Oxidative stress in chronic diseases: causal inference from observational studies

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CHAPTER 7

Associations of metabolomic profiles with circulating vitamin E and urinary vitamin E metabolites in middle-aged individuals

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Abstract

Vitamin E (α-tocopherol, α-TOH) is transported in lipoprotein particles in the blood, but little is known about the transportation of its oxidized metabolites. In the Netherlands Epidemiology of Obesity Study, we aimed to investigate the associations of 147 circulating metabolomic measures obtained through targeted nuclear magnetic resonance (NMR) with serum α-TOH and its urinary enzymatic (α-CEHC) and oxidized (α-TLHQ) metabolites from 24-hour urine quantified by LC/MS-MS. Multivariable linear regression analyses, in which multiple testing was taken into account, were performed to assess associations between metabolomic measures (determinants; standardized to mean = 0, SD = 1) with vitamin E metabolites (outcomes), adjusted for demographic factors. We analyzed 474 individuals (45% men) with mean (SD) age of 55.7 (6.0) years. Out of 147 metabolomic measures, 106 were associated ($p < 1.34E-3$) with serum α-TOH [median beta (IQR): 0.416 (0.383, 0.466)], predominantly lipoproteins associated with higher α-TOH. The associations of metabolomic measures with urinary α-CEHC are in similar directions as those with α-TOH, but effect sizes were smaller and non-significant [median beta (IQR):0.065 (0.047, 0.084)]. However, associations of metabolomic measures with urinary α-TLHQ were markedly different from the associations of metabolomic measures with both serum α-TOH and urinary α-CEHC, with negative and small-to-null relations to most VLDL and amino acids. Therefore, our results highlight the differences of the lipoproteins involved in the transportation of circulating α-TOH and oxidized vitamin E metabolites. This indicates that circulating α-TOH may be representative of the enzymatic but not to the antioxidative function of vitamin E.

Introduction

Vitamin E (α-tocopherol, α-TOH) is a fat-soluble and essential component in the diet. Vitamin E is important in many physiological processes including fetal development and reproduction, neurodevelopment, and cognitive function; deficiency in vitamin E will induce defects in several developing organs, including primary manifestation in the central nervous system of cerebellar ataxia [1-3]. In addition, vitamin E also has chain-breaking antioxidant activities by competitively reacting with lipid peroxyl radicals to ameliorate lipid peroxidation induced damage [4].

Observational studies have shown associations between higher dietary intake or circulating levels of vitamin E with lower risk of lipid peroxidation-related diseases, such as cardiovascular and neurodegenerative diseases [5-9]. However, there is no evidence for a causal effect based on randomized clinical trials with vitamin E supplementation [10-13] or mendelian randomization [14,15]. A potential explanation lies in the bioactivity of vitamin E that can be catabolized via either hepatic enzymatic pathways or oxidized in the periphery [16]. In the hepatic pathway, vitamin E is enzymatically converted to a spectrum of metabolites of carboxymethyl-hydroxy-chroman (CEHC), with successive shortening of the phytyl side chain, and then eliminated mainly via urine. Alternatively, α-tocopherol reacts with lipid peroxyl radicals, with the opening of the chromanol ring, and generates α-tocopherol quinone (α-TQ); thereafter, α-TQ captures hydrogens converting into α-tocopherol hydroquinone (α-THQ), following the β-oxidation and cyclization of the phytyl side chain, α-tocopheronic acid and α-tocopherol lactone (α-TL) are generated; finally, α-TL are excreted as polar conjugates of α-TL hydroquinone (α-TLHQ) [17-19]. Therefore, circulating vitamin E levels might not represent the authentical antioxidative effect.

The intestinal absorption, hepatic metabolism, and cellular uptake of vitamin E largely follow similar processes as lipids. Briefly, vitamin E is emulsified by digestive enzymes to form micelles and was subsequently absorbed by the intestine via passive diffusion or receptor-mediated transport, followed by the circulation and distribution to target organs and tissues. Once internalized into the enterocytes, lipoproteins are the carriers of vitamin E vascular transportation, independent of the types of isomers. Vitamin E is absorbed and secreted in chylomicrons into the lymphatic system and then is transformed into remnants acquired by the liver parenchymal cells via LDL receptor-mediated uptake [20]. In the liver, different forms of vitamin E were sorted; the highly expressed α-TOH transport protein (α-TTP) selectively readily binds to α-TOH for secretion in VLDL and favors the discrimination of α-TOH among other isomers, protecting α-TOH from excessive degradation and excretion; the final acquisition of vitamin E by tissues is through chylomicron and VLDL catabolism, LDL uptake via LDL receptor or lipoprotein transfer to membranes. Apart from the major portion of circulating α-TOH carried by LDL particles, some of the VLDL-derived α-TOH can also be transferred to HDL during lipolysis. HDL is important for the delivery of α-TOH to extrahepatic tissues, particularly to the central nervous system, and to facilitate the transport of α-TOH from the circulation back to the liver [21]. Therefore, vitamin E utility depends on mechanisms underlying lipoprotein metabolism and relies on lipoprotein-mediated production, processing, and uptake [22]. In addition to their role as vitamin E carriers, lipoproteins, and in particular LDL, are also

susceptible to oxidative modifications that require antioxidants protection. However, it is unclear to what extent vitamin E is catabolized via hepatic enzymatic pathways or oxidized, and what lipoproteins are involved in the transportation of these two metabolism processes.

In the present study, we aim to investigate the cross-sectional associations between circulating metabolomic profiles with circulating α-TOH and urinary enzymatic and oxidized α-TOH metabolites in middle-aged individuals in the Netherlands Epidemiology of Obesity study (NEO).

