Metabolite-predicted biological age acceleration and associations with oncologic, neurodegenerative, and adiposity-related endpoints in multi-ancestry populations

Samin Kamal

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Burcu F. Darst

Alexander P. Reiner

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Abstract

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Samin Kamal

Chair of the Supervisory Committee:

Burcu F. Darst

Department of Epidemiology

Chronologic age is widely understood as a significant risk factor for many chronic diseases and is frequently used in risk stratification to guide selective screening. However, for age-related diseases, chronological age alone may not accurately reflect the underlying heterogeneity in biological aging that contributes to disease risk. Using prospectively collected data from the Women's Health Initiative (WHI), the Multiethnic Cohort (MEC), and the Wisconsin Registry for Alzheimer's Prevention (WRAP), we developed statistical models to estimate biological age acceleration from untargeted LC/MS metabolomics data and evaluated associations with, primarily, breast and prostate cancer, cognitive function scores, and BMI. Additionally, we conducted pathway analyses to identify metabolic pathways significantly associated with chronologic age. Model performance was consistent across cohorts, with RMSE ranging from [5.36, 6.41] years. We did not find evidence of a statistically significant association between a 10-year increase in metabolite-predicted biological age acceleration and disease risk, including for any oncologic outcomes, cognitive scores, or BMI. We detected several enriched metabolic pathways across cohorts, including C21-steroid hormone biosynthesis and metabolism, urea cycle/amino acid group metabolism, and carnitine shuttle.

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I. Introduction

Metabolites, ultra-low weight molecular compounds produced as intermediary or end-stage products of biochemical processes, can reflect both endogenous and exogenous perturbations to biological systems with high sensitivity. As such, metabolomics, the study of the complete set of metabolites, is a powerful tool for studying complex traits regulated by both genetic and environmental components in population based epidemiologic studies. Research over the past decade has demonstrated that changes in metabolomic profiles are associated with chronological age, suggesting that the metabolome could be an appropriate endophenotype to investigate the underlying physiological mechanisms mediating aging and/or age-related traits.[1,2] In other words, the metabolome may be a useful marker of biological age, which is particularly relevant given that traditional aging markers (i.e., chronological age) may not fully capture the heterogeneity of aging, especially in the context of age-related diseases.

Similar to how epigenetic approaches have used DNA methylation markers to create 'epigenetic clocks' predictive of biological age,[3] it is also possible to develop similar models using metabolomic data to assess metabolic age. Previous research has shown that epigenetic age acceleration is significantly associated with a variety of disease related phenotypes, including cognitive decline, cardiovascular disease, malignant neoplasms, and all-cause mortality.[4–6] Given that the metabolome is further down on the genotype-phenotype pathway, *divergence* between chronologic and metabolic age may be novelly associated with traits characterized by distinct metabolic features, as compared to those associated with epigenetic age.

Risk stratification methods based on metabolite-predicted age acceleration may be especially relevant for disease states with metabolic signatures indicative of broader network dysregulation, e.g., enrichment of certain lipid-related metabolites/subclasses in triple negative BCa tissue compared to normal breast tissue.[7] Similar metabolite-trait associations have also been reported for neurodegenerative conditions including Alzheimer's disease and related dementias.[8] Prior clock-based studies have reported early evidence for associations between metabolite-predicted age acceleration and various aging-related phenotypes/conditions, including self-rated health and frailty, all-cause mortality, and certain cancer types, though evidence remains preliminary due to limited replication across diverse populations and lack of mechanistic follow-up.[9]

Research to date on modeling biological age as a function of metabolomic markers has been restricted by small sample sizes, limited ancestral diversity, and the use of statistical approaches that do not account for complex interactions (i.e., non-linearity) between metabolic features. Given that metabolomic profiles can vary substantially across populations, these limitations raise concerns about the generalizability and clinical utility of existing models. To better address and contextualize these limitations, the current analysis sought to assess differences in predictive performance between a non-linear gradient boosting method (LightGBM) and the more conventional penalized regression approach (ElasticNet), evaluate subsequent associations with disease outcomes of interest, and perform systems-based analyses to elucidate metabolic pathways implicated in aging.

II. Methods

i. Participant selection

Data were aggregated across three longitudinal, prospective cohort studies – the Multiethnic Cohort (MEC), the Women's Health Initiative (WHI), and the Wisconsin Registry for Alzheimer's Prevention (WRAP) – each with untargeted plasma or serum metabolomic profiling available for a subset of participants. Details regarding the original design and recruitment methods of each cohort are discussed extensively elsewhere.[10–12] For the current analysis, we included all subjects with available metabolomic data. Participants were selected for metabolomic profiling based on characteristics related to cohort-specific research questions of interest. In MEC, participants were selected for a nested case-control study of prostate cancer (PCa) among African American men; in WHI,[13] African American women were selected for profiling based on known sickle cell trait (SCT) status; and in WRAP, participants were selected using a convenience sampling approach, irrespective of any particular exposure status or disease outcome.

ii. Outcome assessment

Several outcomes of interest were evaluated for associations with metabolic age acceleration, contingent on the cohort from which the data originated. (i) In MEC, we evaluated associations with overall PCa and aggressive PCa, compared to both controls and non-aggressive PCa cases. Cancer outcomes in MEC are defined exclusively based on linkage with the Surveillance,

Epidemiology, and End Results (SEER) program. Aggressive PCa was defined as prostate cancer-specific death, stage II-VII disease, a Gleason score of 8 or higher, or, among cases diagnosed before 2004, a tumor grade of 3 or 4. We defined non-aggressive PCa as stage I disease with a Gleason score less than or equal to 7 or, if diagnosed before 2004, a tumor grade of 1 or 2, with no evidence of high-grade features, prostate cancer-specific death, and aggressive disease classification. (ii) For WHI, primary analyses focused on associations with overall breast cancer (BCa) and a composite endpoint encompassing any cancer diagnosis. Secondary analyses evaluated specific cancer subtypes and features, including invasive BCa (vs. no BCa), SEER staging (regional/distant vs. no BCa and regional/distant BCa vs. in situ/localized BCa), hormone receptor status (estrogen receptor-positive vs. negative and progesterone receptor-positive vs. negative), and HER2/neu receptor status (positive vs. negative). WHI outcomes are ascertained by annual participant self-report, followed by medical record abstraction and document review for select conditions (including pathology reports for cancer outcomes). (iii) In WRAP, we focused on validated measures of cognitive aging associated with dementia risk. Items from several neuropsychological batteries were combined into three primary domain-specific composite scores: learning, delayed recall, and executive function. Each cognitive outcome composite score was calculated as the mean z-score across a series of subtests, with a higher z-score interpreted as better test performance.[14]

Across all studies, we additionally investigated the association between metabolomic age acceleration and markers of overall and/or abdominal adiposity, including body mass index (BMI) and waist-to-hip ratio (WHR). BMI was calculated as kg/m^2 based on anthropometric measurements collected at enrollment, time of blood draw, or visit. WHR, available only in WHI and WRAP, was defined as the ratio of waist circumference (cm) to hip circumference (cm).

iii. Metabolite quantification, quality control, and data pre-processing

Metabolite quantification was performed by Metabolon using their Global Untargeted Discovery Platform. Samples were analyzed with a Waters ACQUITY ultra-high performance liquid chromatography (UPLC) system coupled to a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer, interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. To enhance coverage, metabolite concentrations were profiled across four UPLC-MS/MS methods – two separate

reverse phase (RP)/UPLC-MS/MS methods using positive ion mode electrospray ionization (ESI), one RP method using negative ion mode ESI, and one hydrophilic interaction liquid chromatography (HILIC) method with negative ion mode ESI.

