Oxidative stress in chronic diseases: causal inference from observational studies

Luo, J.

CHAPTER 6

Urinary oxidized, but not enzymatic vitamin E metabolites are inversely associated with measures of glucose homeostasis in middle-aged healthy individuals

Jiao Luo, Fleur L Meulmeester, Leon G Martens, Nadia Ashrafi, Renée de Mutsert, Dennis O Mook-Kanamori, Frits R Rosendaal, Ko Willems van Dijk, Saskia le Cessie, Kevin Mills, Raymond Noordam, Diana van Heemst

Clin Nutr. 2021 Jun;40(6):4192-4200

Abstract

Background & aims: Damage induced by lipid peroxidation has been associated with impaired glucose homeostasis. Vitamin E (α-tocopherol, α-TOH) competitively reacts with lipid peroxyl radicals to mitigate oxidative damage, and forms oxidized vitamin E metabolites. Accordingly, we aimed to investigate the associations between α-TOH metabolites (oxidized and enzymatic) in both circulation and urine and measures of glucose homeostasis in the general middle-aged population.

Methods: This cross-sectional study was embedded in the population-based Netherlands Epidemiology of Obesity (NEO) Study. α-TOH metabolites in plasma (α-TOH and α-CEHC-SO_3) and urine [sulfate (SO_3) and glucuronide (GLU) of both α-TLHQ (oxidized) and α-CEHC (enzymatic)] were quantified by liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS). Measures of glucose homeostasis (HOMA-B, HOMA-IR, Insulinogenic index, and Matsuda index) were obtained from fasting and postprandial blood samples. Multivariable linear regression analyses were performed to assess the associations of α-TOH metabolites and measures of glucose homeostasis.

Results: We included 498 participants (45% men) with mean (SD) age of 55.8 (6.1) years who did not use glucose-lowering medication. While plasma α-TOH was not associated with measures of glucose homeostasis, urinary oxidized α-TOH metabolites (α-TLHQ-SO_3/GLU) were associated with HOMA-IR and Matsuda index. For example, a one-SD higher α-TLHQ-SO_3 was associated with 0.92 (95% CI: 0.87, 0.97) fold lower HOMA-IR and 1.06 (1.01, 1.11) fold higher Matsuda index, respectively. Similar results were obtained for the urinary α-TLHQ to α-CEHC ratio as a measure of oxidized-over-enzymatic conversion of α-TOH.

Conclusion: Higher urinary levels of oxidized α-TOH metabolites as well as higher oxidized-to-enzymatic α-TOH metabolite ratio, but not circulating α-TOH or enzymatic metabolites, were associated with lower insulin resistance. Rather than circulating α-TOH, estimates of the conversion of α-TOH might be informative in relation to health and disease.

Introduction

Impaired glucose homeostasis is one of the first steps in the pathogenesis of type 2 diabetes mellitus (T2D). In addition to classical risk factors such as (central) obesity, lipid peroxidation, which is an autocatalytic chain reaction induced by free radicals reacting with lipids, has been implicated to play an important role in the impairment of glucose homeostasis [1].

Vitamin E (α-tocopherol, α-TOH) has been unequivocally demonstrated as an effective lipophilic radical scavenger to prevent the chain propagation in lipid peroxidation [2]. In oxidative catabolism, α-TOH is initially oxidized to α-tocopheroxyl radical by one-electron oxidation, and further reacts with lipid peroxides, opening the chromanol ring and consecutively forming α-tocopheryl quinone (α-TQ), α-tocopheryl hydroquinone (α-THQ), and α-tocopheronic acid. Subsequently, followed by β-oxidation and cyclization of the phytyl side chain, α-tocopherono lactone (α-TLHQ) will be generated (illustrated in our previous review [3]). Alternatively, in the hepatic enzymatic catabolism, α-TOH starts with ω-hydroxylation, and successively shortens the phytyl chain via multiple β-oxidation, generating α-carboxymethyl-hydroxychroman (α-CEHC). Thereafter, α-TLHQ and α-CEHC are both conjugated with glucuronic acid or sulfate to form water-soluble polar metabolites and are excreted mainly via urine. The group of oxidized metabolites α-TLHQ, therefore, reflects the extent of lipid peroxidation and α-TOH bioactivity.

Despite multiple studies having investigated the associations between vitamin E and glucose homeostasis in both observational studies and clinical trials yet with inconsistent results [4-19], studies scarcely addressed the most important aspect of functional α-TOH, i.e. to what extent α-TOH acts as antioxidants. Interestingly, oxidized α-TOH metabolites have been linked to increased risk of multiple diseases with elevated levels of lipid peroxidation [20-22]. Theoretically, impaired glucose homeostasis related to excessive lipid peroxidation is likely to have increased demand for antioxidants protection via α-TOH, and consequently, lead to higher levels of urinary oxidized metabolites. Of note, the only study that specifically explored the association between oxidized metabolites and diabetics identified increased levels of α-TLHQ in diabetic children compared with age- and sex-matched healthy controls [23]. No data so far are available about the association of α-TOH oxidized metabolites with measures of glucose homeostasis in the general population.

We hypothesized that oxidized urinary vitamin E metabolites, but not circulating α-TOH or enzymatic metabolites, would positively associate with worse glucose homeostasis measures. In the present study, we aimed to investigate the associations between α-TOH metabolites and measures of glucose homeostasis in a cross-sectional study of middle-aged healthy individuals embedded in the Netherlands Epidemiology of Obesity study (NEO).

Methods

Study design and Study population

This study was embedded in the population-based prospective Netherlands Epidemiology of Obesity (NEO) study, which is designed to investigate the pathways that are responsible for obesity-related disorders. The NEO study started in 2008 and includes 6,671 individuals aged 45-65 years, with an oversampling of individuals with a self-reported body mass index (BMI) of 27 kg/m^2 or higher. Besides, all inhabitants aged between 45 to 65 years from the municipality of Leiderdorp were invited irrespective of their BMI. The study was approved by the medical ethical committee of the Leiden University Medical Center (LUMC), and all participants gave written informed consent. Detailed information on the study design and data collection have been described previously [24].