Method

Study population

The present study was embedded in the population-based prospective cohort Netherlands Epidemiology of Obesity (NEO) study, which aims to study the pathways that lead to obesity-related disorders. The NEO study was initiated in 2008, comprising 6671 participants aged between 45-65 years. Detailed information on the study design and data collection has been described previously [23]. In brief, inhabitants with a self-reported body mass index (BMI) of 27 kg/m^2 or higher living in the greater area of Leiden were eligible to participate. In addition, all inhabitants from the municipality of Leiderdorp were invited irrespective of their BMI. Participants were invited to the NEO study center of the Leiden University Medical Center (LUMC) for one baseline study visit. Blood samples were drawn after an overnight fast and were separated into serum, and aliquots were stored at -80 °C for later measurements. Participants were asked to collect their urine over 24h and completed a general questionnaire at home with their demographic, lifestyle, and clinical data as well as specific questionnaires on diet and physical activity before their first visit. The urine sample was aliquoted and stored at -80 °C for later analyses of urinary vitamin E metabolites. Additionally, participants were asked to bring all medication (prescribed medication including blood pressure-lowering medication, lipid-lowering medication, and glucose-lowering medication, etc., as well as self-medication such as supplements) they were using one month preceding the study visit. The study was approved by the medical ethical committee of the LUMC, and all participants gave written informed consent.

A random subset of 35% baseline Leiderdorp participants (N = 599) was included in this cross-sectional analysis. We excluded individuals with urine collection less than 20 hours or for whom urinary vitamin E metabolites measurements failed (n = 61). Given that the platform used for measurements contained a considerable number of lipoproteins, participants who were taking statins at the time of blood sampling (n = 38) were consequently excluded. We further consecutively excluded participants with implausible metabolites measurement (concentration ≤ 0, n = 4), missingness or outliers of either urinary vitamin E metabolites (n = 7) or serum vitamin E (n = 4) or metabolomic measures (n = 2), and missing data on confounding factors (n = 9). Therefore, the final number of participants included in the present study was 474.

Metabolomic measures profiling

The lipoprotein profiles were quantified using high-throughput ^{1}H-NMR metabolomics (Nightingale Health, Helsinki, Finland). This platform provides simultaneous quantification of 229 metabolites and ratios. After excluding the calculated ratios from the dataset, we included 147 metabolites from 11 classes: lipoprotein subclasses (n = 98), lipoprotein particle sizes (n = 3), apolipoproteins (n = 2), fatty acids (n = 10), cholesterol (n = 9), and also glycerides and phospholipids (n = 9), amino acids (n = 8), ketone bodies (n = 2), inflammation (n = 1), glycolysis related metabolites (n = 3), and fluid balance (n = 2). Lipoprotein subclasses were defined according to the particle sizes as follows: chylomicrons and extremely large VLDL (XXL-VLDL) with particle diameters from 75 nm upwards, five subclasses for VLDL (XL-VLDL: 64 nm, L-VLDL: 53.6 nm, M-VLDL: 44.5 nm, S-VLDL: 36.8 nm, XS-VLDL: 31.3 nm); IDL (28.6 nm), three subclasses for LDL (X-LDL: 25.5 nm, M-LDL: 23 nm, S-LDL: 18.7 nm), and four subclasses of HDL (XL-HDL: 14.3 nm, L-HDL: 12.1 nm, M-HDL: 10.9 nm, S-HDL: 8.7 nm). Detailed information, including quality assurance measures and applications of the platform, have been described elsewhere [24].

Vitamin E and vitamin E metabolites measurements

Circulating serum α-TOH was detected and quantified by Metabolon, Inc. (Durham, NC, USA) on a platform encompassing four liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) methods (LC-MS/MS negative, LC-MS/MS positive early, LC-MS/MS positive late and LC-MS/MS polar). Given the relative quantification, the absolute amount of α-TOH per liter was not quantified, and therefore data was not expressed as a concentration, but as peak height relative to an internal standard per sample to allow for comparisons between individuals. More information about the quantifications has been described previously [25,26].

Urinary α-CEHC and oxidized α-TOH metabolites (α-TLHQ), presented as their sulfate or glucuronide conjugates, were measured by LC-MS/MS at University College London, the UK between March and May 2019 [19]. The final concentrations of α-TLHQ and α-CEHC were the sum of their corresponding sulfate and glucuronide isoforms.

Briefly, 100µl thawed urine (from 24-hour urine) was centrifuged for 10 min at 14 000 g at room temperature and spiked with 10µl of the internal standards (100µmol/L), lithocholic acid sulfate (LA) and androsterone D4-glucuronide (AD4), and 10 µl was subsequently injected into the LC-MS/MS for detection. Metabolites separation was performed by a Waters ACQUITY UPLC BEH C8 column (1.7µm particles, 50mm x 2.1mm; Waters Corp, Manchester, UK) plus a guard column containing an identical stationary phase. To minimize system contamination and carryover, UPLC eluents before and after the sample elution were discarded and an additional blank sample (H_2O: MeCN) was run between each detection urine sample. Two separate peaks were observed for both α-TLHQ and α-CEHC conjugated with glucuronide, corresponding to major and minor isoforms. These isoforms had been previously described thoroughly by Pope et al [27]. and Sharma et al. [18,19]. The different elution time (minutes) for internal standards (LA 4.33, AD4 2.7) and each metabolite (2.39, 2.12 and 2.29 for α-TLHQ sulfate,

Table 1 Characteristics of the study population[1]

