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Original Research

Slower Pace of Epigenetic Aging and Lower Inflammatory Indicators in Females Following a Nutrient-Dense, Plant-Rich Diet Than Those in Females Following the Standard American Diet

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A B S T R A C T

Background: Plant-based diets are associated with lower inflammatory biomarkers and reduced risk of age-related chronic diseases. Epigenetic biomarkers of aging are DNA methylation-based tools that estimate biological age and rate of aging, providing insights into age-related health risks. Healthy diet and lifestyle indicators correlate with slower epigenetic aging.

Objectives: Neither inflammatory biomarkers nor epigenetic aging has yet been studied in the nutrient-dense, plant-rich (Nutritarian) diet, a plant-based diet that emphasizes specific plant foods, such as cruciferous vegetables, beans and other legumes, onions and garlic, mushrooms, berries, nuts, and seeds. We aimed to compare inflammatory status and epigenetic age acceleration in females following a Nutritarian diet with those of females following a standard American diet (SAD).

Methods: We investigated dietary inflammatory potential, epigenetic age acceleration using first, second, and third-generation clocks, and additional health-related epigenetic biomarkers in this retrospective cohort study of 48 females who habitually (≥5 y) follow a Nutritarian diet and 49 females without obesity who habitually (≥5 y) follow a SAD. Participants completed a series of online questionnaires and provided a blood sample.

Results: Epigenetic age acceleration, indicated by the third-generation clock DunedinPACE, was significantly slower in the Nutritarian group than that in the SAD group ($P = 4.26 \times 10^{-6}$). The Nutritarian diet group showed lower dietary inflammatory potential, as indicated by Empirical Dietary Inflammatory Pattern and Dietary Inflammatory Index. We observed differences in methylation-predicted immune cell subsets (lower neutrophils and higher T regulatory cells) and a lower epigenetic biomarker proxy for C-reactive protein, both of which suggested a lower inflammatory status in the Nutritarian group. Epigenetic biomarker proxies for LDL cholesterol, body mass index (BMI), insulin-like growth factor binding protein 5, and blood glucose were also lower in the Nutritarian group.

Conclusions: Our findings suggest the Nutritarian diet could help reduce chronic inflammation and slow epigenetic aging.

Keywords: epigenetic clocks, DNA methylation, plant-based diet, immune system, inflammation

Introduction

The standard American diet (SAD) is a term used to describe the macronutrient and micronutrient composition of the food the majority of Americans customarily eat, based on a consensus of nutrition experts drawing on data from the National Health and Nutrition Examination Survey (NHANES). The USDA Dietary Guidelines for Americans define the SAD as being too low in fresh fruits and vegetables, whole grains, lean protein, and healthy fats and too high in red meat, high-fat dairy products,

Abbreviations: AFFQ, Arizona Food Frequency Questionnaire; CRP, C-reactive protein; DII, Dietary Inflammatory Index; DMR, differentially methylated region; DNAm, DNA methylation; EAA, epigenetic age acceleration; E-DII, Energy-adjusted Dietary Inflammatory Index; EDIP, Empirical Dietary Inflammatory Pattern; EWAS, epigenome-wide association study; FDR, false discovery rate; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor–binding protein NDPR, nutrient-dense, plant-rich; REDCap, Research Electronic Data Capture; SAD, standard American diet; Treg, T regulatory cell.

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processed and fast foods, refined carbohydrates, added sugars, salt, and calories [1]. Plant-based diets rich in vegetables, legumes, nuts and seeds, fruits, and whole grains or diets higher in plant protein and lower in animal protein have been associated with a reduction in risk of cardiovascular disease, diabetes, cancers, and all-cause mortality [2–10].

The Nutritarian diet—also called a nutrient-dense, plant-rich (NDPR) diet—is a plant-based diet that emphasizes specific foods with evidence for antioxidant, anti-inflammatory, cardioprotective, and antitumor properties and aims for a low-to-moderate glycemic load. Beans and other legumes are emphasized because of their high viscous and fermentable fiber content, including resistant starch [11]. Substances derived from cruciferous vegetables, the onion and garlic family, berries, flax and chia seeds, and mushrooms have demonstrated anticancer properties in vitro [12–18]. Most of the fat in the Nutritarian diet is derived from nuts, seeds, and avocado. Nuts are associated with healthy lipid levels and lower risk of cardiovascular disease and all-cause mortality [19,20]. Studies have demonstrated the anti-inflammatory mechanisms of these foods or their constituent phytochemicals [21–24]. Moreover, dietary interventions increasing legumes, garlic, berries, almonds, or flaxseed have led to reduced levels of circulating inflammatory markers, such as C-reactive protein (CRP) or TNF-α [25–30].

Previous research on the Nutritarian diet suggests that adopting this diet improves cardiovascular disease risk markers, glycemic control in those with diabetes, and weight loss [31–33]. Participants also reported a reduction in uncomfortable symptoms associated with hunger, such as fatigue, light-headedness, and irritability [34].

Chronic inflammation is recognized as a contributor to biological aging and age-related chronic diseases, but diet can modulate inflammation [35]. Additionally, obesity is a contributor to chronic inflammation [36]. Healthful plant-based diets—higher in vegetables, fruits, legumes, whole grains, and nuts and lower in refined grains, potatoes, and sweets—have been associated with lower levels of inflammatory markers such as CRP [37,38]. Studies on associations between food groups or food components and inflammatory markers have been used to generate scoring systems evaluating dietary inflammatory potential, including the Empirical Dietary Inflammatory Pattern (EDIP) and Dietary Inflammatory Index (DII). Several foods and food components considered anti-inflammatory in these scoring systems are emphasized in a Nutritarian diet, including leafy green vegetables, fiber, onions, garlic, anthocyanidins, and ω-3 (n–3) fatty acids [39,40]. A higher (more proinflammatory) DII score has been associated with increased risk of frailty, type 2 diabetes, higher total and LDL cholesterol concentrations, cancers, and all-cause mortality in observational studies [41–46]. Likewise, a higher (more proinflammatory) EDIP score has been associated with increased risk of several cancers, hip fracture, nonalcoholic fatty liver disease, and all-cause mortality [47–51].

Biological aging encompasses a range of interrelated processes thought to underlie most age-related chronic diseases, such as type 2 diabetes, cardiovascular disease, and cancer. Unlike chronologic aging, biological aging involves mechanisms such as oxidative damage to DNA and proteins, protein dysfunction (e.g., aggregation and misfolding), cellular senescence, chronic inflammation, mitochondrial dysfunction, telomere attrition, and a reduced capacity for tissue repair [52–54].

