only on post-menopausal women residing from two New York state counties and included mostly white women. Therefore, these findings may not be representative of premenopausal women or women from different geographical or racial/ethnic backgrounds. Only a single blood sample was analyzed, and the results may not reflect fluctuations in metabolite profiles. Nevertheless, this study was conducted using the richly characterized participants from the LIBCSP, including extensive lifestyle and dietary characterization. Since samples

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Environ. Health 2024, 2, 401−410

were collected from 1996 to 1997, it is possible that some of the metabolites have degraded with time. Nevertheless, all samples were collected within 2 years, so comparisons between cases and controls should be equivalent even if the measured levels do not reflect true values at the time of collection. The merge of positive and negative modes might introduce false positives when coeluted, but not identical chemicals just share the mass distances of 2.02 Da in different modes. Meanwhile, the AMN discovery process should be treated as exploratory data analysis, and validation with analytical standards and using complementary approaches is required. Finally, we could confidently annotate, via MS/MS confirmation, only a limited number of metabolites in the BC−AMN. In addition, the semiquantitative measures limit interpretation of a biologically plausible effect size. Finally, we could confidently annotate, via MS/MS confirmation, only a limited number of metabolites in the BC−AMN, while quantification of those metabolites is missing due to the lack of standard to check the statistical power or biological effect size. The absence of many common metabolites that are typical to our in-house library in the BC−AMN suggests that future studies that utilize a panel of only the most common metabolites are likely to miss the relationships. Additional large-scale studies that include untargeted panels and targeted analysis of inflammation metabolites and biomarkers are needed to further investigate these relationships.

Interestingly, we found no direct significant associations between breast cancer and lifestyle factors in our study population, which is a small subset of the LIBSCP. This is likely due to reduced power in the modest sample size of women. Nevertheless, several gatekeeper-linked lifestyle factors to breast cancer were identified, even when direct associations were absent, demonstrating that the metabolites can be used as a read out for lifestyle choices. This may be because direct associations were masked by antagonist relationships, and molecular level changes such as in the BC−AMN are more sensitive than testing direct exposure biomarkers to health outcomes when study power is limited. Meanwhile, other machine learning models could be used to build the links between molecular and lifestyle habits/disease status as long as they can tell the associations or show the importance between exposure and certain metabolites.

In conclusion, AMN showed that lifestyle factors influence breast cancer through metabolite level changes, especially through the active metabolites connected by correlation networks. Thus, AMN is a powerful tool to build molecular connections and generate hypotheses between exposures and health outcomes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/envhealth.3c00218.

> Annotation results of MS2 from GNPS; active metabolite network in plasma and 71 metabolites for breast cancer−AMN; table of the population demographics and associations among gatekeepers, breast cancer, and selected lifestyle factors; data analysis script in R is provided for reproducible purposes (PDF)

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Notes

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Environ. Health 2024, 2, 401−410