		N = 474
Demography		
Age (years)	Mean (SD)	55.7 (6.0)
Sex (male)	Frequency (proportions)	213 (45%)
BMI (kg/m^2)	Median (IQR)	25.3 (23.1, 27.8)
Lifestyle factors		
Dutch healthy diet index	Mean (SD)	59.7 (8.4)
Energy intake (KJ/day)	Median (IQR)	9106 (7326, 11078)
Physical activity (MET-h/week)	Median (IQR)	28.9 (16.0, 48.8)
Smoking		
Current	Frequency (proportions)	50 (10%)
Former	Frequency (proportions)	222 (47%)
Never	Frequency (proportions)	202 (43%)
Vitamin E supplement use (yes)[2]	Frequency (proportions)	124 (26%)
Vitamin E metabolites measurements		
Blood (log10-transformed)		
α-tocopherol	Mean (SD)	8.5 (0.1)
Urinary		
α-TLHQ (nmol/mmol creatinine)	Median (IQR)	1864.9 (1347.1, 2770.3)
α-CEHC(nmol/mmol creatinine)	Median (IQR)	271.0 (181.4, 439.4)
α-TLHQ (log10-transformed)	Mean (SD)	3.3 (0.2)
α-CEHC (log10-transformed)	Mean (SD)	2.4 (0.3)

[1]SD: standard deviation; IQR: interquartile range.
[2]Vitamin E supplement use was defined as either vitamin E supplement use or multiple vitamin supplement use.
BMI, body mass index; CEHC, carboxymethyl-hydroxychroman.

glucuronide minor and major, 2.64, 2.50, 2.56 for α-CEHC sulfate, glucuronide minor and major) guaranteed that all metabolites could be separated in a single chromatographic run. Metabolites analyses were then performed by MS using a Waters ACQUITY UPLC coupled to a triple-quadrupole Xevo TQ-S fitted with electrospray ionization in negative ion mode. Using multiple reaction monitoring (MRM) mode, specific parent and daughter ions were determined in scan mode and following collision activated dissociation with argon. These ions were then used to quantify each α-TOH metabolite from transitions that corresponded to their molecular masses. Since creatinine concentration is frequently used as a proxy of kidney function, and in cases of severe renal dysfunction, the creatinine clearance rate will be "overestimated" because the active secretion of creatinine will account for a larger fraction of the total creatinine. Therefore, to correct the dilution effect, urinary creatinine concentrations (mmol/L) were also measured by triple-quadrupole Micro Quattro mass spectrometry (MicroMass, Waters, UK). Therefore, the final concentration of α-TOH metabolites was in nmol per mmol of creatinine. A quality control (QC) assessment was performed throughout the

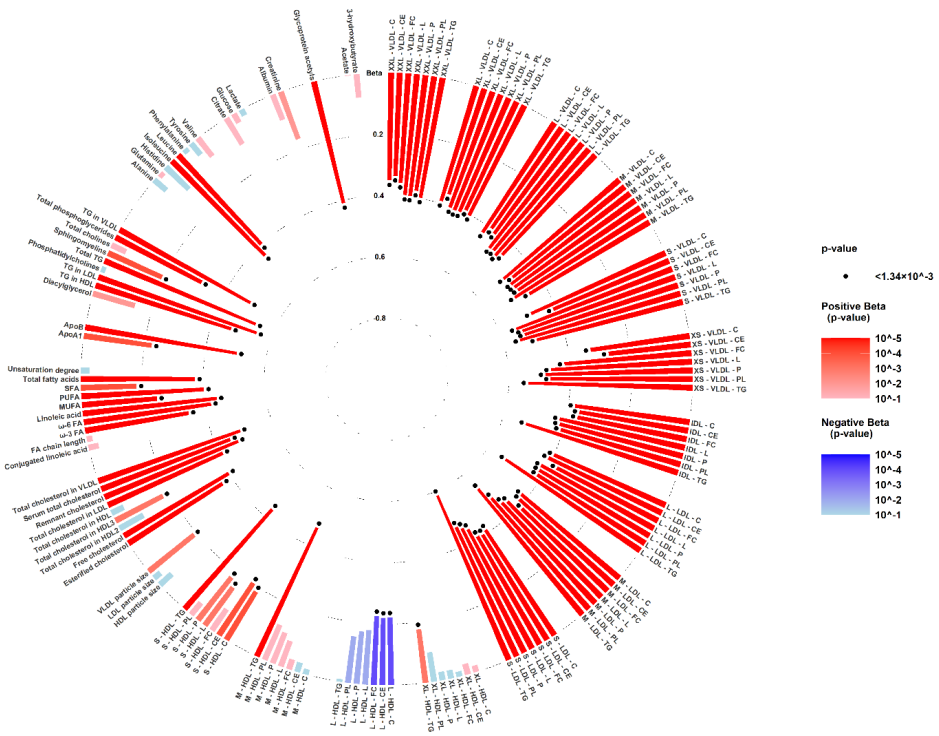

Figure 1 Associations between 147 circulating metabolomic measures with circulating α-tocopherol

Associations were derived from a multivariable linear regression model in the study population (N = 474) adjusted for age, sex, BMI, smoking status, Dutch Healthy Diet Index, energy intake and physical activity.

Effect estimates refers to the coefficients for different metabolomic measures obtained from the linear regression models. we standardized the log10-transformed metabolomic measures and the serum and urinary vitamin E measures (mean = 0, SD =1), so that the regression coefficient with its corresponding 95% confidence interval (CI) can be interpreted as the mean change in SD of the outcome with respect to a one-SD change in the determinant (standardized concentrations of the metabolomic measures). Red and blue colors indicate the directions of the estimates, namely the positive and negative coefficients respectively, and the gradients suggests different significant levels. A p-value of below 1.34×10^{-3} (0.05/37, 37 is the number of independent metabolomic measures) is considered significant, presented as a black dot above each bar. Full names and descriptive information of metabolomic measures are listed in Supplementary Table.

quantification to deal with the variations in sample quality and UPLC-MS/MS performance. Detailed information on the measurements has been described previously [28].