Epigenetic age and epigenetic age acceleration (EAA) are indicators of biological aging, assessed by a variety of algorithms measuring DNA methylation (DNAm) at CpG sites across the genome. These metrics correlate with chronologic age, as well as lifestyle factors and chronic diseases. Different rates of epigenetic aging have been observed within groups of individuals of the same chronologic age [55,56]. Accelerated epigenetic age indicators have been associated with all-cause, cardiovascular, and cancer mortality, as well as chronic diseases, cognitive dysfunction, and frailty [56–60]. In contrast, healthy dietary and lifestyle behaviors such as vegetable intake, biomarkers of fruit and vegetable intake, exercise, smoking cessation, and higher scores on healthy diet indices have been associated with lower epigenetic age or EAA [61–67].

Several epigenetic age algorithms can be found in the literature. The first-generation Horvath and Hannum epigenetic clocks were developed using machine learning models trained to predict chronologic age [68,69]. The second-generation GrimAge and PhenoAge clocks were trained to predict aging-associated factors other than chronologic age, such as mortality or incidence of chronic disease [63,70]. DunedinPACE, a third-generation clock, was trained on data from 3 time points over 12 years to detect DNAm patterns associated with the pace of aging. This clock estimates the rate of biological aging in years of epigenetic aging per chronologic year, rather than providing a static biological age at the time of measurement [71]. The different clocks often show limited agreement with one another, suggesting that they may be indicators of different aspects of the biological aging process [72].

Nutritarian diet guidelines are derived from the literature and aim to reduce chronic inflammation and enhance lifespan; however, indicators of inflammation or biological aging have not been evaluated in previous studies. Because vegetable intake and healthy diet indices have been associated with slower epigenetic aging, we hypothesized that indicators of EAA and chronic inflammation would be lower in Nutritarian diet–consuming participants than those in SAD-consuming participants. Thus, we investigated EAA measured by first-generation, second-generation, and third-generation clocks in 2 groups of nonsmoking females between the ages of 40 and 75 y: 47 females who had adhered to an NDPR diet for 5 y or more and 49 who followed a SAD. Secondary outcomes included dietary inflammatory potential, methylation-predicted immune cell populations, and epigenetic biomarker proxies (EBPs).

Methods

Study population

This was a retrospective cohort study where participants were recruited and expected to provide a 2-h time commitment. Compensation was complimentary epigenetic test results provided to the participants. Participants were recruited from previous studies; existing databases; direct email; and social media and enrolled between February and August 2023, and data were collected until October 2023. Each participant was screened for eligibility via e-mail and follow-up phone calls.

Inclusion criteria included being female, a United States citizen, aged 40 to 75 years, nonpregnant, and nonsmoking. Participants were excluded if they had a history of cancer diagnosis,

were being treated for an autoimmune disease, or were taking prescription medications other than those for hypothyroidism. Additional inclusion criteria for the SAD group were 5 y or more habitually following a standard American dietary pattern; not currently or within the past 2 years following any weight loss plan, BMI (in kg/m^2) of $<$30.0; and no more than 8 alcoholic drinks per week. Additional inclusion criteria for the Nutritarian group were 5 y or more habitually following a Nutritarian dietary pattern with self-reported adherence 90% or greater; BMI of $\leq$23 for $\geq$2 y, and no more than 2 alcoholic drinks per week. We aimed for the BMI criteria to reflect BMI of real-world females habitually following plant-based and typical Western diets. For the Nutritarian group, the BMI criterion was based on studies of females on long-term plant-based diets: in the EPIC-Oxford study, the mean BMI of vegan females was 21.9, and in the Swedish Mammography Cohort, the mean BMI of vegan females was 23.3 [73,74].

A Nutritarian diet was defined as emphasizing cruciferous vegetables, beans and legumes, the onion and garlic family, mushrooms, berries, nuts, and seeds and avoiding or minimizing animal foods, oils, and refined carbohydrate foods. A study coordinator discussed the definition of the diet with prospective Nutritarian group participants via telephone screening to confirm their understanding of the definition of a Nutritarian diet. Participants confirmed via telephone screening and electronic questionnaires the length of time they had been following the diet and self-reported adherence. A SAD was defined as including variety of meat and dairy foods, grain products (pasta, breads, cereals, and baked goods), fruits, and vegetables with no restrictions on specific foods or nutrients (such as carbohydrates or fats). Eligibility of SAD group participants was also confirmed via telephone screening before inclusion in the study.

Diet, lifestyle, and well-being questionnaires

Participants reported health and demographic information, including age and gender, ethnicity, education, height and weight, caffeine and alcohol use, a history of tobacco use, a medical history, and current medications. Demographic, health, and lifestyle information were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted by the Nutritional Research Foundation [75–77]. REDCap is a secure, web-based software platform designed to support data capture for research studies, providing *1*) an intuitive interface for validated data capture; *2*) audit trails for tracking data manipulation and export procedures; *3*) automated export procedures for seamless data downloads to common statistical packages; and *4*) procedures for data integration and interoperability with external sources.

Self-reported physical activity was assessed with the International Physical Activity Questionnaire [78]. Diet was assessed using the Arizona Food Frequency Questionnaire (AFFQ), a semiquantitative 175-item food frequency questionnaire, in which respondents reported how often they usually consume each particular food and whether their usual portion size of that food was small, medium, or large. Multivitamin and other dietary supplement use information was also collected as part of the AFFQ [79,80].

The inflammatory potential of participants' diets was calculated using the EDIP and DII [39,40]. EDIP was developed based on correlations between intake of 18 food groups (9 considered proinflammatory and 9 considered anti-inflammatory) and circulating inflammatory biomarkers CRP, IL-6, and TNFαR2, as described previously. The EDIP scoring system indicates the inflammatory potential of an individual's diet with more negative scores indicating anti-inflammatory diets and more positive scores indicating proinflammatory diets [39].