To ensure data quality and consistency across cohorts, we applied several exclusion criteria at both the sample and metabolite levels. At the individual level, we excluded participants who fasted for less than 8 hours prior to blood draw, had missing information on fasting duration, lacked information on age at blood draw, or were missing values across more than 50% of metabolites. We excluded individual metabolites with constant variance or missingness in 50% or more of samples. In WRAP, metabolomics data were longitudinally collected, with a median of 2 (maximum 3) samples per participant. Since neither ElasticNet nor LightGBM natively support mixed-effects modeling, we used the median of each participants metabolite values across visits as a summary measure. Correspondingly, we also used median age at sample draw as the outcome variable for model training and the predictor for MWAS. We performed all analyses using pre-processed data from Metabolon, which were median-normalized by batch and had missing values imputed with the limit of detection.

iv. Statistical analysis

(a) Estimating age acceleration

We first divided each of the three datasets into separate 60-40 train-test splits. The training data was used to fit both a (i) gradient boosting decision tree (LightGBM)[15] and (ii) ElasticNet model to predict chronological age from metabolomic markers. The LightGBM model was trained on the raw metabolite data since it is robust to variable scale, while metabolite features were standardized to mean zero and unit variance prior to training the ElasticNet model. For the latter, the hyperparameter alpha was fixed at 0.5 and the optimal value of lambda was selected using a grid search procedure with 10-fold cross-validation. Given the complexity of the hyperparameter space in the LightGBM implementation, we focused our efforts on tuning a few high-impact parameters. Specifically, a coarse grid search tandem three-fold cross validation approach was used to tune values for L1 and L2 penalties, learning rate, and number of trees (iterations). For both ElasticNet and LightGBM, the root mean squared error (RMSE) served as the primary performance metric in selecting the optimal hyperparameter set. We then evaluated the best-performing model on the held-out test set to get an estimate of model performance on unseen

data using primarily RMSE and R^2, which represents the improvement in predictive accuracy compared to a null model that predicts the mean value of the outcome for all observations, secondarily.

Following model optimization, we used the best performing LightGBM model to generate predictions of chronologic age on the combined dataset (training and test data) to use in downstream analyses. The final measure of age acceleration was defined by subtracting chronological age from metabolite-predicted age. As such, positive values of this residual indicate age acceleration (metabolite-predicted age greater than chronologic age) and negative values indicate age deceleration (metabolite-predicted age less than chronologic age).

We also performed tree-based feature selection in order to identify metabolites most useful in predicting chronological age. Among the available importance metrics, we specifically focused on gain-based measures, which quantify the improvement in model performance (i.e., reduction in error) attributable to a given feature. Default implementations of these importance metrics provide a value for each feature instead of performing explicit feature selection; since individual feature scores appeared to substantially decrease beyond the top-ranked features, we limited our focus to the top 10 metabolites.

(b) Phenotype associations

For all associational analyses, the primary predictor of interest was the previously defined age acceleration measure scaled to represent the effect of a 10-year (approximately 1-standard deviation) increase in metabolite-predicted age acceleration. Given the high correlation between the predicted age acceleration measure and chronologic age, all downstream analyses adjusted for chronologic age as a potential confounder. Binary disease outcomes were analyzed using multivariable logistic regression and continuous outcomes using linear regression. Resulting point estimates are presented as either odds ratios or beta coefficients, along with corresponding 95% model-based confidence intervals. In WRAP, we fit linear mixed-effect models with a random intercept term for each participant to leverage longitudinal cognitive measure data and account for within-person correlation over time. We also included an additional random effect term for family since WRAP includes related individuals.

For certain outcomes, we also adjusted for a set of potential confounders selected *a priori* based on available data. In WHI, associations with the overall BCa outcome were adjusted for smoking status (ever vs. never), BMI, age at menopause, age at menarche, alcohol consumption (servings/week), and duration of oral contraceptive use (in years). Associations with the composite (i.e., any cancer) endpoint were adjusted only for smoking status. In MEC, analyses of the prostate cancer outcome were adjusted for sample collection and birth years as design-based matching variables. In WRAP, models for each neurocognitive outcome measure were adjusted for visit number (as a proxy for practice effects), sex, years of education, and an apolipoprotein E (*APOE*) risk score.[16]

With respect to the BMI and WHR outcomes, we observed substantial variability (i.e., range) in the distributions and had inclination to believe that values at either extrema of the distribution may be implausible or an otherwise outlier with high leverage. As such, we truncated values at the 1st and 99th percentiles. We additionally conducted sensitivity analyses in which we applied an explicit statistical correction on the model predictions (prior to calculating age acceleration) in order to deal with systematic overprediction of age in younger individuals and underprediction in older individuals. We followed the approach described by G. de Lange et al.,[17] where we first regressed metabolite-predicted age on chronologic age (via a linear model) and used the corresponding intercept and coefficient term to scale the original predictions. This process was performed separately for each cohort.

(c) Biological pathway enrichment

To investigate metabolomic pathways impacted by the aging process, we performed pathway and network analyses using *mummichog*, a high throughput, untargeted metabolomics tool that predicts functional activity from unannotated metabolomics data.[18] As mummichog requires summary statistics as input, we first performed a metabolome-wide association study (MWAS) in each cohort, testing associations between each individual metabolite (outcome) and chronologic age (predictor) in unadjusted linear regression models. Prior to conducting the MWAS, all metabolite values were standardized to mean zero and unit variance. We present significant findings from the MWAS after applying the Benjamini-Hochberg procedure to control the false discovery rate associated with multiple testing. We did not apply a multiple testing correction prior to inputting features into mummichog, as the method implicitly accounts for this at the

pathway level by using permutation-based testing. Note, we retained all unidentified metabolites across cohorts, except in WHI, as we did not have available m/z and RI information for these compounds in this cohort.

The summary statistics were then split based on whether the corresponding metabolites were detected in positive or negative ionization modes. Since mummichog does not account for chromatographic differences, we grouped data solely by ionization mode (positive vs. negative) regardless of chromatographic platform. Each set was analyzed independently, using default adduct settings (positive: M[1+], M+H[1+], M+Na[1+]; negative: M-H[-], M-2H[2-], and M-H20-H[-]). Statistically significant pathway enrichment results are presented separately for each cohort.