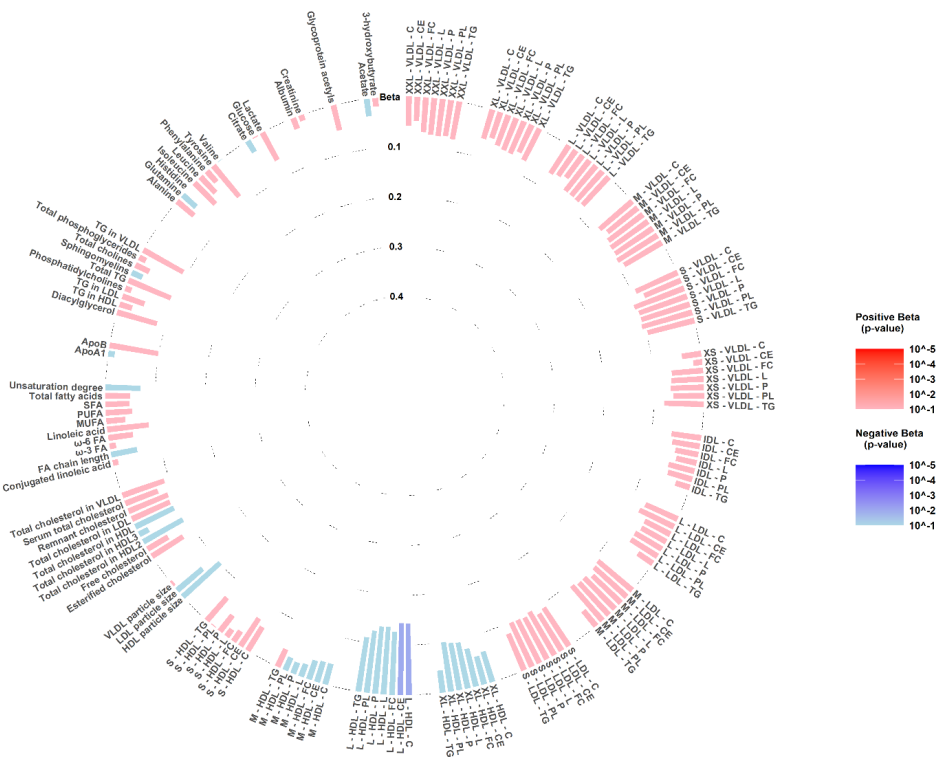

Figure 2 Associations between 147 circulating metabolomic measures with urinary α-CEHC

Associations were derived from a multivariable linear regression model in the study population (N = 474) adjusted for age, sex, BMI, smoking status, Dutch Healthy Diet Index, energy intake and physical activity. Figure legend is the same with Figure 1, except that no significant associations [$p > 1.34 \times 10^{-3}$ (0.05/37, 37 is the number of independent metabolomic measures)] were detected.

Confounding factors

To determine BMI (kg/m^2), body weight was measured without shoes, and one kilogram was subtracted for the correction of clothing weight. Smoking status was categorized into the current smoker, former smoker, and non-smoker. Physical activity levels (in MET-hours per week, MET: Metabolic equivalent of task) were estimated based on the frequency and duration of leisure physical activity over the past 4 weeks reported by participants on the Short Questionnaire to Assess Health-enhancing physical activity (SQUASH) [29]. A semiquantitative food frequency questionnaire was used to assess food and beverage intake. Total energy intake (in kJ) and Dutch Healthy Diet Index (DHD-index) were subsequently estimated based on dietary intake [30]. Vitamin E supplement use was collected via questionnaires and was defined as either vitamin E supplement use only or multivitamin supplement use (yes/no).

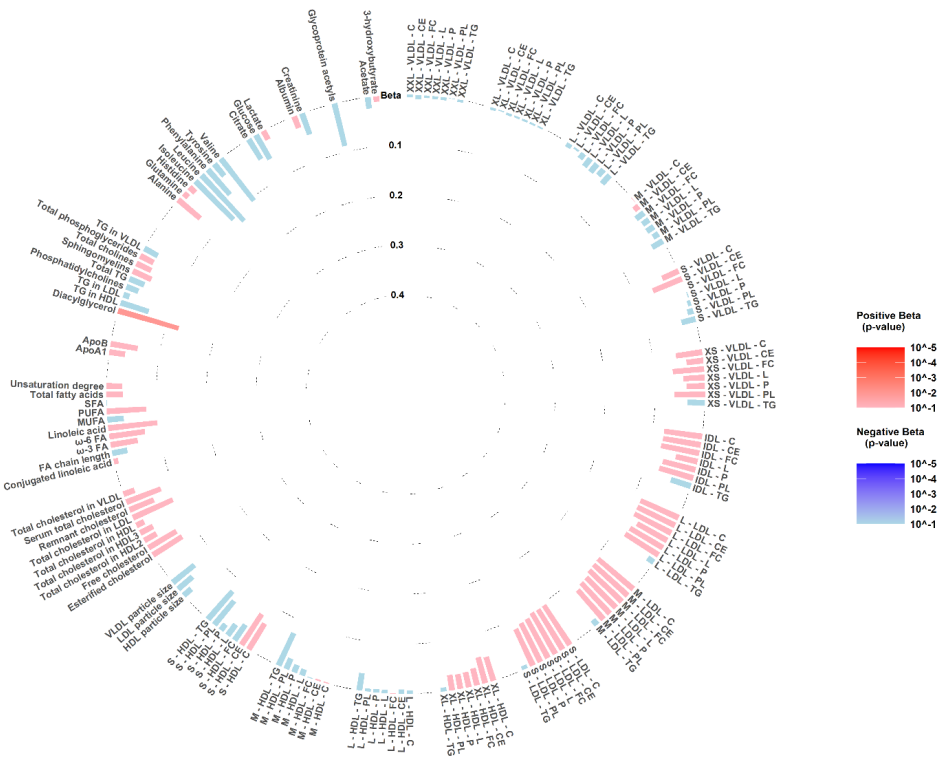

Figure 3 Associations between 147 circulating metabolomic measures with urinary α-TLHQ