The DII was developed to quantify the inflammatory potential of individuals' diets on a scale from maximally anti-inflammatory (most negative score) to maximally proinflammatory (most positive score), as described in detail previously [40]. Briefly, the DII scoring algorithm is based on correlations between 45 food parameters and 6 inflammatory biomarkers: IL-1, IL-4, IL-6, IL-10, TNF-α, and CRP. Self-reported values for 38 of the 45 food parameters in the DII were available from the AFFQ. These values were translated into *z*-scores using a global comparative database consisting of data from 11 countries by subtracting from the individual's self-report value the mean of the global database and then dividing by the standard deviation. These scores were then converted to proportions (i.e., with values ranging from 0 to 1) and centered on zero by doubling each and subtracting 1. These centered proportions were then multiplied by their respective coefficients (overall food parameter-specific inflammatory effect scores) to obtain DII scores for each food parameter. These were summed to obtain the overall DII score. Energy-adjusted Dietary Inflammatory Index (E-DII) scores were also calculated by calculating DII per 1000 kcal consumption [81,82]. DII and E-DII scores have a potential range from approximately -9 to $+8$. For this study, the following 38 of the 45 food parameters were used to calculate an individual's overall DII score: energy, carbohydrate, fiber, total fat, monounsaturated fat, polyunsaturated fat, ω-3 fatty acids, ω-6 fatty acids, saturated fat, trans fat, protein, alcohol, tea, caffeine, onions and garlic, peppers, anthocyanidins, cholesterol, flavan-3-ols, flavones, flavonols, flavanones, iron, isoflavones, magnesium, niacin, riboflavin, thiamin, vitamin B-6, vitamin C, vitamin E, zinc, β-carotene, folate, selenium, vitamin A, vitamin B-12, and vitamin D [81].

The study was approved by the Institute of Regenerative and Cellular Medicine Institutional Review Board (IRCM-IRB) and was conducted in compliance with the Belmont Report and Good Clinical Practice Guidelines (IRB: IRCM-2022-335). Informed consent was collected using REDCap [83]. All participants received their personal test results after their involvement in the study. All shared personal information was deidentified.

DNAm assessment

Participants were shipped a test kit, which included a lancet, blood spot card, and mailer to return the sample to TruDiagnostic. DNA extraction was performed, and 500 ng of DNA was subjected to bisulfite conversion using the EZ DNA Methylation kit from Zymo Research, following the manufacturer's protocol. The bisulfite-converted DNA samples were then randomly allocated to designated wells on the Infinium HumanMethylationEPIC BeadChip or the Infinium HumanMethylationEPICv2 BeadChip. The samples were amplified, hybridized onto the array, and subsequently stained. After washing steps, the array was imaged using the Illumina iScan SQ instrument to capture raw image intensities, enabling further analysis.

The *Minfi* R package was used for preprocessing DNAm data [84]. In the sample quality control, we did not identify any

samples with aberrant methylation levels or background signal levels (mean P value > 0.05).

Statistical analyses and reproducibility

DNAm clocks and related measures

We used DNAm data to calculate a series of measures broadly known as epigenetic clocks. We computed 4 clocks designed to predict the chronologic age of the donor—Horvath Pan Tissue [69], Horvath skin and blood [69], and Hannum [68]; 3 clocks designed to predict mortality—the DNAmPhenoAge [84], GrimAge [70], and OMICmAge clocks [85]; a clock to measure telomere length—DNAmTL [86]; and a DNAm measure of the rate of deterioration in physiological integrity, the DunedinPACE [87].

Nonprincipal component (non-PC)-based Horvath, Hannum, and DNAmPhenoAge epigenetic metrics were calculated using the methyAge function in the *ENMix* R package [88]. To calculate the PC-based epigenetic clock for the Horvath multitissue clock, Hannum clock, DNAmPhenoAge clock, GrimAge clock, and telomere length, we used the custom R script available via GitHub (https://github.com/MorganLevineLab/PC-Clocks). The OMICmAge clock was calculated using a custom script. The pace of the aging clock, DunedinPACE, was calculated using the PACEProjector function from the DunedinPACE package available via GitHub (https://github.com/danbelsky/DunedinPACE).

To calculate the EAA of the age-based clocks (all except DunedinPACE because it measures the pace of aging), we fit a regression model between the chronologic age of the individuals and the different epigenetic age measures. We also included the array type and BMI to control for potential batch effects and differences due to BMI. The *t*-tests were performed between groups at a significance level of P value of <0.05. Most of the metrics followed the assumptions of the *t* test because the samples were independent; we evaluated normality using the Shapiro test and checked the homogeneity of the variance using the Bartlett test.

Twelve immune cell subsets were estimated: basophils, memory B cells, naïve B cells, memory $CD4^+$ T cells, naïve $CD4^+$ T cells, memory $CD8^+$ T cells, naïve $CD8^+$ T cells, eosinophils, monocytes, neutrophils, natural killer, and T regulatory cells (Tregs). These immune cells were estimated from methylation data using a previously constructed DNAm reference matrix for blood cell subtypes, which R^2 was 0.96 or above to immune cell subsets measured by RNA-seq and flow cytometry [89].

EBPs for 109 proteins, 266 metabolites, and 21 clinical variables were estimated using DNAm. To this end, we used the models previously developed by Chen et al. [85].

Diet, lifestyle, and demographic data

Analysis of continuous variables (e.g., age and physical, psychosocial, dietary, and activity characteristics) included calculating means and SDs. At the same time, frequencies and percentages were determined for categorical variables (e.g., race, ethnicity, marital status, and education level). Independent-sample *t*-tests were used to analyze the differences in outcomes between the 2 diets (i.e., Nutritarian and SAD groups). IBM SPSS Statistics, version 29, was used to analyze diet, lifestyle, and demographic data.

Epigenome-wide association study

We conducted an epigenome-wide association study (EWAS) using the *limma* package to identify significant differences between diets in each CpG site across the genome. We adjusted all models by age, array type, BMI, beadchip, and immune cell types that were significantly associated with the outcome (memory $CD8^+$ T cells, memory B cells, Tregs, and neutrophils). We identified as significant those probes with a nominal P value of $<1 \times 10^{-4}$ to account for multiple comparisons. We also created a Q–Q plot and estimated the λ value to assess the inflation of the model.

Differentially methylated region analysis

After evaluating each probe separately, we assessed differentially methylated regions (DMR) using the *DMRcate* package. We set the minimum number of CpG sites for being a DMR to 5 with a maximum of 1000 bp between CpG sites. For each DMR, we obtained information on the annotated coordinates, the number of CpG sites belonging to the DMR, the global and minimum false discovery rate (FDR), the maximum and mean coefficients, and the genes overlapped with the region. We identified as significant those with a global FDR of <0.05.