III. Results

i. Sample characteristics

After sample and metabolite exclusions, the final analytic datasets included: 714 participants and 1143 metabolites in MEC; 2929 participants and 1040 metabolites in WHI; and 1200 participants and 1029 metabolites in WRAP. Select participant characteristics are presented in **Table 1**. Self-reported race and ethnicity was predominantly non-Hispanic African American in MEC and WHI (as a result of sampling), and primarily non-Hispanic White in WRAP. By design, WHI and the MEC sub study were restricted to exclusively female and male participants, respectively, as a result of the research questions of interest. Among the included WRAP participants, a majority self-identified as female (69%). Participants in the MEC cohort had a higher median age at sample draw (67; IQR: 62-73) compared to those in WHI (61; IQR: 56-67) and WRAP (63; IQR: 58-68). Across all cohorts, the youngest participant was 39 years old (in WRAP), and the oldest was 93 (in WHI). Of the metabolites included, most were quantified with known structure, while a small proportion were unidentified (i.e., unique detectable mass, unknown chemical structure). Across all cohorts, the proportion of unidentified metabolites ranged from 18-20%. Of the metabolites passing quality control criteria, 808 were measured across cohorts (16% of which were unidentified).

ii. Predicted age acceleration

Across all cohorts, LightGBM performed similarly to ElasticNet on the held-out test data, with RMSE ranging from [5.36, 6.41] years compared to [5.02, 6.80] years for ElasticNet (**Figure 1**). Similarly, R^2 (coefficient of determination) values varied from [0.25, 0.42] in ElasticNet to between [0.30, 0.40] for LightGBM. There was considerable correlation between ElasticNet- and LightGBM-predicted metabolic age in all cohorts ($r > 0.70$). Pearson correlation coefficients between LightGBM-predicted and chronologic age ranged from [0.55, 0.65] (in WHI and MEC, respectively), compared to [0.50, 0.64] (in WHI and WRAP, respectively) for ElasticNet. Given the slightly higher predictive ability of LightGBM-predicted metabolic age in MEC and WHI, LightGBM models were used for downstream analyses. The top 10 contributors to predictive accuracy in the LightGBM models, identified using default *gain-based* feature importance scores, are summarized in **Figure 2**. The difference between metabolite-predicted and chronologic age (i.e., age acceleration) had a fairly normal distribution across the cohorts and ranged from -30 years younger to 20 years older (**Figure 3**).

iii. Advanced aging and disease outcome associations

Across MEC and WHI cohorts, we did not find evidence of a statistically significant association between a 10-year increase in metabolite-predicted age acceleration and overall cancer risk (**Table 2**), including for prostate cancer (OR: 1.30; 95% CI: [0.80, 2.11]), breast cancer (OR: 1.01; 95% CI: [0.69, 1.50]), or any cancer (OR: 1.11; 95% CI: [0.84, 1.46]). Notably, in MEC, metabolomic age acceleration was associated with 53% lower odds of aggressive vs. non-aggressive PCa (95% CI: [0.19, 1.10]; p-value = 0.0886). Additionally, in WHI, age acceleration was positively associated with both ER (OR: 2.30, 95% CI: [0.85, 6.47]) and PR (OR: 2.02, 95% CI: [0.82, 5.17]) positive tumors, and negatively associated with HER2/neu tumor positivity (OR: 0.326, 95% CI: [0.007, 1.39]). However, none of the associations reached statistical significance at the $p < 0.05$ threshold.

In WRAP, metabolic age acceleration was positively associated with learning ($\beta = 0.00744$, 95% CI: [-0.16, 0.18]), delayed recall ($\beta = 0.0359$, 95% CI: [-0.14, 0.21]), and executive function ($\beta = 0.0532$, 95% CI: [-0.12, 0.23]) z-scores, though none were statistically significant. Additionally, our analysis did not provide support for a consistent effect of metabolic age acceleration on BMI (MEC: 0.118, 95% CI: [-0.93, 1.2]; WHI: -0.217, 95% CI: [-0.92, 0.48]; WRAP: 0.783, 95% CI:

[-0.39, 2.0]) or WHR (WHI: 0.00733, 95% CI: [0.00. 0.02]; WRAP: -0.0190, 95% CI: [-0.04, 0.00]).

In sensitivity analyses that applied an explicit statistical correction (**Supplementary Figure 1**) for chronologic age (compared to covariate adjustment), resulting inference based on hypothesis tests remained consistent with the primary approach. However, on average, results were slightly more attenuated towards the null. Observed effect estimates were generally in the same direction as the primary analysis, with the exception of learning and delayed recall cognitive function scores in WRAP. After statistical adjustment for chronologic age, age acceleration was negatively associated with learning (β = -0.0343, 95% CI: [-0.21, 0.15]) and delayed recall (β = -0.000767, 95% CI: [-0.18, 0.18]) (**Supplementary Table 1**). Associations with executive function remained directionally consistent and of similar magnitude.

iv. Pathway analyses and MWAS

We also evaluated individual metabolites and biological pathways in association with chronologic age. At the individual metabolite level, we performed feature selection using both traditional MWAS and tree-based feature selection methods. Using the MWAS approach, many metabolites were associated with age, including 338 (30%) in MEC, 626 (60%) in WHI, and 570 (55%) in WRAP (**Figure 4**). Of these significant metabolites, a substantial portion were unidentified, including 60 (18%) in MEC, 110 (18%) in WHI, and 137 (24%) in WRAP. A total of 151 metabolites were significantly associated with age across the three cohorts, of which 30 (20%) were unidentified. As per Metabolon's provided annotations, these cross-cohort significant metabolites mapped to lipid (n = 53), amino acid (n = 37), and nucleotide metabolism (n = 11) related super pathways. Within these, the most commonly represented sub-pathways included fatty acid metabolism (acyl carnitine) (n = 18), androgenic steroids (n = 9), urea cycle arginine and proline metabolism (n = 9), and fatty acid (dicarboxylate and monohydroxy) metabolism (n = 12).

Using gain-based feature importance methods, we also identified the top 10 metabolites contributing most to predictive accuracy in LightGBM in each cohort (**Figure 2**). All metabolites identified using feature selection methods were also detected as significant metabolites in MWAS, with the exception of dimethyl sulfone in WRAP. Additionally, tree-based feature

selection identified several unnamed metabolites across all cohorts, ranging from 10-40% of the 10 most significant features, compared to 0-30% of the top metabolites in MWAS.

At the pathway level, we observed evidence of several significantly enriched pathways potentially implicated in or reflective of the biological aging process (**Table 3**). Many pathways only reached significance in a given cohort, though several associations were consistent across at least two populations, including for C21-steroid hormone biosynthesis and metabolism, urea cycle/amino acid group metabolism, and carnitine shuttle. We also observed repeated associations with distinct but metabolically related pathways, including the pentose phosphate pathway and pentose and glucuronate interconversions.