Participants were invited to come to the NEO study center of the LUMC for one baseline study visit after an overnight fast. Prior to this study visit, participants collected their urine over 24h and completed a general questionnaire at home in terms of their demographic, lifestyle, and clinical data in addition to specific questionnaires on diet and physical activity. Medication use within one month prior to the visit was asked to bring with participants and was recorded by research nurses. Fasting blood samples were drawn, and within five minutes later, a 400 mL, 600 kcal mixed meal (energy derived from protein, carbohydrate and fat were 16%, 50%, and 34%, respectively) was consumed. Postprandial blood samples were then drawn at 30 and 150 minutes after the meal.

The present study cross-sectionally analyzed the baseline measurements. We included a random subset of 35% of the Leiderdorp participants from Western European ancestry with imaging and genomics information collected (N = 599). In total, 536 participants were eligible with urine collected for at least 20 hours. We excluded participants for the main analyses with: 1) glucose-lowering medication use (n = 12) or no glucose homeostasis measures (n = 1); 2) sample failed to measure urinary metabolites (n = 1); 3) biologically implausible urinary metabolites measures due to sample problem (no TLHQ metabolites, n = 2); outliers (see 2.5. Statistical analysis) on plasma (α-TOH) or urinary metabolites (n = 9); 3) missing data (n = 1) or outliers on HOMA-B or HOMA-IR (n = 1); 4) missing data on potential confounders including physical activity, Dutch health diet index, education level and body fat percentage (n = 11, 1 overlap with vitamin E metabolites outliers). Consequently, a total of 498 individuals were used in the fasting analyses. In addition, we excluded individuals with (1) uncompleted mixed meal challenge (n = 33); (2) missing data on Matsuda index or Insulinogenic Index (n = 20, 3 overlapped with uncompleted mix meal), leaving a total of 448 participants in the postprandial analyses. Summary of the participant exclusions is presented in **Supplementary Figure 1**.

Alpha-tocopherol metabolites measures

Alpha-tocopherol (metabolite) measurements in plasma

In fasting plasma samples, α-TOH and α-CEHC sulfate conjugates (α-CEHC-SO_3) were detected and quantified by untargeted metabolomics provider 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). More detailed descriptions have been described previously [25,26].

Alpha-tocopherol metabolite measurements in urine

Urinary oxidized α-TOH metabolites (α-TLHQ) and enzymatic metabolites (α-CEHC), presented as their sulfate or glucuronide conjugates (α-TLHQ-SO_3, α-TLHQ-GLU, α-CEHC-SO_3, α-CEHC-GLU), were measured by LC-MS/MS at the University College London, UK.

Prior to the measurement, urine samples were thawed, and 100µl fresh urine was then centrifuged in Eppendorf tubes at 14 000 g for 10 min at room temperature and spiked with 10µl of the internal standards (100µmol/L), lithocholic acid sulfate (LA), and androsterone D4-glucuronide (AD4). Subsequently, samples were vortexed and transferred into screw-cap glass vials. 10 µl was injected into the LC-MS/MS for detection.
The metabolites were separated using 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. The mobile phase was a gradient elution of solvent A (99.98% water; 0.01% (v/v) formic acid) and solvent B (99.98% acetonitrile/MeCN; 0.01% (v/v) formic acid), which were LC-MS grade or equivalent (Sigma-Aldrich Co. Ltd). The flow rate was set to 0.8mL/min and the LC gradient was established by coordinating the solvents as follows: 95% solvent A plus 5% solvent B for 0 to 0.40 min; 80% solvent A plus 20% solvent B for 2 min; 0.1% solvent A plus 99.9% solvent B for 3.01 to 4 min; 95% solvent A plus 5% solvent B for 4.01 to 5 min. In order to minimize system contamination and carryover, the MS diverter valve was set up to discard the UPLC eluent before and after the sample elution, at 0 to 0.40 min and 4.01 to 5 min, respectively, as well as an additional run of a blank sample (H_2O: MeCN) between each run of urine samples. Two peaks were observed for α-TLHQ and α-CEHC glucuronide conjugates, corresponding to major and minor isoforms. The different elution time (min) for internal standards (LA 4.33, AD4 2.7) and each metabolite (2.39, 2.12 and 2.29 for α-TLHQ sulfate, 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.

After separation, the metabolites were then analyzed by MS using a Waters ACQUITY UPLC coupled to a triple-quadrupole Xevo TQ-S fitted with electrospray ionization in negative ion mode. The gas temperatures persisted at 600°C for desolvation. In addition, nitrogen was used as the nebulizing gas with a 7.0 bar. α-CEHC and α-TLHQ are isobaric because of the same molecular mass ($C_{15}H_{22}O_4$). The cone voltages were set at 56 V and 54 V, and the collision voltages at 28 eV and 30 eV for sulfate conjugates and glucuronide conjugates, respectively.

Running time for each sample is 5 minutes with a 20μL injection volume together with a partial loop with needle overfill mode. Using multiple reaction monitoring (MRM) mode, specific parent and daughter ions were determined in scan mode and the following collision activated dissociation (CAD) with argon. These ions were then used to quantify each α-TOH metabolite from transitions previously established by Sharma et al. [27] (glucuronide conjugates 453.3>113.0 m/z and sulfate conjugates, 357.1>79.9 m/z) that corresponded to their theoretical molecular masses.