Associations were derived from a multivariable linear regression model in the study population (N = 474) adjusted for age, sex, BMI, smoking status, Dutch Healthy Diet Index, energy intake and physical activity. Figure legend is the same with Figure 1, except that no significant associations [$p > 1.34 \times 10^{-3}$ (0.05/37, 37 is the number of independent metabolomic measures)] were detected.

Statistical analysis

Descriptive characteristics of the study population were presented as mean (standard deviation, SD), median (interquartile range, IQR) for normally distributed variables and skewed variables respectively, and frequency (proportions) for categorical variables.

Vitamin E metabolites and metabolomic measures were log10-transformed to approximate a normal distribution. Observed metabolites concentrations located beyond 4 standard deviations from the mean after log10-transformation were classified as outliers and further excluded. Since missing data on the metabolomic measures were most likely due to concentrations that were lower than the limit of detection, these missing values were imputed by giving them the value of half of the minimum observed value for each metabolite. In addition, we assessed

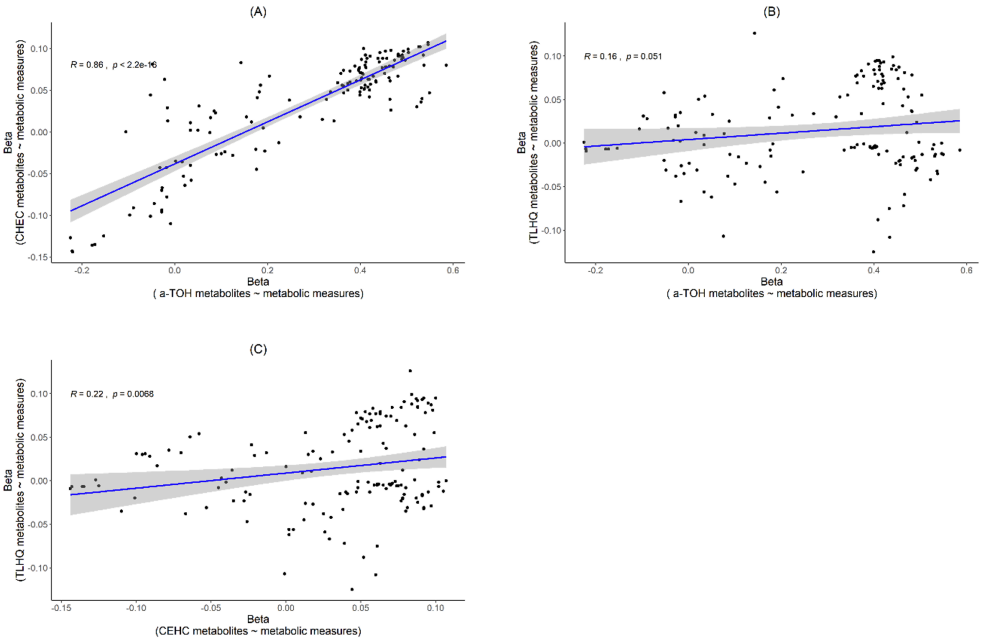

Figure 4 Correlations of effect estimates between metabolomic measures with circulating α-tocopherol, urinary α-CEHC and α-TLHQ

(A) the correlation of regression coefficients of metabolomic measures with circulating α-tocopherol and metabolomic measures with urinary α-CEHC. (B) the correlation of regression coefficients of metabolomic measures with circulating α-tocopherol and α-TLHQ. (C) the correlation of regression coefficients of metabolomic measures with urinary α-CEHC and α-TLHQ.

Axis represents the beta coefficient derived from multiple linear regression of metabolomic measures (determinants) and vitamin E metabolites (outcomes). Correlation coefficients and p value were derived from Pearson correlation.

the percentage of missingness for each metabolomic measure. To compare the effect estimates, i.e. the coefficients for different metabolomic measures obtained from the regression models, we standardized the log10-transformed metabolomic measures and the serum and urinary vitamin E measures (mean = 0, SD =1), so that the regression coefficient with its corresponding 95% confidence interval (CI) can be interpreted as the mean change in SD of the outcome with respect to a one-SD change in the determinant (standardized concentrations of the metabolomic measures).

Multivariable-adjusted linear regression models were fitted, with metabolomic measures as determinants, confounding factors as covariates, and vitamin E metabolites as outcomes. Based on prior knowledge, confounding factors included age, sex, BMI (kg/m^2), physical activity (MET-hours per week), smoking habits (non-smoker, current smoker, or former smoker), Dutch Healthy Diet Index, and total energy intake (kJ/day). Scatter plots were used to visualize the difference in both direction and effect sizes of the estimated associations among

metabolomic measures with different vitamin E metabolites, and we calculated Pearson correlations between the effect estimates derived from the regression results. Therefore, the correlation indicates the similarity of those associations.