IV. Discussion

i. Context

In this study, we leveraged untargeted LC/MS metabolomics data from three deeply phenotyped cohorts to investigate whether metabolite-predicted biological age acceleration was associated with several clinically relevant oncologic, neurodegenerative, and adiposity-related endpoints of interest. To do so, we constructed an age acceleration measure by developing separate metabolomic clocks in each cohort using LightGBM to account for complex, non-linear interactions between metabolic features. Our analysis additionally expanded the application of metabolomic clocks to African American ancestry populations, allowing assessment of metabolomic aging across potentially heterogeneous risk profiles. To contextualize and benchmark performance of LightGBM, we compared predictive performance to ElasticNet, a prevalent approach in the literature. We observed relatively similar performance across both of these estimation approaches, with LightGBM slightly outperforming ElasticNet in WHI and MEC.

Notably, LightGBM performed worse than ElasticNet in the WRAP cohort. While we are unsure why this cohort-specific deterioration in predictive accuracy occurs, it is unlikely to be driven by sample size issues (since WRAP has a greater number of observations than MEC) or different sample sources (plasma vs. serum), given metabolomic measurements in WHI and WRAP were both quantified in plasma. One possible explanation for the observed discrepancy is the use of

median metabolite concentrations across visits as a summary measure. We used this approach to leverage all of the available longitudinal metabolomics and outcome data in WRAP while applying modeling techniques that did not support mixed-effects structures. However, LightGBM may have been more sensitive to this approach. Unlike ElasticNet, which assumes linear relationships and is relatively robust to lower within-feature variability, LightGBM relies on this variation to identify meaningful partitions (i.e., feature splits). These more fine-scale patterns may have been masked as a result of our aggregation procedure. For a given estimation approach (ElasticNet or LightGBM), performance was also relatively consistent across cohorts. Some of the observed difference may partly reflect metabolomic differences by ancestry and/or sex, as WRAP included male and female participants of primarily European descent, whereas WHI (only female) and MEC (only male) participants were of African American descent.

Across all analyses, we did not find evidence of statistically significant associations between metabolite-predicted biological age acceleration and various age-related diseases/phenotypes, including for several cancers/cancer subtypes, adiposity markers, and measures of cognitive function. In the primary analysis, many effect estimates, though not statistically significant, were in the opposite direction to which we hypothesized *a priori* (i.e., metabolic age acceleration would be associated with increased risk of negative/more aggressive outcomes). For example, in WHI, age acceleration was associated with lower odds of HER2/neu tumor receptor positivity, typically indicative of advanced disease severity and poor prognosis.[19] Additionally, age acceleration was positively associated with greater odds of ER and PR tumor positivity – subtypes that, while potentially leading to poor outcomes if left untreated, are more effectively targeted with hormone therapies and associated with better prognostic outcomes (compared to ER/PR negative tumors).[20] In conjunction, these two results may suggest that metabolic age acceleration is associated with underlying biophysiological changes which result in a decreased likelihood of developing fast-growing, aggressive disease. This post-hoc hypothesis is also consistent with our findings in MEC, where metabolic age acceleration was associated with lower odds of aggressive vs. non-aggressive PCa. However, these results should be interpreted with caution, as they are not statistically significant at the pre-specified threshold, are based on underpowered subgroup analyses with small event counts, and do not take into account multiple hypothesis testing.

Although our findings were largely null, previous literature has reported age acceleration to be associated with generally poorer health outcomes. For example, using NMR-based data from the UK Biobank (UKB), Mutz et al.[21] estimated that metabolic age acceleration was positively associated worse self-rated health, frailty, and hazard of all-cause mortality. Application of such approaches to oncologic outcomes (especially disease subtypes) has been somewhat limited, though Shang et al.[22] investigated associations between metabolic age acceleration with several oncologic endpoints in UKB and observed null associations with both overall breast (HR: 1.01, 95% CI: [0.99, 1.02]) and prostate (HR: 0.99, 95% CI: [0.97, 1.00]) cancer, both of which are consistent with our results. However, a statistically significant association was detected for esophageal cancer (HR: 1.06, 95% CI: [1.02-1.11]) and a composite other cancer endpoint (HR: 1.01, 95% CI: [1.00, 1.02]).[22] In addition, a positive effect of age acceleration on BMI has also been observed in several studies.[22,23] Our analysis was not suggestive of an association with BMI or WHR, as estimates were not consistently in one direction and, often times, in the opposite direction.

We also more broadly explored metabolites associated with chronologic age, both at the individual feature and pathway level. At the individual metabolite level, our results showed significant concordance between features identified using traditional MWAS and tree-based importance metrics. Notably, tree-based metrics identified a slightly greater number of unidentified metabolites as among the top 10 most significant features compared to MWAS. This may reflect the tendency of MWAS to select metabolites that are marginally associated with chronological age, while tree-based methods prioritize features involved in higher-order interactions that may not exhibit strong marginal associations on their own. We identified several metabolic pathways which correspond to known metabolic changes associated with the aging process,[24] notably several sex steroid related pathways (androgen and estrogen biosynthesis metabolism and C21-steroid hormone biosynthesis and metabolism). A similar analysis of metabolomic age acceleration in human cerebrospinal fluid (CSF) also reported significant associations for the carnitine shuttle pathway,[25] an association which has also been shown in other cross-sectional samples.[26] Signaling pathways activated by amino acids (corresponding to urea/amino acid group metabolism) have also been posited to play a role longevity and anti-oxidative stress in animal models.[27]

ii. Strengths

A notable strength of the current analysis is the inclusion of unidentified metabolites in the age acceleration estimation procedure. Despite their unknown chemical structure and function, these metabolites are still important predictors of age and, subsequently, age acceleration, as evidenced by large gain scores in tree-based feature importance rankings and significant associations in univariate MWAS. This observation appears to be relatively consistent with findings in the published literature, in which approaches retaining unidentified compounds (typically from LC/MS experiments) consistently explain a larger proportion of the variance in chronologic age compared to more targeted approaches like nuclear magnetic resonance (NMR).[28,29]

Additionally, our analysis expanded the application of metabolomic clocks to include African American participants, attempting to address the underrepresentation of non-European ancestry populations in prior work. Future work should aim to include greater numbers of participants from underrepresented groups so to ensure corresponding results are generalizable to diverse and clinically relevant populations and to increase the transferability of developed metabolite clocks between populations.

iii. Limitations

There are several limitations to the present analysis which should frame the interpretation and generalizability of our results. First, there may be temporality issues between when metabolomic data were collected with respect to the incidence of disease outcomes. Although we focused on incident breast and prostate cancer diagnoses (i.e., metabolomic profiling occurred before disease diagnosis), it is possible that onset of these conditions preceded diagnosis, possibly resulting in metabolic perturbations associated with disease progression which distort the biological age estimation process. This reverse causality issue makes it difficult to determine whether the measure of age acceleration truly preceded (and influenced the risk of) disease onset, or whether biophysiological changes associated with pre-clinical disease disrupted the metabolic profile and, hence, resulting measure of age acceleration.