Urinary creatinine concentrations (mmol/L) were measured to correct dilution differences for each metabolite, by triple-quadrupole Micro Quattro mass spectrometry (MicroMass, Waters, UK) using deuterated creatinine as the internal standard. Therefore, the concentrations of α-TOH metabolites are expressed as nmol per mmol of creatinine. A quality control (QC) assessment was performed throughout the quantification both in creatinine and α-TOH metabolite assays to deal with the variations in sample quality and UPLC-MS/MS performance over time. Four QC samples were systematically interleaved every 50 urine samples to limit the amount of sample loss. The whole measurement protocol was developed and further modified by the detection group in London [23,28].

The final concentrations of glucuronide conjugates for α-TLHQ and α-CEHC were the sum of their corresponding major and minor isoforms. In addition to the measured single metabolite, total, glucuronide and sulfate conjugates ratios were further determined to reflect the α-TOH antioxidative capacity as well as lipid peroxidation levels taking α-TOH status into consideration, namely as the α-TLHQ-to-α-CEHC ratio, α-TLHQ-GLU-to-α-CEHC-GLU ratio, and α-TLHQ-SO_3-to-α-CEHC-SO_3 ratio.

Glucose homeostasis measures

For blood samples, plasma glucose concentrations were obtained by enzymatic and colorimetric methods (Roche Modular Analytics P800, Roche Diagnostics, Mannheim, Germany; CV < 5%), while serum insulin concentrations were detected via an immunometric method (Siemens Immulite 2500, Siemens Healthcare Diagnostics, Breda, The Netherlands; CV < 5%). All these measurements were performed in the central clinical chemistry laboratory of the LUMC. Homeostatic model of insulin resistance (HOMA-IR), a marker of hepatic insulin resistance, homeostatic model of and β-cell function (HOMA-B), a measure of β-cell to glucose-stimulated insulin secretion were then calculated by using the formula (fasting glucose × fasting insulin)/22.5 and (20 × fasting insulin)/(fasting glucose - 3.5) respectively [29,30]. Matsuda index, which represents both hepatic and peripheral tissue sensitivity to insulin, was generated as $10000/\sqrt{}$ (fasting glucose × fasting insulin) ($glucose_{mean(0\text{-}150min)}$ × $insulin_{mean(0\text{-}150min)}$) [31]. Moreover, Insulinogenic Index also reflects β-cell function but specifically, the first-phase insulin response to glucose challenge was calculated with the formula ($insulin_{30min}$ - fasting insulin)/($glucose_{30min}$ - fasting glucose) [32].

Covariates

Education level was grouped into high (including higher vocational school, university, and postgraduate education) and low based on the Dutch Education system. Smoking habits were reported in three categories: current smoker, for-

mer smoker and never smoke. Familial diabetes history (yes/no) was collected through a general questionnaire and defined as having a father, mother, or brother or sister with a diagnosis of diabetes mellitus. The frequency and duration of leisure physical activity over the past 4 weeks were reported by participants on the Short Questionnaire to Assess Health-enhancing physical activity (SQUASH), which is expressed as metabolic equivalent (MET-hours per week). A semiquantitative food frequency questionnaire was used to assess food and beverage intake, and total energy intake. Dutch Healthy Diet Index (DHD-index) was then calculated based on dietary intake [33]. Percentage body fat was measured by Bio Impedance Balance (TBF-310, Tanita International Division, UK). Total cholesterol levels (mmol/L) were obtained in fasting blood samples in our central clinical chemistry laboratory using standard assays. Lipid-lowering medication defined as the use of statins fibrates and other lipid-lowering medication was recorded by medicine inventory by the research nurses. Information on the use of vitamin E supplements was collected through questionnaires as the combination of vitamin E supplements only or the use of multivitamin supplements.

Statistical analysis

Main analysis

Descriptive characteristics at the NEO baseline were presented as mean (standard deviation, SD) for normally distributed variables, median (interquartile range) for skewed variables, and frequency (proportions) for categorical variables. As the distribution of both plasma and urinary vitamin E metabolites and the measures of glucose homeostasis (HOMA-B, HOMA-IR, Insulinogenic Index, and Matsuda index) were skewed, these were natural log-transformed. Outliers (defined as located more than 4 SD from the mean) were removed after natural log transformation, comprising approximately 2% of the total observations.

In order to explore the α-TOH metabolism process, pairwise Pearson correlations were performed between circulatory and urinary metabolites. Multivariable linear regression analyses were performed to examine the association between α-TOH metabolites (determinant) and measures of glucose homeostasis (outcome). To facilitate interpretation and comparison between measures of glucose homeostasis, determinants were then z-transformed (mean = 0, SD = 1), so that the regression coefficient with its corresponding 95% confidence interval (CI) can be interpreted as the fold difference in the outcome with respect to a one-SD change in the determinant. For each outcome and determinant, four models were fitted. The basic regression model was adjusted for age and sex (Model 1). Model 2 was additionally adjusted for potential confounders based on biological knowledge and previous studies including educational level (high or low), familial history of diabetes (yes or no), physical activity (MET-hours per week), smoking habits (never smoke, current smoker or former smoker), Dutch Healthy Diet Index and total energy intake (kJ/day). Total cholesterol level (mmol/L) and lipid-lowering medication use (yes/no) were also included in Model 2 in models on the fasting levels for plasma α-TOH only since the transportation and uptake of α-TOH are largely dependent on lipoproteins [34]. Of the lipoproteins, it was shown before that specifically (but not exclusively) LDL cholesterol is acting as α-TOH transporter [35]. Therefore, we performed additional analyses with adjustment of LDL-cholesterol only in Model 2(A), LDL, and HDL-cholesterol in Model

2(B) instead of the adjustment of total cholesterol levels. Moreover, obesity has been shown to directly have an influence on glucose homeostasis, especially insulin resistance [36], and meanwhile, obesity also increases lipid peroxidation levels [37] and might thus affect the conversion of α-TOH in the body. Therefore, we included total body fat (Model 3) as an indicator of fat content to explore the underlying mechanisms.