Results

Characteristics and dietary inflammatory potential of participants in Nutritarian and SAD groups

Forty-seven participants in the Nutritarian diet group and 48 in the SAD group completed all questionnaires and epigenetic aging blood test kits (Figure 1). One participant in the Nutritarian group and 1 in the SAD group completed the diet, health, and lifestyle questionnaires but did not successfully complete the blood test kit by the end of the data collection period. These 2 participants were not included in DNAm analyses. As shown in Table 1, education level and age were not significantly different between groups, with mean ages of ~59 and ~57 y in the Nutritarian and SAD groups, respectively. However, menopausal status was significantly different between groups, with 61.7% of the Nutritarian group and 12.5% of the SAD group reporting they had been through menopause. Although energy intake and physical activity did not differ between groups, body weight [121.60 compared with 152.49 lb (kg); $P < 0.001$] and BMI (20.49 compared with 25.33; $P < 0.001$) were significantly lower in the Nutritarian group than those in the SAD group, as expected based on the inclusion criteria for each group. Mean time spent sitting in the SAD group was greater than that of the Nutritarian group, but the difference was not statistically significant. Sleep duration was significantly different between groups; however, data on sleep were missing for approximately one-fourth of the Nutritarian group and two-thirds of the SAD group.

We used 2 dietary scoring methods to indicate the inflammatory potential of participants' diets: EDIP and DII. EDIP is based on food groups (such as leafy green and dark yellow vegetables), whereas the DII scoring algorithm is based mainly on nutrients and food components (such as polyunsaturated fatty acids, zinc, and isoflavones). EDIP scores in the Nutritarian group were significantly lower than those in the SAD group (mean difference of −0.39; $P < 0.001$) (Table 1), indicating a

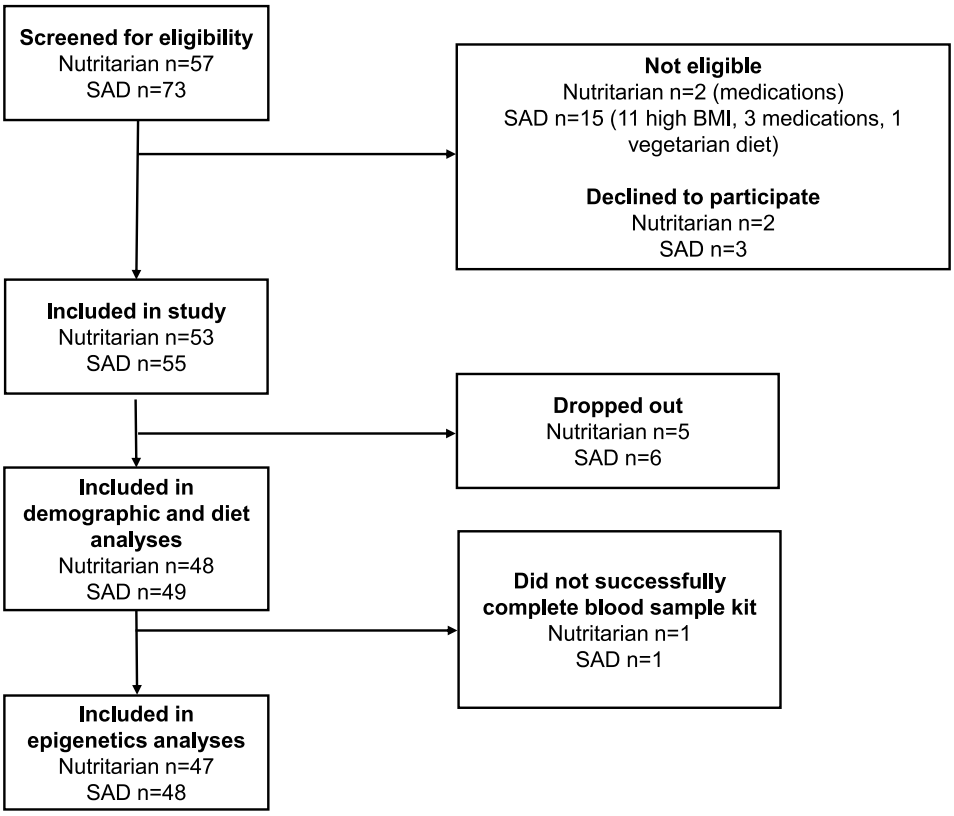

FIGURE 1. Participant flowchart. SAD, standard American diet.

lower dietary inflammatory potential. Likewise, DII scores and E-DII scores were lower in the Nutritarian group (mean differences of −3.38 and −2.44, respectively; both $P < 0.001$) (Table 1). Additional sociodemographic, health, and lifestyle characteristics are presented in Table 1.

Epigenetic age acceleration

EAA was calculated based on several epigenetic clocks that have been previously described: PC-corrected Horvath, PCHannum, PCGrimAge, PCPhenoAge, OMICmAge, as well as DNAm-predicted telomere length (PCDNAmTL). We also calculated DunedinPACE, a measure not in years but a pace of aging. Although none of the EAA metrics was significantly different between groups, DunedinPACE was significantly lower in those participants who followed the Nutritarian diet than that in those who followed SAD (mean Nutritarian diet group = 0.8116; mean SAD group = 0.9086; $P = 4.27 \times 10^{-6}$) (Figure 2). To account for outliers, we removed those samples with DunedinPACE beyond 3 SDs from the mean, and we observed the same effect (mean Nutritarian diet group = 0.8116; mean SAD group = 0.8984; $P = 2.02 \times 10^{-6}$). We also regressed out menopausal status, BMI, and array type from DunedinPACE to evaluate whether the difference was consistent. Adjusting for menopausal status only, the reduction in the Nutritarian group remained significant ($P = 0.00883$). Adjusting for array only, the difference remained significant ($P = 0.00433$). After adjustment for both array and BMI, DunedinPACE remained lower in the Nutritarian group than that in the SAD group; however, the difference between groups was no longer significant.

Methylation-predicted immune cell subsets

We used EpiDISH (2023) to quantify 12 different immune cell subsets and assess differences between both groups (Figure 3 and Supplemental Figure 1). Compared with the SAD group, the Nutritarian group showed higher estimates of CD8T memory cells ($P = 0.001$), B memory cells ($P = 6.34 \times 10^{-5}$), and Tregs ($P = 0.025$). Conversely, the estimated number of neutrophils was lower in the Nutritarian group than that in the SAD group ($P = 0.030$). Because there were some values that were out of range for these immune subsets, we performed a sensitivity analysis after removing those values that were outside the range mean ± 3 SDs. In this case, CD8T memory cells and B memory cells were still significant after removing the outliers ($P = 0.002$ and $P = 5.99 \times 10^{-5}$, respectively). However, Tregs and neutrophils were no longer significant ($P = 0.0732$ and $P = 0.0535$, respectively).

Epigenetic biomarker proxies

EBPs for multiple proteins, metabolites, and clinical variables were estimated. A Wilcoxon test was used to compare the estimates between diet groups.