Measures of metabolic age acceleration and associations with disease phenotypes may also exhibit significant heterogeneity by biological media or tissue type. Metabolite profiles significantly differ based on the biological specimen analyzed (e.g., plasma vs. serum vs. urine)[30]

and whether the relevant matrix captures global or localized metabolism. Across all cohorts included in the present analysis, metabolites were quantified in either blood plasma or serum, reflecting a global, system-wide metabolic profile. However, this systemic metabolic profile may not fully capture metabolic alterations relevant to specific disease outcomes of interest. In other words, it may be more informative to construct age acceleration measures from biological specimens etiologically relevant to a given disease outcome, e.g., cerebrospinal fluid for neurodegenerative outcomes.

A notable limitation of our pathway enrichment approach is that *mummichog* infers metabolite identities using observed m/z and RI features, which may result in inaccurate metabolite assignments. As such, there is no guarantee that the inferred metabolite identities correspond exactly to the biochemical compounds matched against Metabolon's proprietary reference libraries. However, many of the significantly enriched metabolic networks we identified have also previously been reported in the literature, providing some degree of confidence in biological interpretation despite annotation uncertainties.

Another potential limitation of the model training procedure and association analyses is insufficient hyperparameter optimization. As a result of computational concerns, we selectively optimized a few high-yield hyperparameters in LightGBM, which may have limited the models predictive performance. A more exhaustive tuning approach may result in more accurate age acceleration estimates, though it is unclear whether improved predictive accuracy of the underlying model actually improves the discriminative ability of the of the age acceleration measure with respect to disease outcomes.

We also excluded all observations with missing exposure, outcome, and/or covariate data instead of handling the data missingness using imputation (i.e., performed a 'complete-case' analysis). The effect of this is likely minor since most variables exhibited relatively low degrees of missingness. Lastly, our analytic approach (particularly for outcomes in WHI) did not fully leverage the longitudinal/time-to-event nature of the data. Instead, we ignored differences in follow-up duration and evaluated associations using logistic regression as if follow-up was of a fixed duration and outcomes observed for all participants at a singular time point.

iv. Conclusions

Our analysis did not find evidence that metabolic age acceleration was associated with any of the disease outcomes/phenotypes of interest in the current investigation. Additionally, many of our effect estimates were in the opposite direction to which we hypothesized. Some of this may be explained by low statistical power and/or potential model calibration issues. Future work should prioritize outcomes with clearer metabolic involvement – e.g., diabetes, cardiovascular disease, dyslipidemia, etc. – to better assess the relevance of metabolomic aging measures. Additionally, integrating metabolomics data with other omics approaches (e.g., proteomic, epigenetic) to construct multi-ome predictors of age acceleration may explain a greater proportion of variation in chronologic age and provide a more biologically relevant understanding of age acceleration.

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VI. Appendix

Table 1. Participant descriptive characteristics by cohort

Characteristic	MEC N = 714[1]	WHI N = 2 929[1]	WRAP N = 1 200[1]
Age	67 (62 – 73)	61 (56 – 67)	63 (58 – 68)
Sex			
Female	0 (0%)	2 929 (100%)	833 (69%)
Male	714 (100%)	0 (0%)	366 (31%)
Unknown	0 (0%)	0 (0%)	1 (<0·1%)
BMI	27·3 (24·8 – 30·1)	29·8 (26·2 – 34·1)	27·8 (24·5 – 32·1)
Unknown	39	26	0
WHR	--	0·82 (0·77 – 0·87)	0·86 (0·79 – 0·95)
Unknown	714	13	0
Pack years	6 (0 – 20)	0 (0 – 13)	--
Unknown	52	116	1 200
Prostate cancer	354 (50%)	--	--
Unknown	0	2 929	1 200
Time to PCa (years)	5·0 (2·0 – 8·0)	--	--
Unknown	360	2 929	1 200
Breast cancer	--	286 (9·8%)	--
Unknown	714	0	1 200
Time to BCa (years)	--	11 (6 – 18)	--
Unknown	714	2 643	1 200
Any cancer	--	649 (22%)	--
Unknown	714	0	1 200
Time to any cancer (years)	--	13 (7 – 20)	--
Unknown	714	2 280	1 200

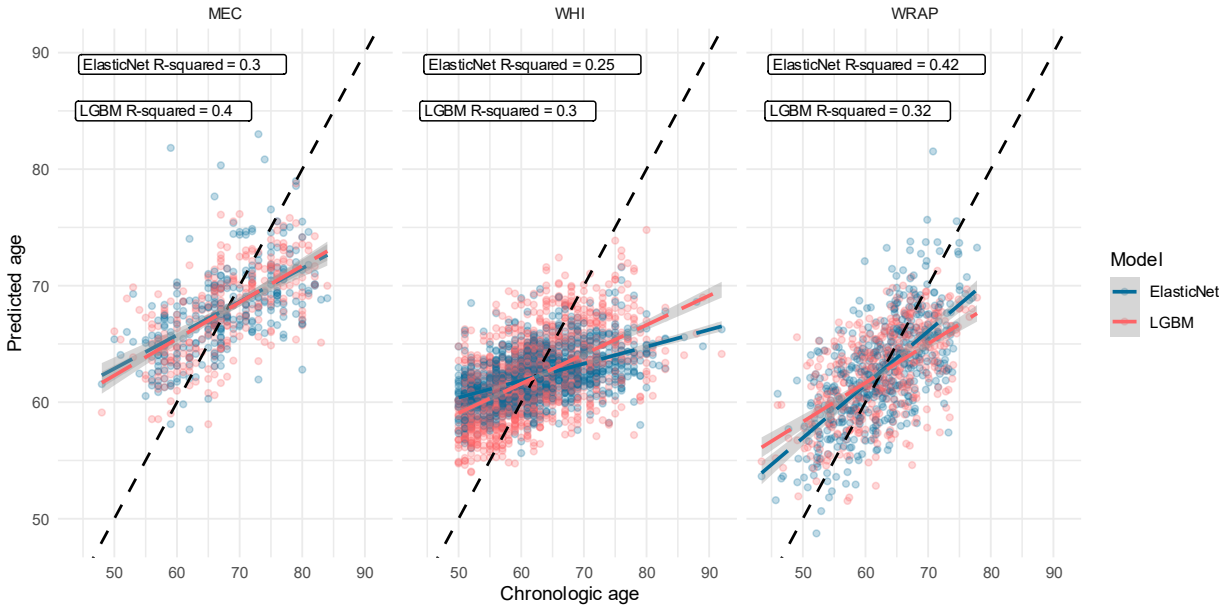
[1]Median (IQR); n (%)