Sensitivity analyses

Urine sample collection missingness and other exclusions

To evaluate whether the current study population is representative of the total study population, the study characteristics among individuals of urine sample collection missingness and other exclusion reasons were compared with the included participants separately. Chi-square test was used for categorical variables, while t-test was used for normally distributed numeric variables and Mann-Whitely U test was used for non-normally distributed numeric variables.

Vitamin E supplement use

Vitamin E supplement use might have an influence on measures of glucose homeostasis as well as vitamin E conversion in the body. Vitamin E supplement use in the current study was defined as either the use of vitamin E supplements only or the use of multiple vitamin supplements. In order to fully rule out the supplement effect, we further performed sensitivity analyses in participants who did not use vitamin E and/or multivitamin supplements.

Censored normal regression

In the main analysis, we excluded all participants with glucose-lowering medication use. However, exclusion might underestimate the effect size of the determinants, reduce power, and could introduce (collider-stratification) bias in the analyses. A reliable approach to correct this dilution is to use censored normal regression model analysis which has been established previously [38]. Therefore, we additionally performed censored normal regression model analysis for the final adjusted model (Model 3) as in the main analysis.

All the analyses were undertaken using R (v3.6.1) statistical software (The R Foundation for Statistical Computing, Vienna, Austria) (https://www.r-project.org/). Pairwise correlation plots were performed using the R package “GGally” (https://CRAN.R-project.org/package=GGally) 39, and the censored normal linear regression models were performed using the R package “survival” (https://CRAN.R-project.org/package=survival) 40.

Results

Characteristics of the study population

A total of 498 participants (45% men) were analyzed, of whom 448 were available for postprandial analyses. The baseline characteristics of the study population are presented in **Table 1**.

Table 1 Characteristics the study population[1]

	N = 498
Demographics	
Age (years)	55.8 (6.1)
Sex (male)	225 (45.2%)
BMI (kg/m^2)	25.4 (23.1, 27.8)
Total body fat (%)	30.9 (8.3)
Education level (high)	253 (50.8%)
Family history of diabetes (yes)	136 (27.3%)
Lifestyle factors	
Dutch healthy diet index	59.8 (8.4)
Energy intake (KJ/day)	9106 (7304, 11053)
Physical activity a (MET-h/week)	29.4 (16.5, 48.8)
Smoking	
Current	53 (10.6%)
Former	235 (47.2%)
Never	210 (42.2%)
Vitamin E supplement use (yes)[2]	128 (25.7%)
Lipid-lowering medication use (yes)	28 (5.6%)
Vitamin E metabolites measurements	
Blood (log-transformed, no unit)[3]	
α-tocopherol	19.7 (0.2)
α-CEHC-SO$_3$[4]	12.1 (0.6)
Urinary	
α-TLHQ-SO$_3$ (nmol/mmol creatinine)	2.6 (1.6, 4.1)
α-TLHQ-GLU (nmol/mmol creatinine)	1822.5 (1339.3, 2744.8)
α-CEHC-SO$_3$ (nmol/mmol creatinine)	165.4 (97.4, 298.3)
α-CEHC-GLU (nmol/mmol creatinine)	91.0 (62.3,139.4)
α-TLHQ/α-CEHC	7.1 (5.1, 9.9)
α-TLHQ-SO$_3$/α-CEHC-SO$_3$	0.016 (0.011, 0.024)
α-TLHQ-GLU/α-CEHC-GLU	20.8 (15.4, 28.5)
Urinary (log-transformed)	
α-TLHQ-SO$_3$	1.0 (0.7)
α-TLHQ-GLU	7.6 (0.6)
α-CEHC-SO$_3$	5.1 (0.8)
α-CEHC-GLU	4.5 (0.6)
α-TLHQ/α-CEHC	1.9 (0.5)
α-TLHQ-SO$_3$/α-CEHC-SO$_3$	-4.1 (0.7)
α-TLHQ-GLU/α-CEHC-GLU	3.0 (0.4)
Glucose homeostasis	
Fasting levels	
HOMA-IR	1.9 (1.3, 2.9)
HOMA-B (%)	89.7 (63.8, 129.6)
Postprandial response[5]	
Matsuda Index	5.6 (3.8, 7.9)
Insulinogenic index	7.2 (5.7, 9.4)

[1]Data are presented as median (interquartile range) for numeric variables, and number (proportions) for categorical variables. [2] Vitamin E supplement use was defined as either vitamin E supplement use or multiple vitamin supplement use. [3] The concentrations were measured by Metabolon platform, and no units were presented. [4] Only complete cases in metabolon platform (n = 263). [5] Participants with completed meal challenge and no missing data on postprandial response were used (N = 448).

Table 2 Associations between circulatory α-tocopherol and measures of glucose homeostasis in the general population[1]

	Fasting measures		Postprandial responses	
	HOMA-B	HOMA-IR	Insulinogenic index	Matsuda Index
Model 1	0.95 (0.91, 1.00)	0.97 (0.92, 1.03)	1.00 (0.96, 1.03)	0.99 (0.94, 1.04)
Model 2	0.98 (0.92, 1.04)	0.98 (0.91, 1.05)	1.01 (0.96, 1.05)	1.00 (0.94, 1.07)
Model 2 (A)	0.96 (0.91, 1.01)	0.96 (0.90, 1.03)	1.00 (0.96, 1.04)	1.01 (0.95, 1.07)
Model 2 (B)	0.97 (0.92, 1.02)	0.98 (0.92, 1.04)	1.00 (0.96, 1.05)	1.00 (0.94, 1.06)
Model 3	0.97 (0.92, 1.03)	0.97 (0.91, 1.03)	1.01 (0.96, 1.05)	1.01 (0.96, 1.06)
Model 3 (A)	0.96 (0.91, 1.01)	0.97 (0.91, 1.02)	1.00 (0.96, 1.04)	1.00 (0.95, 1.05)
Model 3 (B)	0.97 (0.92, 1.02)	0.97 (0.92, 1.03)	1.00 (0.96, 1.05)	1.00 (0.95, 1.05)