Of 396 EBPs estimated, we identified 90 with an FDR of <0.05 (Supplemental Table 1). EBPs with highly significant differences between groups included proxies for bone morphogenetic protein 1, eukaryotic translation termination factor 1, insulin-like growth factor–binding protein (IGFBP)-5, and LDL cholesterol. Although bone morphogenetic protein 1, TLL1, LDL cholesterol, and IGFBP-5 had lower concentrations in the Nutritarian group than those in the SAD group, higher

TABLE 1
Descriptive characteristics of participants ($N = 97$).

Participants' characteristics	Nutritarian group ($n = 48$)	Standard American diet group ($n = 49$)	P
Sociodemographic			
Age (y) ($n = 48$, Nutritarian; $n = 49$, SAD), mean (SD), range	58.96 (8.06), 42–72	56.51 (8.89), 42–73	0.079
Race/ethnicity ($n = 47$, Nutritarian; $n = 48$, SAD)			
Caucasian/White	43 (91.5)	40 (83.3)	0.190
African American/Black	2 (4.3)	1 (2.1)	
Asian American/Asian	1(2.1)	0 (0.0)	
Hispanic/Latino	0 (0.0)	4 (8.3)	
Native American/Alaskan Native	1 (2.1)	1 (2.1)	
Middle Eastern	0 (0.0)	2 (4.2)	
Marital status ($n = 38$, Nutritarian; $n = 15$, SAD)			
Single	3 (7.9)	2 (13.3)	0.233
Married	26 (68.4)	9 (60.0)	
Widowed	5 (13.2)	0	
Divorced/separated	4 (10.5)	4 (26.7)	
Education ($n = 47$, Nutritarian; $n = 48$, SAD)			
High school/equivalent	3 (6.4)	2 (4.2)	0.192
Some college	3 (6.4)	3 (6.3)	
Associate's degree	4 (8.5)	7 (14.6)	
Bachelor's degree	19 (40.4)	12 (25.0)	
Technical degree	0 (0.0)	4 (8.3)	
Master's degree	13 (27.7)	18 (37.5)	
Professional (PhD, MD, DDS, etc.)	5 (10.6)	2 (4.2)	
Physical, mean (SD), range			
Weight [lb (kg)] ($n = 48$, Nutritarian; $n = 49$, SAD)	121.60 (12.84), 99–150	152.49 (16.78), 120–198	<0.001
BMI (kg/m^2) ($n = 48$, Nutritarian; $n = 49$, SAD)	20.49 (1.50), 16.0–23.0	25.33 (2.77), 19.4–29.9	<0.001
Diets ($n = 48$, Nutritarian; $n = 49$, SAD)			
Total energy (kcal)	2049 (1877), 297–12,162	2275 (2062), 425–13,590	0.287
Dietary Inflammatory Index (DII)	−5.57 (2.40), −8.07 to 4.8	−2.19 (3.56), −7.04 to 4.98	<0.001
DII—energy adjusted	−5.41 (0.92), −6.8 to −3.26	−2.97 (2.32), −6.19 to 3.75	<0.001
Empirical Dietary Inflammatory Pattern	−0.61 (0.64), −3.75 to 0.17	−0.22 (0.37), −1.84, 0.46	<0.001
Activity ($n = 48$, Nutritarian; $n = 49$, SAD)			
Walking (met-min)	2999.70 (5728.96), 0–29,106	1916.83 (2635.60), 0–12,128	0.117
Moderate (met-min)	1634.17 (2229.07), 0–8400	1286.12 (2593.79), 0–14,280	0.240
Vigorous (met-min)	4481.67 (18,531.24), 0–129,600	2098.12 (3828.92), 0–22,080	0.190
Total (met-min)	9115.53 (19,127.86), 0–130,542	5301.07 (8310.40), 0–48,488	0.102
Sitting (min)	404.58 (233.64), 20-1080	516.96 (473.58), 90–3300	0.071
Sleep ($n = 38$, Nutritarian; $n = 15$, SAD)			
6 h or less	4 (10.5)	7 (46.7)	0.009
6–8 h	30 (78.9)	8 (53.3)	
>8 h	4 (10.5)	0	
Other characteristics			
Weekly alcohol use ($n = 48$, Nutritarian; $n = 49$, SAD)			
3–5 times weekly	0 (0.0)	7 (14.3)	<0.001
Never	31 (64.6)	11 (22.4)	
On special occasions	12 (25.0)	18 (36.7)	
Once per week	4 (8.3)	10 (20.4)	
Regularly	0 (0.0)	2 (4.1)	
Menopause ($n = 47$, Nutritarian; $n = 48$, SAD)	29 (61.7)	6 (12.5)	<0.001
Parents, grandparents, or great grandparents live to 100 years of age or more ($n = 47$, Nutritarian; $n = 48$, SAD)	7 (14.9)	5 (10.2)	0.511

Values are n (%) unless otherwise specified. Owing to missing data, values for individual variables vary and are noted in the table.
Abbreviations: SAD, standard American diet.

concentrations were observed for eukaryotic translation termination factor 1.

Additional EBPs significantly lower in the Nutritarian group included those for CRP, total cholesterol, BMI, and glucose. An EBP for ergothioneine, an antioxidant derived mainly from dietary mushrooms, was higher in the Nutritarian group ($P = 0.023$), although with an FDR >0.05 (FDR = 0.08).

Interestingly, several EBPs for proteins involved in the insulin-like growth factor (IGF) signaling pathway significantly differed between groups. The IGFBPs regulate the actions of IGF by binding IGF-1 and/or IGF-2 with high affinity, preventing IGFs from binding to the IGF-1 receptor. EBPs for IGFBP-5, IGFBP-2, and IGFBP-6 were significantly lower in the Nutritarian group than those in the SAD group. An EBP for IGF-1 was significantly lower in the Nutritarian group based on P value (=0.026), although with an FDR > 0.05 (FDR = 0.09).