Vitamin E supplement use may have an influence on metabolomic measures and vitamin E conversion and will potentially distort the associations. However, given the high heterogeneity of vitamin E supplement use, as either vitamin E only or multivitamin use, as well as limited information on frequency and dosage, natural or modified vitamin E acetate, we therefore additionally performed the regression analyses in participants who did not take vitamin E or multivitamin supplements (N = 350). Furthermore, in order to test the effect modification by obesity status, we stratified participants into normal weight (BMI < 25kg/m^2, N = 217) and overweight (since only 59 were obese with BMI above 30 kg/m^2, we combined individuals with BMI >= 25kg/m^2, N = 257), and all multivariable regression models in the main analyses were conducted in each stratum.

Given that most of the metabolomic biomarkers, especially lipid subclasses, were highly correlated, conventional correction for multiple testing (e.g., Bonferroni) is too stringent. Therefore, the 'effective number' (Meff) procedure was used which identified independent metabolomic traits [31]. The final significance threshold of the p-value was then defined as 0.05/37 = 1.34E-3. All the analyses were undertaken using R (v3.6.1) statistical software (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Characteristics of the study population

474 participants were included in the current study after excluding participants with missing data or outliers in serum or urinary vitamin E measures; characteristics of the study population are presented in **Table 1**. The mean (SD) age was 55.7 (6.0) years with a median (IQR) BMI of 25.3 (23.1, 27.8) kg/m^2; 45% of the participants were male. Approximately 124 (26%) participants used vitamin E supplements, and 50 (10%) were current smokers. Summaries of metabolomic biomarkers are presented in **Supplementary Table 1**. The percentages of missing data of individual metabolomic measures were all below 30%, with 9 out of 147 metabolites having 20% or more missingness, of which 7 were extremely large VLDL-characteristics (**Supplementary Figure 1**).

3.2 Main analyses

In the multivariable-adjusted linear regression model, 106 out of 147 metabolomic measures were associated with serum α-TOH with p < 1.34E-03 [median effect size (IQR): 0.416 (0.383, 0.466)] (**Figure 1** and **Supplementary Table 1**). Three of the 106 associations were negative: higher levels in total cholesterol, cholesterol ester, and free cholesterol in large HDL were associated with lower mean serum α-TOH [effect estimates -0.221 (95% confidence interval, CI: -0.330, -0.112), -0.220 (95% CI: -0.329, -0.112) and -0.225 (95% CI: -0.332, -0.118), respectively, per 1 SD higher level of the metabolomic measure]. In all other cases, higher levels of the metabolic measures were associated with higher α-TOH.

These associations include VLDL, IDL, LDL, and small HDL, total cholesterol (not in HDL and HDL2) particles and its components, Apo-A and Apo-B, glycerides and phospholipids, and glycoprotein acetyls, with effect sizes ranging from 0.154 (95% CI: 0.063, 0.244) SD for total lipids in small HDL to 0.585 (95% CI: 0.509, 0.660) SD for triglycerides in small-LDL. Moreover, per-SD higher level of leucine and isoleucine were associated with a 0.399 (95% CI:0.290, 0.507) and 0.434 (95% CI:0.327, 0.540) SD higher α-TOH.

Figure 2 and **Figure 3** present the associations between metabolomic measures with urinary α-CEHC and α-TLHQ metabolites, separately. None of the analyses were statistically significant upon correction for multiple testing. Nevertheless, for α-CEHC, the direction of the associations with metabolomics measures was similar to those of α-TOH with metabolomics measures, the effect sizes, however, were much smaller [median effect size (IQR): 0.065 (0.047, 0.084)], as shown in **Figure 2**. For α-TLHQ, the strongest association was with diacylglycerol 0.126 (95%CI: 0.037, 0.215, p = 0.006). The direction of the associations was different substantially from those of α-TOH with metabolomics measures, and only very small VLDL, IDL, LDL, XL-HDL, and fatty acids showed associations in the same direction.

The effect estimates between metabolomic measures with circulating α-TOH were strongly correlated with the effect estimates of metabolomic measures with urinary α-CEHC ($r = 0.86$, $p < 0.001$). However, the correlations between the effect estimates of metabolomic measures with α-TOH and with urinary α-TLHQ, and the correlations between the effect estimates of metabolomic measures with α-CEHC and with urinary α-TLHQ were very weak, $r = 0.16$ ($p = 0.05$) and $r = 0.22$ ($p = 0.007$), respectively (**Figure 4**).

Sensitivity analyses

Excluding vitamin E supplement users

We excluded 124 participants with either vitamin E or multivitamin supplementation, leaving 350 participants for further analyses. The associations between metabolomic measures and vitamin E metabolites in this group were generally consistent with the analyses in the whole study population (**Supplementary Figure 2**). However, several effect sizes became larger. Notably, a one-SD higher level of total cholesterol and cholesterol esters in medium LDL and small LDL particles were associated, even after correction for multiple testing, with higher levels of α-CEHC with effect sizes of 0.176 (95% CI: 0.071, 0.280), 0.175 (95% CI: 0.070, 0.280), 0.180 (95% CI: 0.075, 0.285) and 0.179 (95% CI: 0.074, 0.284) SD, respectively. Furthermore, 22 out of the 147 metabolomic measures (most notably 4 IDL, 16 LDL, total cholesterol, and total cholesterol in LDL) were associated with α-TLHQ levels. Specifically, higher levels of LDL cholesterol sub-particles, except for the amount of triglycerides in LDL particles, were associated with higher levels of α-TLHQ, with effect sizes ranging from 0.186 (95% CI: 0.079, 0.293) SD for large LDL particles to 0.227 (95% CI: 0.121, 0.333) SD for cholesterol esters in small LDL. In addition, a one-SD higher level of total cholesterol, total cholesterol in LDL were associated with 0.186 (95% CI: 0.076, 0.295) and 0.207 (95% CI: 0.102, 0.313) SD higher α-TLHQ, respectively.