[1] In total, 498 participants with available α-tocopherol were used for fasting HOMA-B and HOMA-IR analysis while 448 for postprandial Insulinogenic index and Matsuda Index analysis. Results are derived from linear regression coefficients with 95% confidence interval (CI) and were expressed as a one-SD change in blood α-tocopherol with corresponding fold difference in log-transformed glucose homeostasis traits. Model 1: age and sex. Model 2: Model 1 + education level (high or low), familial history of diabetes (yes or no), physical activity (MET hours per week), smoking habits (never smoke, current smoker or former smoker), Dutch Healthy Diet index (DHD-index), energy intake (KJ/day), total cholesterol level (mmol/L) and lipid lowering medication (yes or no). Model 2 (A): Model 1 + education level (high or low), familial history of diabetes (yes or no), physical activity (MET hours per week), smoking habits (never smoke, current smoker or former smoker), Dutch Healthy Diet index (DHD-index), energy intake (KJ/day), LDL cholesterol level (mmol/L) and lipid lowering medication (yes or no). Model 2 (B): Model 1 + education level (high or low), familial history of diabetes (yes or no), physical activity (MET hours per week), smoking habits (never smoke, current smoker or former smoker), Dutch Healthy Diet index (DHD-index), energy intake (KJ/day), LDL and HDL cholesterol level (mmol/L) and lipid lowering medication (yes or no). Model 3: Model 2 + body fat percentage (%). Model 3 (A): Model 2 (A) + body fat percentage (%). Model 3 (B): Model 2 (B) + body fat percentage (%).

Main analysis

Pairwise correlation between plasma and urinary metabolites

Correlations between circulatory and urinary α-TOH metabolites are shown in **Figure 1**. In all participants, plasma α-TOH was weakly associated with any urinary metabolite (r: 0.18 - 0.23), while plasma α-CEHC-SO_3 was moderately correlated with urinary metabolites (r: 0.31 - 0.49,) with the highest correlation with urinary α-CEHC-SO_3 (r = 0.49). Urinary metabolites were highly correlated with each other with r ranging from 0.44 to 0.73.

Circulatory α-TOH and glucose homeostasis measures

In plasma, we did not find evidence that α-TOH was associated with measures of glucose homeostasis after fully adjusting for potential confounders, as shown in **Table 2**.

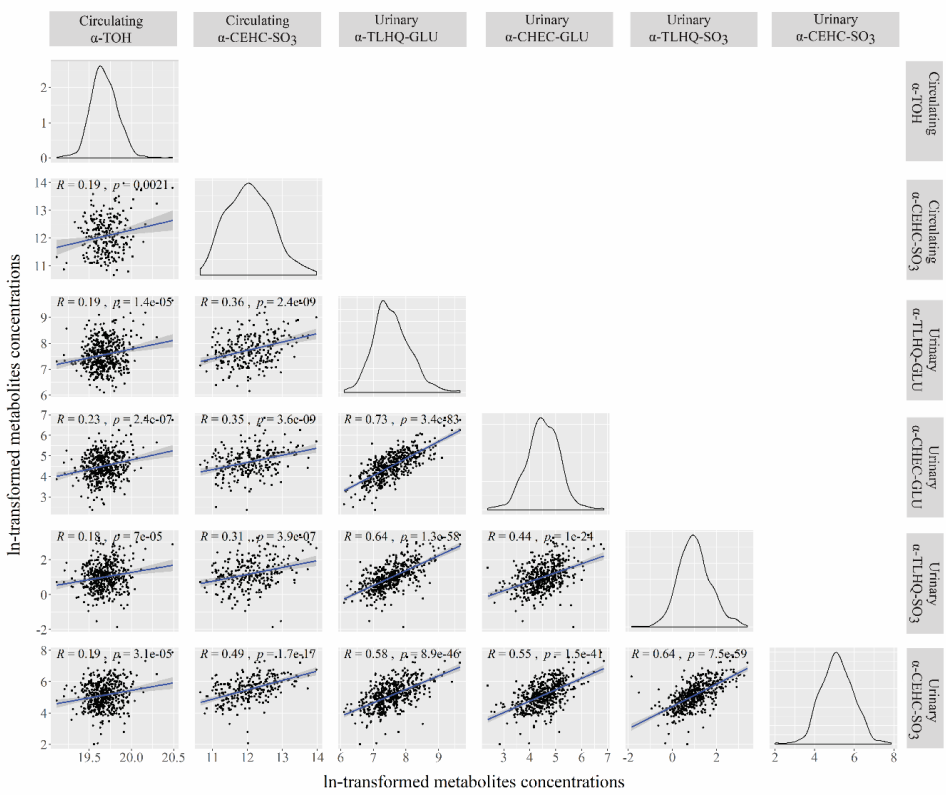

Figure 1 Pairwise correlation

Pairwise correlation of α-tocopherol metabolites in blood and urine (N = 498). Pearson correlations were calculated after natural log-transformation of metabolites. For blood α-CEHC-SO_3, only the complete cases in the metabolon platform were used (n = 263).