Table 2. Associations with disease phenotypes by cohort

	Effect estimate	95% CI	p-value	N	Events
WHI					
Oncologic outcomes					
Breast cancer (overall – unadjusted)	1.01	0.69, 1.50	0.948	2929	286
Breast cancer (overall – adjusted)[1]	1.08	0.71, 1.65	0.716	2615	256
Any cancer[2]	1.11	0.84, 1.46	0.475	2899	641
Subtype and tumor characteristics					
Invasive breast cancer[3]	0.938	0.61, 1.44	0.768	2929	231
Regional/distant breast cancer[3]	1.17	0.49, 2.79	0.721	2697	54
Regional/distant breast cancer[4]	1.26	0.45, 3.53	0.663	269	54
Estrogen-receptor assay[5]	2.30	0.85, 6.47	0.105	248	185
Progesterone-receptor assay[5]	2.02	0.82, 5.17	0.132	244	154
HER2/neu assay[5]	0.326	0.07, 1.39	0.135	194	29
HER2+/HR+ vs. HER2-	0.532	0.11, 2.56	0.437	187	22
HER2+/HR- vs. HER2-	0.0546	0.00, 1.22	0.0946	172	7
Adiposity markers					
Body mass index	-0.217	-0.92, 0.48	0.543	2903	--
Waist-to-hip ratio	0.00733	0.00, 0.02	0.0829	2916	--
MEC					
Oncologic outcomes					
Prostate cancer (overall)[6]	1.30	0.80, 2.11	0.291	714	352
Aggressive/lethal prostate cancer[6]	0.885	0.43, 1.81	0.739	465	105
Aggressive/lethal prostate cancer[7]	0.470	0.19, 1.10	0.0886	315	105
Prostate-specific antigen	-3.47	-7.9, 0.95	0.125	546	--
Adiposity markers					
Body mass index	0.118	-0.93, 1.2	0.826	675	--
WRAP					
Cognitive outcomes					
Learning	0.00744	-0.16, 0.18	0.932	4595	--
Delayed recall	0.0359	-0.14, 0.21	0.688	4595	--
Executive functioning	0.0532	-0.12, 0.23	0.549	4595	--
Adiposity markers					
Body mass index	0.783	-0.39, 2.0	0.192	4590	--
Waist-to-hip ratio	-0.0190	-0.04, 0.00	0.0748	4588	--

1. model adjusted for smoking status, BMI, age at menopause, age at menarche, alcohol consumption, and oral contraceptive; 2. model adjusted for smoking status; 3. versus no breast cancer; 4. versus in situ/localized breast cancer; 5. positive versus negative assay result; 6. versus controls; 7. versus non-aggressive prostate cancer

Figure 1. Metabolite-predicted age vs. chronologic age at blood draw



Black dashed line corresponds to $y = x$. Blue and red dashed lines correspond to linear regression fits for ElasticNet and LightGBM predictions, respectively (95% CI depicted in gray).

Figure 2. Top features by gain score. A: MEC; B: WRAP; C: WHI.