EWAS and DMRs

We also assessed global differences of DNAm between groups. To this end, we performed a EWAS comparing the methylation

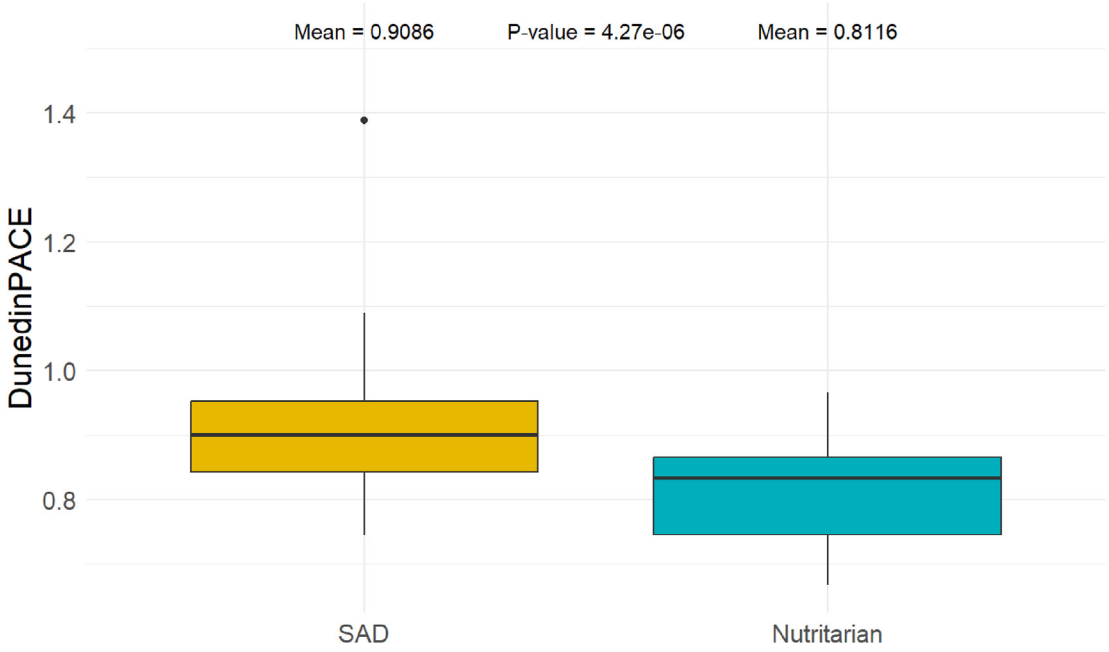

FIGURE 2. DunedinPACE. Boxplot shows the pace of epigenetic aging indicated by DunedinPACE for the SAD (left) and Nutritarian (right) groups. Mean values and *P* values of the *t* test are displayed above. SAD, standard American diet.

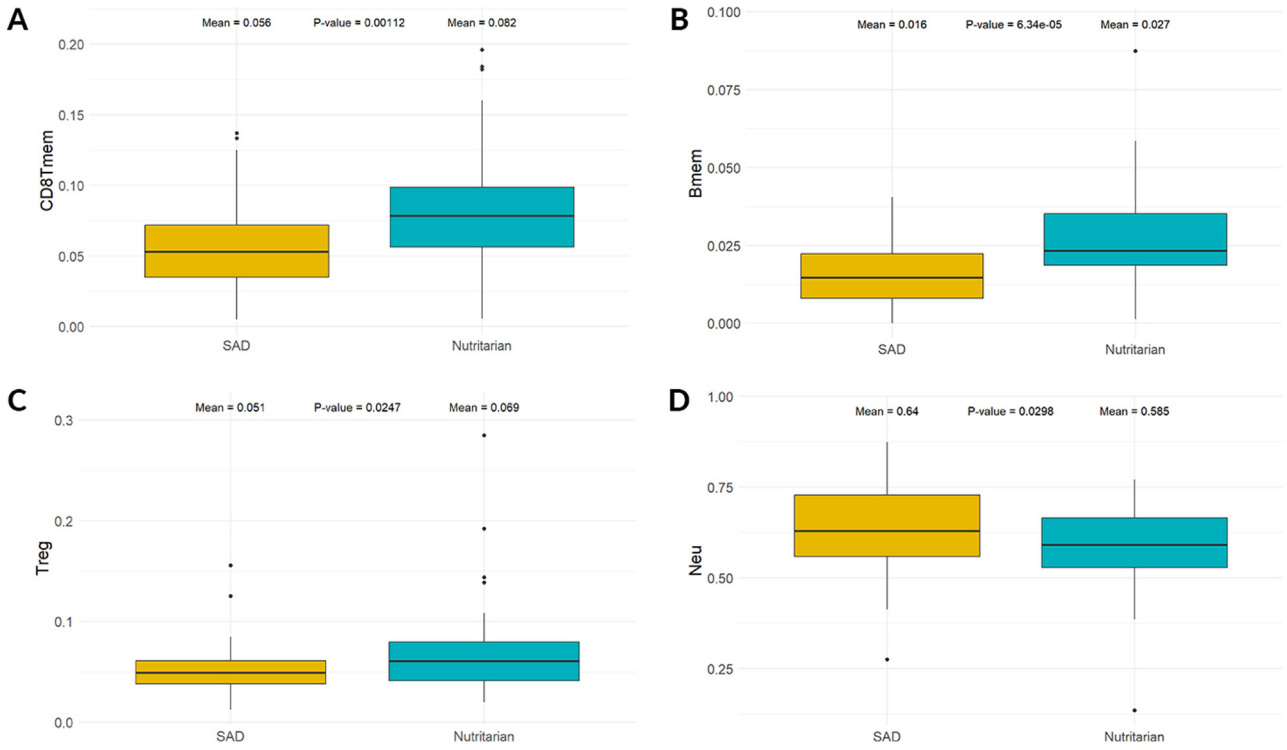

FIGURE 3. Methylation-predicted immune cell subsets. Boxplots show populations of $CD8^+$ T memory cells (A), B memory cells (B), T regulatory cells (C), and neutrophils (D) as predicted from DNA methylation for the SAD (left) and Nutritarian (right) groups, with mean values and *P* values from *t*-tests. Additional immune cell subsets are shown in Supplemental Figure 1. SAD, standard American diet.

levels for all CpG sites in the genome between the individuals in the SAD group and those in the Nutritarian diet group. The λ was 1.11, indicating no inflation of the model.

We first used the most restrictive threshold, defining significant CpG sites as those with an FDR of <0.05. However, we identified only 1 significant probe differentially methylated

(cg22039458), mapped to *HTRA2* and *AUP1* genes, coding for high-temperature-requirement A2 and ancient ubiquitous protein-1, respectively. This CpG site was hypomethylated in the Nutritarian group compared with that in the SAD group. To increase the number of CpG sites, a less restrictive threshold for significance (P value $< 1 \times 10^{-4}$) was used. In this case, we identified 131 differentially methylated sites distributed throughout the genome (Supplemental Table 2 and Figure 4). Among them, 32 were hypermethylated, and 120 were hypomethylated in the Nutritarian diet group. The genes involved in the top CpG sites were *TP73*, *LCLAT1*, *BSX*, *HPCA*, and *CDH23*, among others.