Urinary metabolites and glucose homeostasis measures

The urinary oxidized metabolites α-TLHQ-SO_3 and α-TLHQ-GLU were associated with HOMA-IR and Matsuda index as visualized in **Figure 2** (summary statistics in **Supplementary Table 2**). For fasting measures (**Figure 2A** and **2B**), in the age- and sex- adjusted basic model (Model 1), a one-SD higher α-TLHQ-SO_3 was associated with 0.93 [95% confidence interval (CI): 0.89, 0.98] fold lower HOMA-B and 0.89 (95% CI: 0.83, 0.94) fold lower HOMA-IR, respectively. However, when we additionally adjusted for other potential confounders including lifestyle factors and adiposity measures (Model 3), the association observed for HOMA-B diminished towards null and α-TLHQ-SO_3 was only associated with HOMA-IR (β: 0.92, 95% CI: 0.87, 0.97). Similarly, α-TLHQ-GLU followed the same pattern, and a one-SD higher α-TLHQ-GLU was associated with 0.93 (95% CI: 0.88, 0.98) fold lower HOMA-IR in the fully adjusted Model 3. This pattern was also observed in postprandial analyses, and α-TLHQ-SO_3 and α-TLHQ-GLU were only associated with Matsuda index (**Figure 2D**), with a one-SD higher α-TLHQ-SO_3 corresponding to 1.06 (95% CI: 1.01, 1.11) fold higher Matsuda

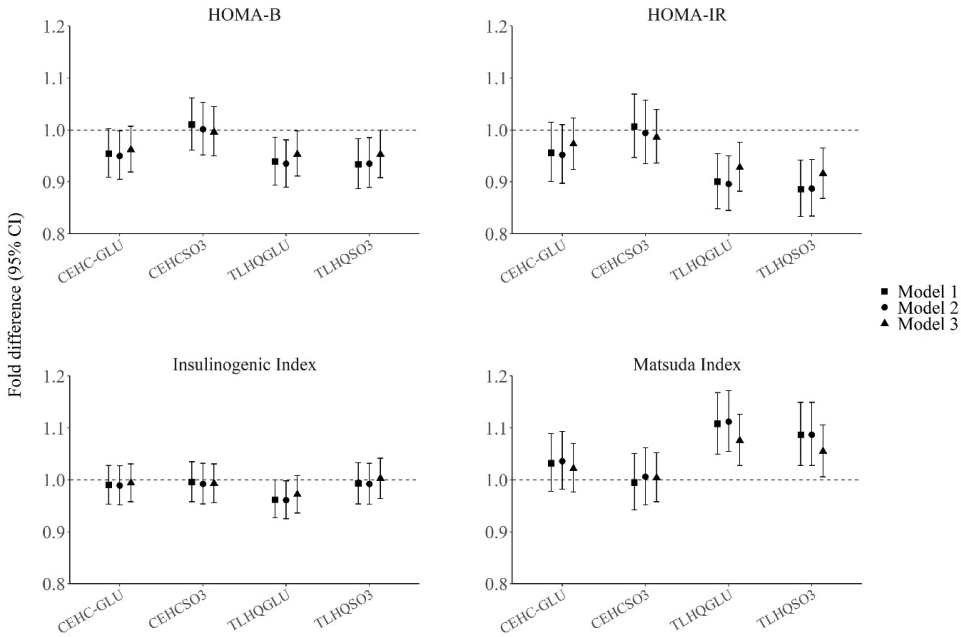

Figure 2 Association between urinary metabolites and glucose homeostasis measurements

Association between urinary metabolites and (A) HOMA-B, (B) HOMA-IR, (C) Insulinogenic Index, and (D) Matsuda Index in general population. Results were based on general population (N = 498 for HOMA-B and HOMA-IR; N = 448 for Insulinogenic Index and Matsuda Index), and were derived from multivariable linear regression coefficient with corresponding 95% confidence interval (CI) and were expressed as fold difference in log-transformed outcomes with a one-standard deviation change in determinants. Model 1: adjust for age and sex. Model 2: Model 1 + educational level (high or low), familial history of diabetes (yes or no), physical activity (MET hours per week), smoking habits (never smoke, current smoker or former smoker), Dutch Healthy Diet Index and total energy intake (KJ/day). Model 3: Model 2 + total body fat percentage (%).

index, and a one-SD higher α-TLHQ-GLU corresponding to 1.08 (95% CI: 1.03, 1.13) fold higher Matsuda index after adjustment for all potential confounders (Model 3). However, no association was found between enzymatic metabolites and any measures of glucose homeostasis.

Higher oxidized-to-enzymatic metabolite ratios showed similar associations as oxidized metabolites with measures of glucose homeostasis (**Figure 3** and summary statistics in **Supplementary Table 3**). For example, in the basic

model (Model 1), a one-SD higher α-TLHQ-to-α-CEHC ratio was associated with 0.90 (95% CI: 0.85, 0.95) fold lower HOMA-IR (**Figure 3B**) and 1.11 (1.05, 1.17) fold higher Matsuda index (**Figure 3D**). After adjustment for potential confounding factors (Model 3A), the association was attenuated, and a one-SD higher α-TLHQ-to-α-CEHC ratio was associated with 0.94 (95% CI: 0.89, 0.99) fold lower HOMA-IR and 1.07 (95% CI: 1.02, 1.12) fold higher Matsuda index. Simi-