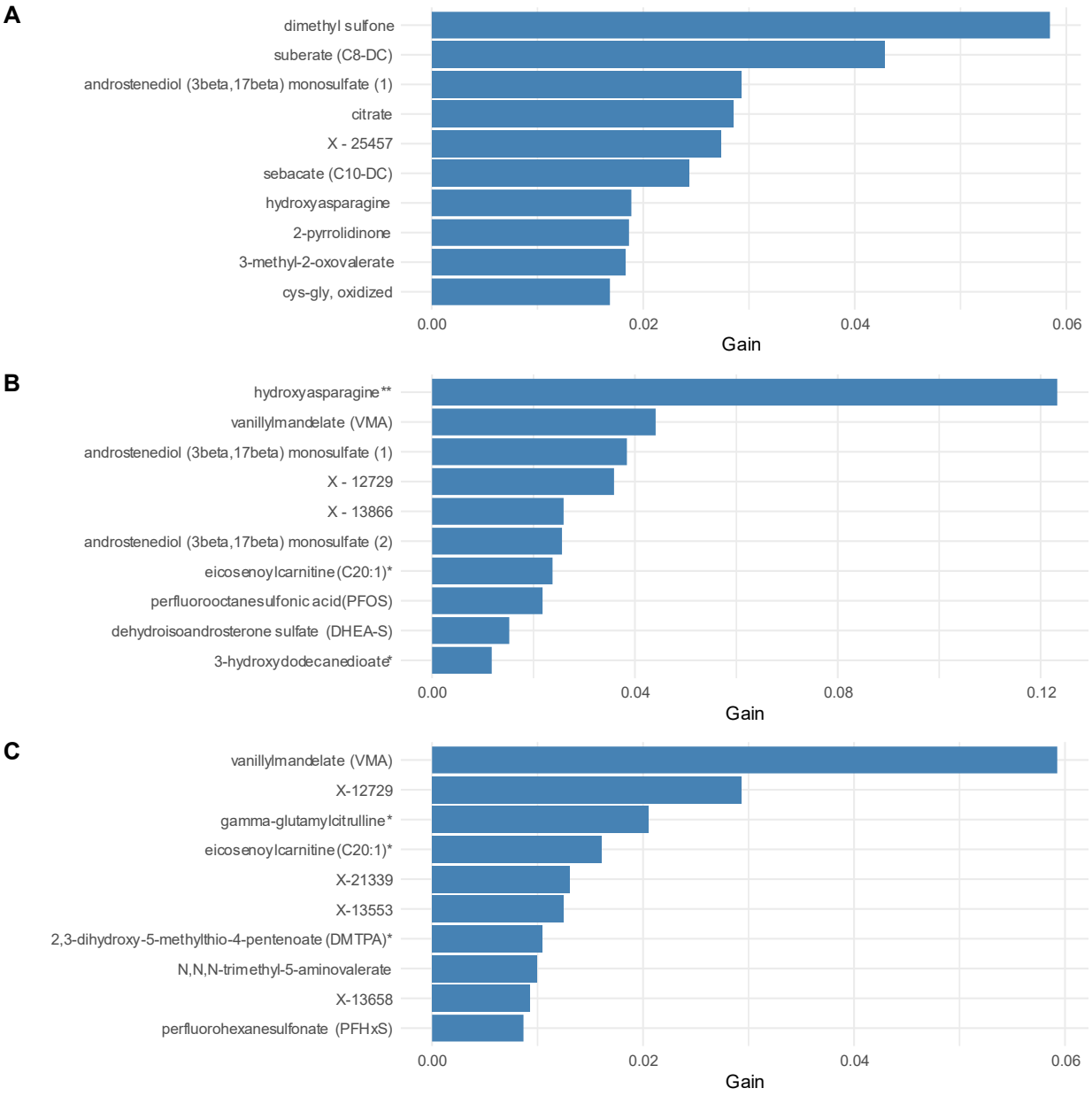


Figure 3. Distribution of predicted age acceleration by method and cohort

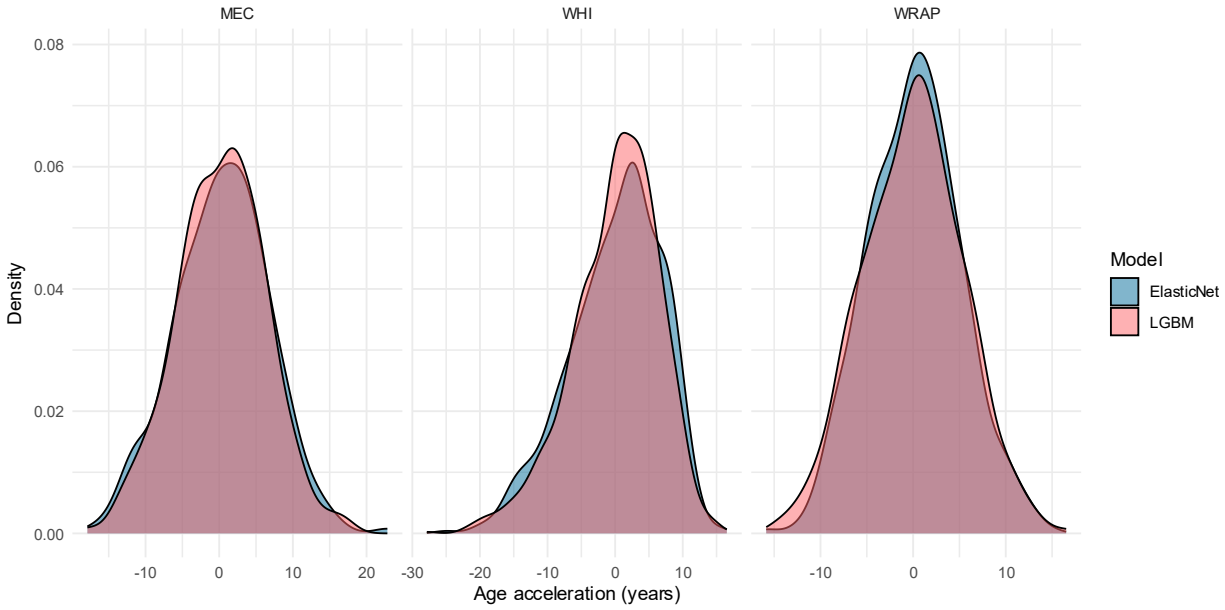
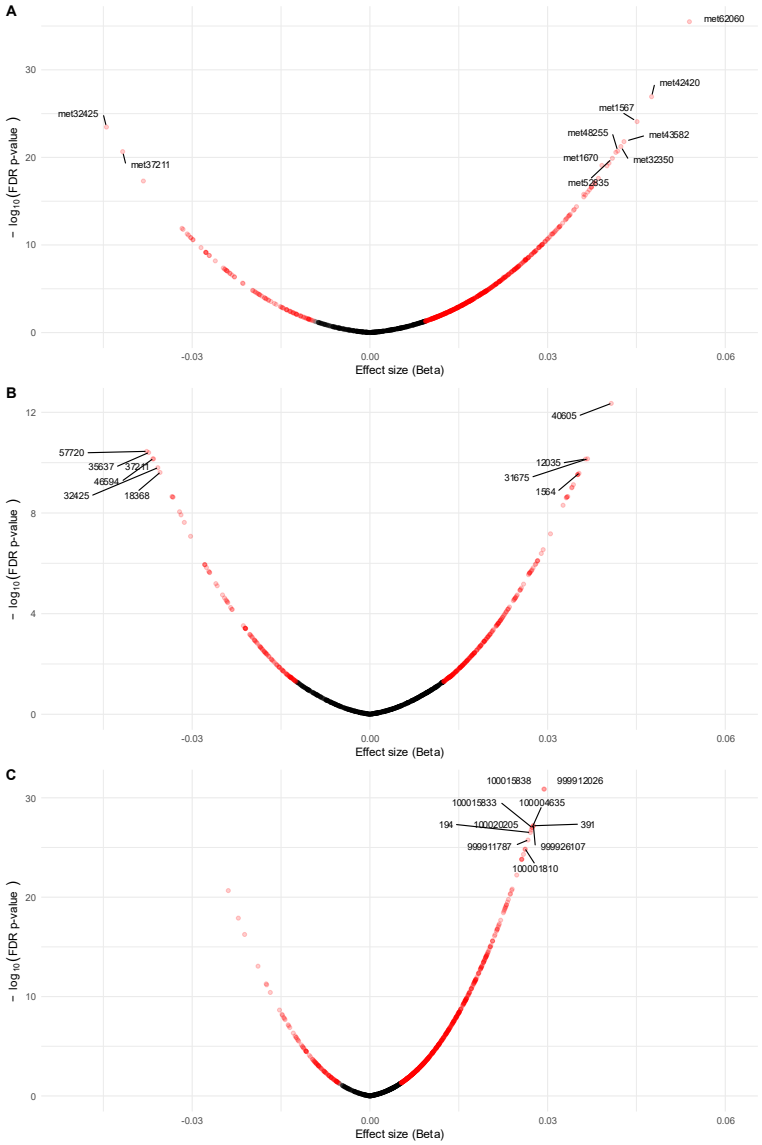


Figure 4. Metabolome-wide association study (MWAS) of chronologic age



A. WRAP metabolites – met1567: vanillylmandelate (VMA); met1670: urea; met32350: 1-methyl-4-imidazoleacetate; met32425: dehydroisoandrosterone sulfate (DHEA-S); met37211: androstenediol (3beta,17beta) monosulfate (1); met42420: erythronate*; met43582: 5-(galactosylhydroxy)-L-lysine; met48255: arabonate/xylonate; met52835: N-carbamoylvaline; met62060: hydroxyasparagine**

B. MEC metabolites – 31675: 2-pyrrolidinone; 46594: X – 11372; 57720: X – 24953; 37211: androstenediol (3beta,17beta) monosulfate (1); 40605: butyrate (4:0); 1564: citrate; 18368: cys-gly, oxidized; 35637: cysteinylglycine; 32425: dehydroepiandrosterone sulfate (DHEA-S); 12035: pelargonate (9:0)