The effect estimates of metabolomic measures with circulating α-TOH were strongly correlated with the effect estimates of metabolomic measures with urinary α-CEHC ($r = 0.69$, $p < 0.001$), whereas no correlation was found between the effect estimates of metabolomic measures with circulating α-TOH and with urinary α-TLHQ ($r = -0.013$, $p = 0.88$). A moderate correlation was observed for the estimates between metabolomic measures with circulating α-CEHC and with urinary α-TLHQ ($r = 0.49$, $p < 0.001$), **Supplementary Figure 3**. However, this correlation was mainly due to the association with LDL ($r = 0.18$, $p = 0.053$ after excluding the LDL subclass from the list of metabolic measures).

Stratification analyses by obesity

In the normal weight subgroup analyses (N = 217), the associations of metabolomic measures and serum α-TOH were analogous to those obtained from the main analyses (**Supplementary Figure 4**). However, the associations with HDL were no longer significant, resulting in slightly fewer (95 out of 147) significant associations. The median significant effect size (IQR) was 0.375 (0.330, 0.414). Similarly, the associations between metabolomic profiles with α-CEHC did not differ materially. Interestingly, diacylglycerol was significantly positively related to α-TLHQ. However, the associations between HDL and α-TLHQ became stronger while the association between XXL- and XL-VLDL and α-TLHQ turned positive and stronger compared to the estimates from the whole population, despite being insignificant.

In the overweight subgroup analyses (N = 257), the magnitude of the estimations from regression analyses was generally larger than those derived from the main analyses in both three vitamin E metabolites analyses (**Supplementary Figure 5**). Particularly, the associations of metabolomic profiles with α-TOH remained in the same directions, and 99 out of 147 associations were significant [median effect size (IQR): 0.497 (0.423, 0.561)]. Likewise, the relationships of metabolomic measures with α-CEHC did not change substantially. However, with regards to α-TLHQ, the direction of the association with HDL, notably large and middle HDL, turned to positive, though insignificant.

Discussion

In this cross-sectional study, we investigated the association between circulating metabolomic measures and urinary vitamin E metabolites. The direction of the estimates from metabolomic measures and circulating α-TOH and urinary α-CEHC were similar but with weaker effect sizes for α-CEHC than α-TOH, whereas α-TLHQ showed distinct associations. 106 out of 147 metabolomic measures were associated with circulating α-TOH after correction for multiple testing, while no significant associations were identified for associations with urinary α-CEHC and α-TLHQ. Sensitivity analyses in participants without vitamin E supplement use generally showed consistent results with the main analyses for each vitamin E metabolite, but with significant associations between IDL, LDL, total cholesterol, and cholesterol content in LDL particle and α-TLHQ.

Our study shows similar associations of metabolomic measures with urinary α-CEHC and with circulating α-TOH. The liver parenchymal cells acquire α-TOH through taking up chylomicron remnants, which contains a major proportion of absorbed α-TOH, and the highly expressed α-TTP facilitate the preferential enrichment of α-TOH in VLDL particles secreted by the liver. These processes are responsible for the regulation and release of α-TOH into circulation and consequently for its delivery to tissues. Therefore, the vitamin E enzymatic activity in the liver is well regulated to maintain a certain level of α-TOH, i.e. higher α-TOH gives higher α-CEHC [32]. However, the different associations observed between metabolomic measures with urinary α-TLHQ and with blood α-TOH indicate that other processes that are not associated with α-TOH regulated the association for α-TLHQ. In addition, we observed that circulating α-TOH is associated with higher levels of most lipoprotein fractions and cholesterols. This observation may be somewhat counterintuitive to previous observations of higher vitamin E circulating levels with a lower risk of atherosclerosis and cardiovascular diseases [9]. Nevertheless, individuals with hyperlipidemia were found to have reduced uptake of the newly absorbed α-TOH into blood; the abnormal lipoprotein metabolism does not necessarily increase α-TOH delivery to the peripheral tissues and this uptake reduction of α-TOH may be relevant to the pathogenesis of atherosclerosis [33]. In addition, our results are in line with previous research which found that α-TOH concentrations were correlated with serum lipids levels and that the retainment of plasma α-TOH was longer with higher serum total lipids [22]. This might be attributed to the notion that higher lipid concentrations can keep vitamin E from reaching peripheral tissues, and the catabolism and uptake of lipoproteins decreases at high concentrations of lipid. However, higher levels of large HDL particles were associated with lower levels of α-TOH. This may be due to that α-TOH content in HDL particles depends not only on tocopherol levels but also on HDL concentrations, and HDL α-TOH retention was found related to a high concentration of HDL fraction [33]. Two branched-chain amino acids (leucine and isoleucine) are positively associated with higher α-TOH. Experimental studies have demonstrated that vitamin E is crucial for the maintenance of energy homeostasis, and its deficiency dysregulated energy metabolism and mitochondrial dysfunction, measured by extracellular oxygen consumption [34-36]. Ketogenic amino acids, particularly leucine, which can be utilized for ketone synthesis, were elevated in vitamin E deficient Zebrafish brains, where there are probably elevated lipid peroxidation and metabolic disruptions [35,36]. Health states that are associated with increased oxidative stress are likely to have a greater antioxidant requirement, which would result in depletion of circulating levels of vitamin E, and higher urinary concentrations of α-TLHQ. The negative though the insignificant association of leucine with α-TLHQ contrasts to the findings from previous experimental studies, understanding the underlying mechanisms, will however require additional efforts.