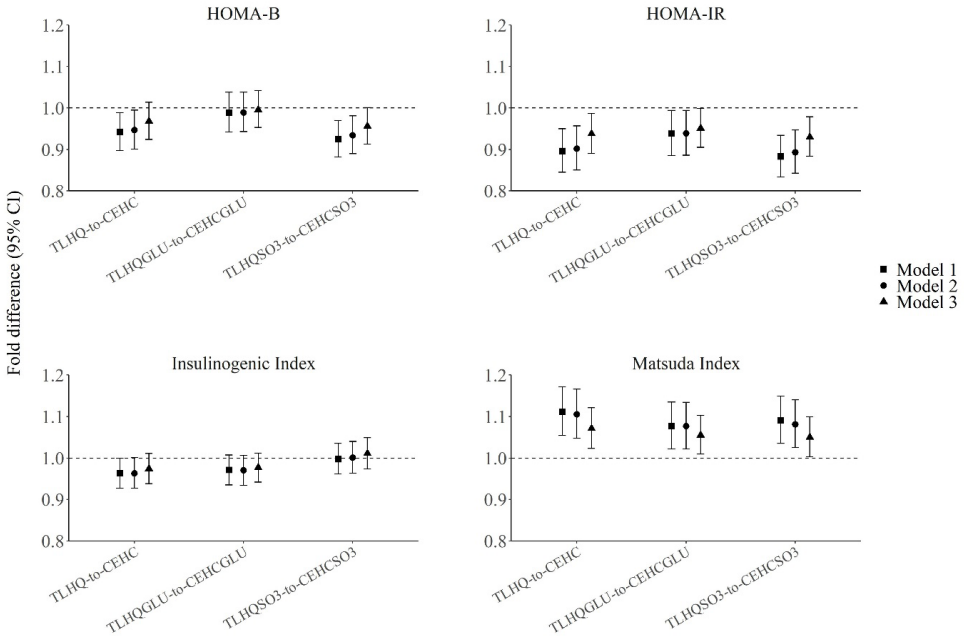

Figure 3 Association between urinary enzymatic-to-oxidized metabolite ratios and glucose homeostasis measurements

Association between urinary metabolite ratios and (A) HOMA-B, (B) HOMA-IR, (C) Insulinogenic Index, and (D) Matsuda Index in general population. Results were based on general population (N = 498 for HOMA-B and HOMA-IR; N = 448 for Insulinogenic Index and Matsuda Index), and were derived from multivariable linear regression coefficient with corresponding 95% confidence interval and were expressed as fold difference in log-transformed outcomes with a one-standard deviation change in determinants. Model 1: adjust for age and sex. Model 2: Model 1 + educational level (high or low), familial history of diabetes (yes or no), physical activity (MET hours per week), smoking habits (never smoke, current smoker or former smoker), Dutch Healthy Diet Index and total energy intake (KJ/day). Model 3A: Model 2 + total body fat percentage (%).

lar associations were observed for the sulfate conjugate ratio and glucuronide conjugate ratio.

Sensitivity analysis

Urine sample collection missingness and other exclusions

Basic characteristics were compared between included participants (N = 498), individuals with less than 20 hours or no urine collection (N = 63) and excluded participants due to other reasons (N = 38), as presented in **Supplementary Table 1**. The participants excluded due to other reasons had higher percentages of family history of diabetes (27.3%, 30.2% versus 47.4%) and lipid-lowering medication use (5.6%, 11.1% versus 29.0%). Moreover, this group had slightly higher age and more male participants.

Vitamin E supplementation
Participants with vitamin E supplementation (n = 128) were further excluded in this sensitivity analysis, leaving 370 individuals for fasting and 337 for postprandial analyses, respectively. The correlation between α-TOH and other metabolites remained low. Plasma α-CEHC-SO_3 was moderately correlated to urinary α-CEHC-SO_3 (r = 0.41) only (**Supplementary Figure 2**). In addition, effect sizes did not change materially in the multivariable regressions after adjusting for confounders compared with the full population analyses (**Supplementary Table 4, Figure 3 and 4**).

Censored normal regression
In censored normal regression models with 12 individuals taking glucose-lowering medication included, 510 participants with complete data were included for fasting analysis, and 460 participants for postprandial analysis. We did not observe substantial differences in Model 3 (Supplementary Table 5) compared with the main analysis.

Discussion
In the present cross-sectional study, we aimed to investigate the association between metabolites of vitamin E (α-TOH) and measures of glucose homeostasis in both fasting and postprandial state. When adjusted for potential confounders, we did not find evidence supporting an association between plasma α-TOH with measures of glucose homeostasis. However, we observed that higher urinary oxidized metabolites of α-TOH, as well as oxidized-to-enzymatic metabolite α-TOH ratios, were associated with reduced insulin resistance. Additional adjustment for total body fat only minimally explained these observations. Sensitivity analysis including restriction to individuals not taking vitamin E or multivitamin supplements and censored normal regression model provided similar results emphasizing the robustness of the observations.

The lack of evidence supporting an association between plasma α-TOH with measures of glucose homeostasis observed in the present study is in accordance with some [8-12], but not all previous observational studies [6,7]. The discrepancies might due to several factors such as study design, sample size as well as used confounders. In line with our study, in a more than 20 years follow-up study of middle-aged Swedish men, plasma α-TOH was not associated with future insulin response or T2D incidence [8]. Interestingly, in some clinical trials, α-TOH supplement only, which is mostly corresponding to an increase of circulating α-TOH [41-43], was not beneficial for the improvement of glucose levels, lipid levels, or insulin sensitivity [17-19]. However, this may be explained by factors such as dosage, timing, duration, and type of vitamin E.

With respect to oxidized metabolites, contrary to our hypotheses, we found associations between higher oxidized metabolites and better measures of glucose homeostasis. Previously, a study particularly identified that urinary α-TLHQ was higher in children with type 1 diabetes mellitus than in healthy controls [23]. Participants with metabolic syndrome also had decreased concentration of uri-

nary α-CEHC due to increased oxidative stress levels and inflammation in spite of rather similar plasma α-TOH concentration compared with healthy individuals, thereby indicating a higher antioxidative demand for α-TOH [44]. Moreover, metabolic syndrome patients had approximately 12% greater static oxidation reduction potential, i.e. oxidants, and 59% lower readily available antioxidant reserves compared with healthy adults [45]. An explanation for the inconsistency identified in our study may lie in the bioavailability and elimination of α-TOH. The bioavailability was shown to be reduced and the elimination delayed in metabolic syndrome patients who have increased lipid peroxidation, observed as lower plasma and urinary α-CEHC, independent of the co-ingested dairy fat amount [46]. In disease conditions where oxidants outweigh antioxidants, more antioxidants are required to diminish the damage caused by oxidative stress. Together with the decreased hepatic turnover, the conversion of α-TOH may shift to a preference for non-enzymatic oxidation. However, in our relatively healthy population, lipid oxidation level is assumed to be relatively low and antioxidants outweigh oxidants with no delayed enzymatic conversion. In addition, other antioxidative systems still have the potential to neutralize oxidants, therefore, the scavenging function of α-TOH might be compensated. Furthermore, the excretion of these metabolites may also alter in different health conditions, and a higher/lower excretion via bile or feces may occur.