After evaluating each probe along the genome, we performed a DMR analysis to see whether additional findings were found. However, none of the DMRs with ≥5 CpG sites reached the significance level.

Discussion

In this study, we explored the relationship between the Nutritarian diet and various biomarkers of epigenetic aging and inflammation, comparing a cohort of females following this dietary pattern with a cohort following a SAD. Our findings indicate that individuals adhering to the Nutritarian diet exhibit significantly slower epigenetic aging, as evidenced by the third-generation epigenetic clock DunedinPACE, alongside a reduction in inflammatory biomarkers. Specifically, the Nutritarian diet group demonstrated lower dietary inflammatory potential, as assessed by the EDIP and DII, and favorable changes in methylation-predicted immune cell subsets and inflammatory status proxies. These results suggest that long-term adherence to the Nutritarian diet may confer protective effects against chronic inflammation and age-related health risks, highlighting the potential of diet in modulating epigenetic aging processes.

Interventions using a plant-based diet, Mediterranean diet, or calorie restriction have reported reductions in epigenetic age indicators [90–93]. In our study, of the 7 epigenetic age algorithms evaluated, a slower pace of epigenetic aging was indicated only by DunedinPACE in the Nutritarian group than that in the SAD group. DunedinPACE, considered a third-generation epigenetic clock, is an indicator of the pace of epigenetic aging rather than a moment-in-time indicator of epigenetic age [71, 87].

The mean DunedinPACE in the Nutritarian group was 0.81 compared with 0.91 in the SAD group. These results align with the CALERIE randomized controlled trial, in which the mean DunedinPACE decreased from 0.95 to 0.93 in the calorie restriction group and did not significantly change in the control group. Moreover, GrimAge and PhenoAge did not change in this trial, following the same trends as our study [93]. Moreover, consistent with our results, an analysis of Women's Health Initiative data found that scores on healthy eating indices were more strongly correlated with DunedinPACE than with EAA measures based on Hannum, Horvath, PhenoAge, and GrimAge clocks [65].

Despite a nonsignificant difference in mean age between groups, a greater proportion of participants in the Nutritarian group (Table 1) had been through menopause. Although the influence of menopausal status on DunedinPACE has not yet been reported in the literature, one study found that increased EAA based on the Horvath epigenetic clock was associated with earlier menopause and a longer time since menopause [94]. In our study, after adjusting for menopausal status, the difference in DunedinPACE remained significant. However, the difference between groups in DunedinPACE was no longer significant after adjusting by BMI and array type. Although it may indicate that the lower DunedinPACE observed in the Nutritarian group may be primarily due to their lower BMI, future studies with groups matched for BMI could provide a clearer understanding of the effects of this diet on biological aging independent of BMI. However, plant-based diets focusing on healthful foods, including the Nutritarian diet, are typically associated with lower BMI or other adiposity indicators [33,95]. Moreover, DunedinPACE may more strongly reflect body fat-associated aspects of biological aging than other clocks [96].

Dietary inflammatory potential, as indicated by EDIP, DII, and E-DII, was lower (more anti-inflammatory) in the Nutritarian group than that in the SAD group (Table 1). However, the mean dietary inflammatory scores of the SAD group in our study were somewhat lower (i.e., more anti-inflammatory) than typical scores reported in the literature for females in Western populations, such as the Nurses' Health Study, Women's Health

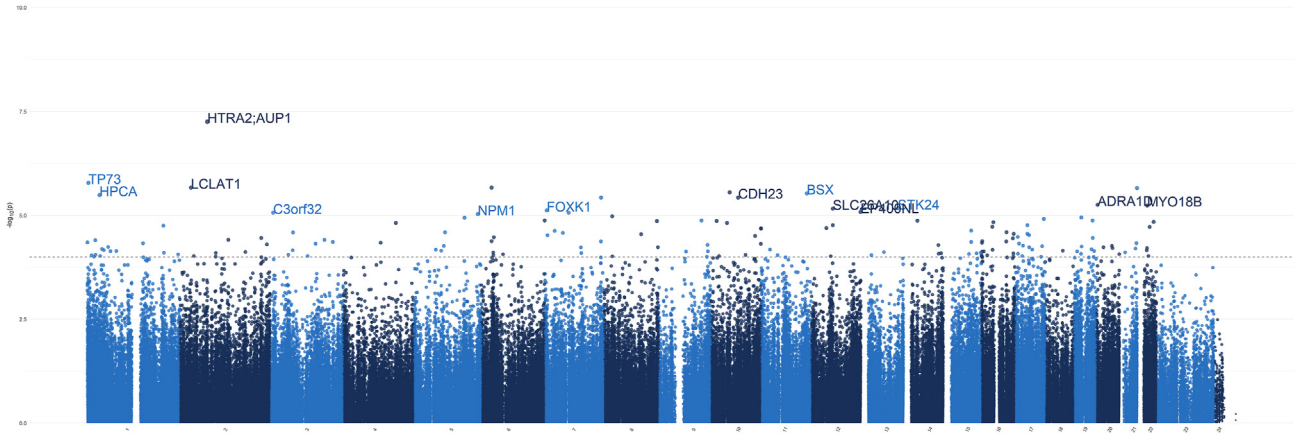

FIGURE 4. Differentially methylated sites. Manhattan plot of the epigenome-wide association study, showing sites differentially methylated between groups. The y axis shows $-\log_{10}(P \text{ value})$, and the horizontal dashed line represents $P = 0.0001$.

Initiative, and NHANES [97–100]. The SAD group in our study may have healthier than typical diet and lifestyle behaviors for American females, based on their mean BMI (25.3, compared with the estimated mean BMI of American females, 29.8) [101], dietary inflammatory status, and absence of prescription medications. The SAD and Nutritarian groups had similar energy intake and physical activity, except for a nonsignificantly higher sitting time in the SAD group (Table 1).

Changes in the numbers, surface receptor expression, and function of immune cells that typically occur with aging simultaneously reduce the effectiveness of the immune response and increase systemic inflammation, leading to increased risk of infection, autoimmune conditions, cancers, and cardiovascular disease [102]. Using a previously constructed DNA methylation reference matrix for blood cell subtypes [89], we found higher methylation-predicted $CD8^+$ T memory cell, B memory cell, and Treg counts in the Nutritarian group than those in the SAD group, as well as a lower predicted neutrophil count. However, after sensitivity analysis removing values 3 or more SDs from the mean, Tregs and neutrophils were no longer significantly different between groups.