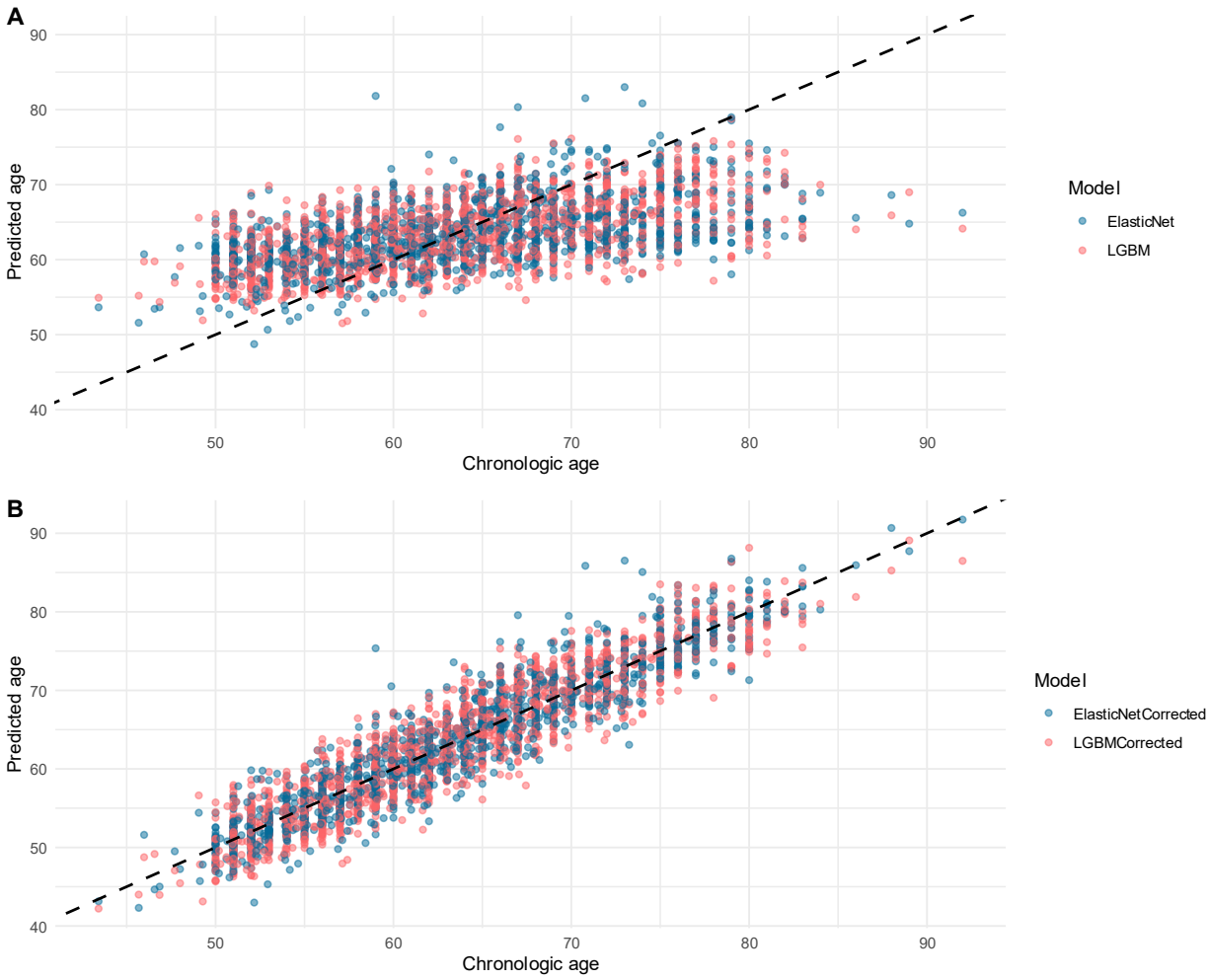
C. WHI metabolites – 194: N-formylmethionine; 391: citrulline; 100001810: dimethylarginine (SDMA + ADMA); 100004635: methionine sulfone; 100015833: arachidoylcarnitine (C20)*; 100015838: eicosenoylcarnitine (C20:1)*; 100020205: hydroxyasparagine**; 999911787: X-11787; 999912026: X-12026; 999926107: X-26107

Table 3. Network analysis for pathway enrichment

	Pathway size	Overlap size	p-value
MEC			
Hexose phosphorylation	9	8	0.0068
Glycine, serine, alanine, and threonine metabolism	30	17	0.0210
C21-steroid hormone biosynthesis and metabolism	10	8	0.0239
Urea cycle/amino acid group metabolism	18	8	0.0239
Androgen and estrogen biosynthesis and metabolism	7	6	0.0262
Pentose phosphate pathway	15	11	0.0292
Galactose metabolism	9	7	0.0399
Porphyrin metabolism	9	7	0.0399
Sialic acid metabolism	6	5	0.0481
WRAP			
Fatty acid metabolism	10	9	0.0097
Alanine and aspartate metabolism	10	9	0.0097
Vitamin B3 (nicotinate and nicotinamide) metabolism	6	6	0.0101
Pentose and glucoronate interconversions	12	10	0.0213
Aminosugars metabolism	5	5	0.0234
Aspartate and asparagine metabolism	18	14	0.0243
Carnitine shuttle	4	4	0.0402
Nitrogen metabolism	4	4	0.0402
WHI			
Carnitine shuttle	24	24	0.0001
TCA cycle	10	10	0.004
C21-steroid hormone biosynthesis and metabolism	6	6	0.0266
Bile acid biosynthesis	14	12	0.0355
Lysine metabolism	16	13	0.0460
Urea cycle/amino acid group metabolism	9	8	0.0487
Methionine and cysteine metabolism	9	8	0.0487

VII. Supplementary materials

Supplementary Figure 1. Predicted age vs. chronologic age with and without statistical age correction



Supplementary Table 1. Sensitivity analyses with separate statistical adjustment for age

	Effect estimate	95% CI	p-value	N	Events
WHI					
Oncologic outcomes					
Breast cancer (overall – unadjusted)	1.01	0.69, 1.48	0.953	2929	286
Breast cancer (overall – adjusted)[1]	1.08	0.72, 1.63	0.702	2615	256
Any cancer[2]	1.10	0.84, 1.45	0.477	2899	641
Subtype and tumor characteristics					
Invasive breast cancer[3]	0.940	0.62, 1.43	0.772	2929	231
Regional/distant breast cancer[3]	1.16	0.50, 2.71	0.736	2697	54
Regional/distant breast cancer[4]	1.25	0.45, 3.52	0.666	269	54
Estrogen-receptor assay[5]	2.33	0.87, 6.54	0.0974	248	185
Progesterone-receptor assay[5]	2.03	0.83, 5.16	0.125	244	154
HER2/neu assay[5]	0.407	0.10, 1.56	0.191	194	29
HER2+/HR+ vs. HER2-	0.623	0.14, 2.82	0.535	187	22
HER2+/HR- vs. HER2-	0.153	0.02, 1.61	0.0987	172	7
Adiposity markers					
Body mass index	-0.224	-0.93, 0.48	0.533	2903	--
Waist-to-hip ratio	0.00734	0.00, 0.02	0.0830	2916	--
MEC					
Oncologic outcomes					
Prostate cancer (overall)[6]	1.32	0.82, 2.15	0.258	714	352
Aggressive/lethal prostate cancer[6]	0.868	0.43, 1.76	0.695	465	105
Aggressive/lethal prostate cancer[7]	0.542	0.24, 1.21	0.139	315	105
Prostate-specific antigen	-1.60	-4.4, 1.2	0.271	546	--
Adiposity markers					
Body mass index	0.146	-0.92, 1.2	0.790	675	--
WRAP					
Cognitive outcomes					
Learning	-0.0343	-0.21, 0.15	0.710	4595	--
Delayed recall	-0.000767	-0.18, 0.18	0.993	4595	--
Executive functioning	0.0367	-0.16, 0.23	0.709	4595	--
Adiposity markers					
Body mass index	0.783	-0.39, 2.0	0.192	4590	--
Waist-to-hip ratio	-0.0189	-0.04, 0.00	0.0766	4588	--

1. model adjusted for smoking status, BMI, age at menopause, age at menarche, alcohol consumption, and oral contraceptive; 2. model adjusted for smoking status; 3. versus no breast cancer; 4. versus in situ/localized breast cancer; 5. positive versus negative assay result; 6. versus controls; 7. versus non-aggressive prostate cancer