Results from sensitivity analyses after excluding supplement users were generally consistent with the main analyses, but with several significant associations of α-TLHQ. Notably, higher levels of substances that are susceptible to oxidative modifications, particularly IDL and LDL particles were significantly associated with higher urinary α-TLHQ. Several fatty acids are also closely related to α-TLHQ though not significantly. The inhibitory effect against lipid peroxidation of vitamin E decreases gradually from polyunsaturated fatty acids (PUFA) to cholesterol.

In accordance, LDL, as the most susceptible particle to oxidative modification compared to the other lipoproteins, was associated with α-TLHQ. However, this does not include triglycerides in LDL particles which are more resistant to lipid peroxidation. Therefore, oxidation, rather than the level of α-TOH, regulated the association of metabolomic measures and α-TLHQ. Despite the strong correlation ($r = 0.48$) between the effect estimates of metabolomic measures with α-CEHC and with α-TLHQ, this is predominantly driven by LDL particles.

In the stratification analyses by obesity, the associations of metabolomic measures with α-TOH and α-CEHC did not differ materially in general, but the effect sizes are larger in the overweight group possibly due to the higher lipids levels in overweight participants compared to the normal-weight individuals. Interestingly, the associations of lipoproteins, particularly VLDL and HDL with α-TLHQ differed. Though none of the associations is significant, VLDL is positively and HDL is negatively associated with α-TLHQ in the strata of normal weight, whereas opposite directions were observed in the strata of overweight. Excessive fat accumulation will certainly lead to elevated oxidative stress, and the supply of vitamin E by HDL might be more important under conditions of oxidative stress due to the independence of regulatory mechanisms of cholesterol metabolism [37]. Previous efforts have demonstrated the exchange of α-TOH between lipoproteins, which may depend on the ratio of HDL/LDL [38]; the discrepancies of VLDL associations might imply a different transfer in obese participants compared to non-obese provided distinct lipid profiles in obese people. However, the underlying mechanisms warrant further investigation.

One strength of the present study is that we simultaneously quantified the concentrations of circulating α-tocopherol and urinary metabolites derived from two metabolic pathways, which facilitates the exploration of the circulating level versus the functional level. In addition, the measurement of urinary α-TLHQ was performed by LC-MS/MS based method, which deliberately avoids artefactual oxidation products of α-CEHC that might result from previous chromatography-mass spectrometry (GC-MS) detection. The method we used measures the intact conjugate with minimal preparation and has been demonstrated with solid reliability and reproducibility [19]. Several limitations should also be noted. Firstly, some associations increased after excluding vitamin E supplement users, suggesting that there might be effect modification by the use of supplements or we might introduce collider stratification bias in the users of these supplements. However, the information on supplement use was very limited, as there were no data available on the dosages and frequency and natural or modified vitamin E acetate, resulting in a highly heterogeneous group. Therefore, further exploration of the effect of vitamin E supplement use on these associations was not feasible. Secondly, within our study population, we are not able to test these associations in different physiological situations that may affect the lipoproteins involved in the transportation process such as fasting or not [39-41], or in individuals with elevated lipid peroxidation that will reduce α-TOH bioavailability such as lower bioavailability identified in metabolomic syndrome patients compared with healthy controls [42]. Thirdly, we might still have insufficient power for some of these associations, particularly in the sensitivity analysis. However, we do not only perform statistical hypothesis testing, but apart from the point estimates obtained from our multivariable adjusted regression analyses illustrated in the main text,

we also calculate confidence intervals (CIs) for each estimate, as shown in the supplementary table. CIs reflect the precision of the estimation in the sample. In addition, we had a specific focus on the similarity of the directionality of the associations between metabolomic measurements with circulating α-TOH and the associations between metabolomic measurements with circulating α-CEHC/α-TLHQ. Lastly, the observational design could not rule out residual confounding.

Conclusion

Associations of metabolomic measures with circulating α-TOH and urinary oxidized vitamin E α-CEHC are very similar in direction, whereas associations of metabolomic measures with α-TLHQ were markedly different from the associations of metabolomic measures with both serum α-TOH and urinary α-CEHC. Our results highlight the differences of the lipoproteins involved in the transportation of enzymatic and oxidized vitamin E metabolites. This indicates that circulating α-TOH may be representative for the enzymatic but not to antioxidative function of vitamin E.

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Supplementary materials

Supplementary Table 1 Summary of metabolomic biomarkers and the associations with serum α-TOH, urinary α-CEHC and α-TLHQ

Supplementary Figure 1 The percentage of observations below detection limit per metabolite in all 147 metabolomic measures.

Supplementary Figure 2 Associations between 147 metabolomic measures with (A) circulating α-tocopherol, (B) urinary α-CEHC, (C) urinary α-TLHQ in non-supplement users

Supplementary Figure 3 Correlations of effect estimates between metabolomic measures with circulating α-tocopherol, urinary α-CEHC and α-TLHQ in sensitivity analysis (N = 350)

Supplementary Figure 4 Associations between 147 metabolomic measures with (A) circulating α-tocopherol, (B) urinary α-CEHC, (C) urinary α-TLHQ in participants with normal weight (BMI < 25kg/m^2)

Supplementary Figure 5 Associations between 147 metabolomic measures with (A) circulating α-tocopherol, (B) urinary α-CEHC, (C) urinary α-TLHQ in overweight participants (BMI >= 25kg/m^2)

The Supplementary materials for this article can be found online at: *https://drive.google.com/drive/folders/1d46G5jf6fIZUUlp6aHpjnz74SnLjzQPd?usp=sharing*