Previous research on methylation-predicted immune cell subsets identified associations between some immune cell subpopulations and age or health outcomes in a cohort of 4386 participants from the Mass General Brigham Biobank. In this analysis, higher neutrophil count was associated with a greater risk of all-cause mortality, type 2 diabetes, and chronic obstructive pulmonary disease, in agreement with previous studies evaluating neutrophil counts or neutrophil-to-lymphocyte ratio [89,103–106]. Neutrophils play a proatherogenic role in the artery wall, and higher neutrophil counts have been associated with increased risk of cardiovascular events [107]. In this study, methylation-predicted neutrophil count was lower in the Nutritarian group than that in the SAD group, suggesting lower inflammatory status and possible benefit to cardiovascular health. A recent randomized controlled trial found a decrease in neutrophil count following 4 weeks of a vegan diet compared with that after a meat-rich diet in healthy volunteers [108]. Furthermore, in clinical experience, patients following a Nutritarian/NDPR diet frequently display low-normal or lower-than-normal range neutrophil counts (Joel Fuhrman, MD, clinical observations).

Tregs play a suppressive role in the immune system, maintaining homeostasis by preventing excessive immune responses. Decreased numbers or impaired function of Treg is implicated in autoimmunity, and Treg numbers decrease with aging [109].

A decrease in Treg has been linked to vulnerable atherosclerotic plaque, and Tregs may play an antiatherogenic role [110]. There is evidence that intake of dietary flavonoids could promote the differentiation of naïve T cells into Tregs [111]. Previous evidence linking a plant-based diet to improvements in autoimmune disease symptoms could potentially reflect dietary effects on Tregs [112]. A high-sodium diet may promote autoimmune inflammation by increasing the T helper 17 to Treg ratio [113]. Nutritarian diet guidelines recommend limiting added sodium to 300 mg/d.

The lower concentration of the EBP for CRP suggests an overall lower level of inflammation in participants in the Nutritarian group, consistent with the methylation-predicted neutrophil and Treg counts and indicators of dietary inflammatory potential. These findings suggest the Nutritarian diet as a strategy for addressing inflammation.

Consistent with the difference between groups in an LDL cholesterol EBP, the Nutritarian diet emphasizes nuts and seeds, which have reduced LDL cholesterol in clinical trials, as well as beans and legumes, which are rich in viscous fiber and have also been shown to lower LDL cholesterol in dietary interventions [20,114]. The Nutritarian guidelines also recommend low-to-moderate glycemic load carbohydrate foods, focusing on beans and other legumes, consistent with the lower EBP for blood glucose.

Essential amino acid intake is a major determinant of circulating IGF-1 concentrations, and higher animal protein intake is associated with higher IGF-1 and lower IGFBP-2 concentrations [115–121]. Elevated IGF-1 concentrations have been associated with greater cancer risk [122–124]. Increased IGF-1 signaling has also been implicated in aging and lifespan [125]. Thus, the Nutritarian diet aims to prevent high IGF-1 concentrations and excessive IGF-1 receptor signaling by recommending limited animal protein intake and focusing on obtaining adequate protein from plant protein sources.

The potential significance to the health of differences in circulating IGFBPs 2, 5, and 6 is unclear [119,126]. Although, in tissues, IGFBPs sequester IGFs, restricting their ability to bind the IGF-1 receptor, IGFBPs increase the half-life of IGFs in the circulation, which may have positive effects on IGF activity. Therefore, a higher or lower concentration of an IGFBP in the circulation may not provide insight into its functional significance [127].

The lower concentrations of EBPs representing multiple IGFBPs and a near significant reduction in an EBP representing IGF-1 may suggest a lower activation of the IGF-1 pathway due to a lower animal protein diet. Our dietary data showed fewer servings of meat products and more servings of vegetables in the Nutritarian group (not shown).

In addition, a proxy for ergothioneine was significantly higher in the Nutritarian group based on P value but not after adjusting by multiple comparisons ($P = 0.023$, FDR $= 0.083$). Ergothioneine is an amino acid and specialized antioxidant that has been proposed as a longevity vitamin [128]. Ergothioneine is found in almost all human cell and tissue types and tends to accumulate in tissues exposed to high levels of oxidative stress [128,129]. Mushrooms are the primary dietary source of ergothioneine, and Nutritarian diet guidelines recommend eating mushrooms daily.

Our study has limitations. As our study included primarily White females, the results may not be generalizable to other racial and ethnic groups. Our cohorts were also highly educated, with ~40% of participants in each group having Master's or doctoral degrees. The compensation of receiving epigenetic age data may have motivated more health-conscious females to participate, leading to a SAD cohort nonrepresentative of typical American females consuming a SAD. Moreover, we were unable to assess the influence of sleep on epigenetic markers because of missing data. Self-report of 5-y retrospective dietary patterns is a limitation. Our epigenetic outcome measures, however, are objective. Although we attempted to limit the influence of BMI on our results by excluding participants with BMI of 30 or greater from the SAD group, the difference in BMI between groups is a limitation.

In conclusion, in this study of females aged between 40 and 75 y, we observed in the Nutritarian diet group a slower pace of epigenetic aging indicated by DunedinPACE, lower EDIP and DII scores, lower methylation-predicted neutrophils and higher T regs, and lower DNAm-CRP than those in the SAD group, despite no significant differences in energy intake or physical activity between groups. Furthermore, EBPs for LDL cholesterol, BMI, IGFBP5, and blood glucose were also lower in the Nutritarian group. The Nutritarian diet may reduce chronic inflammation and slow epigenetic aging.

Author contributions

The authors' responsibilities were as follows – JHF: formulated the study concept; JHF, JTS, DMF: designed the research plan; DMF, CAS, JTS, AAP: conducted the research; NVL, RS, NC-G, VBD: analyzed data and performed statistical analysis; DMF: wrote the original manuscript with contributions from NC-G, JTS, and NVL; DMF: had primary responsibility for final content; and all authors: participated in revising the manuscript and reviewed and approved the final manuscript.

Conflict of interest

JHF is the President of the Nutritional Research Foundation and the author of multiple books and the owner of a website providing guidelines for following a nutrient-dense, plant-rich/Nutritarian diet. DMF is an employee of JHF. RS, NC-G, VBD, and AAP are employees of TruDiagnostic.

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Data availability

Data described in this manuscript will be made available upon request pending approval by TruDiagnostic and the Nutritional Research Foundation. In order to protect data privacy of the individuals represented in this cohort, individual applications will be reviewed by TruDiagnostic, and in case TruDiagnostic is willing to share data, a data sharing agreement will be set up.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cdnut.2024.104497.

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