In the pairwise correlation analysis, the moderate correlation of α-CEHC-SO_3 between plasma and urine is a validation of the measurement. However, a very weak correlation was found between plasma α-TOH and oxidized metabolites indicating that the excretion of oxidized metabolites does not increase with the increase of circulating α-TOH. This raises the argument that despite of an increased level of circulating α-TOH, the body does not fully make use of this α-TOH as antioxidant. Taken together, the circulating level of α-TOH may not reflect the antioxidative capacity and may not affect glucose homeostasis.

One of the strengths of the present study is the general population-based setting and the various confounding factors considered. Former studies of oxidized α-TOH metabolites and health outcomes have been conducted in relatively small patient cohorts, limiting the generalization of the results. Another strength is the measurement of oxidized metabolites. Previous chromatography-mass spectrometry (GC-MS) based method required long sample preparation of deconjugation, extraction, and derivatization, and only detected free unconjugated metabolites [47,48]. These may result in artefactual oxidation products of α-CEHC during sample preparation, α-TOH acid, and α-tocopherono lactone, better known as Simon metabolites [49,50]. The LC-MS/MS based method used in our study was developed and validated previously to have solid reliability and reproducibility [27,28], and the intact conjugate with minimal preparation ensures that the metabolites are unlikely due to artifact formation [27].

There are several limitations in this study. Firstly, a considerable number of individuals had no urine collection for at least 20 hours, however, study characteristics were comparable with our study population. Secondly, since multiple urinary metabolites and study outcomes were used in the analyses, there might be multiple testing and a chance of false-positive results. However, our exposures and outcomes were highly intercorrelated, and conventional corrections

for multiple testing (e.g, Bonferonni) are too conservative. Thirdly, the habitual dietary intake of all participants was estimated by using a self-administered, semiquantitative 125-item food frequency questionnaire (FFQ). As this FFQ did not contain complete information on certain food items (micronutrients), we had very limited information to calculate dietary intake of alpha-tocopherol, therefore, we are not able to perform any analysis based on intake of alpha-tocopherol. Lastly, because of the cross-sectional design, we are not able to rule out residual confounding or reverse causation.

In conclusion, the present study suggests that, in the middle-aged population, circulating α-TOH is not associated with glucose homeostasis measures. However, higher amounts of urinary oxidized α-TOH metabolites and oxidized-to-enzymatic metabolite ratios are associated with lower insulin resistance. This finding supports the hypothesis that, rather than circulating α-TOH, its conversion might be more informative in relation to health and diseases. These findings also highlight the importance of disentangling the conversion preference of α-TOH in different health states in future studies.

References

1. S. Sasson. Nutrient overload, lipid peroxidation and pancreatic beta cell function. Free Radic Biol Med 2017; 111: 102-9.
2. E. Niki. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence. Free Radic Biol Med 2014; 66: 3-12.
3. J. Luo, K. Mills, S. le Cessie, R. Noordam, D. van Heemst. Ageing, age-related diseases and oxidative stress: What to do next? Ageing Res Rev 2020; 57: 100982.
4. E. S. Eshak, H. Iso, I. Muraki, A. Tamakoshi. Fat-soluble vitamins from diet in relation to risk of type 2 diabetes mellitus in Japanese population. Br J Nutr 2018: 1-18.
5. C. Zhou, L. Na, R. Shan, et al. Dietary Vitamin C Intake Reduces the Risk of Type 2 Diabetes in Chinese Adults: HOMA-IR and T-AOC as Potential Mediators. PLoS One 2016; 11(9): e0163571.
6. E. J. Mayer-Davis, T. Costacou, I. King, D. J. Zaccaro, R. A. Bell. Plasma and dietary vitamin E in relation to incidence of type 2 diabetes: The Insulin Resistance and Atherosclerosis Study (IRAS). Diabetes Care 2002; 25(12): 2172-7.
7. T. Costacou, B. Ma, I. B. King, E. J. Mayer-Davis. Plasma and dietary vitamin E in relation to insulin secretion and sensitivity. Diabetes Obes Metab 2008; 10(3): 223-8.
8. J. Arnlov, B. Zethelius, U. Riserus, et al. Serum and dietary beta-carotene and alpha-tocopherol and incidence of type 2 diabetes mellitus in a community-based study of Swedish men: report from the Uppsala Longitudinal Study of Adult Men (ULSAM) study. Diabetologia 2009; 52(1): 97-105.
9. A. Reunanen, P. Knekt, R. K. Aaran, A. Aromaa. Serum antioxidants and risk of non-insulin dependent diabetes mellitus. Eur J Clin Nutr 1998; 52(2): 89-93.
10. H. Z. Pan, L. Zhang, M. Y. Guo, et al. The oxidative stress status in diabetes mellitus and diabetic nephropathy. Acta Diabetol 2010; 47 Suppl 1: 71-6.
11. L. A. Da Costa, B. Garcia-Bailo, C. H. Borchers, A. Badawi, A. El-Sohemy. Association between the plasma proteome and plasma alpha-tocopherol concentrations in humans. J Nutr Biochem 2013; 24(1): 396-400.
12. I. Mehmetoglu, F. H. Yerlikaya, S. Kurban. Correlation between vitamin A, E, coenzyme Q(10) and degree of insulin resistance in obese and non-obese subjects. J Clin Biochem Nutr 2011; 49(3): 159-63.
13. G. Paolisso, A. D'Amore, D. Galzerano, et al. Daily vitamin E supplements improve metabolic control but not insulin secretion in elderly type II diabetic patients. Diabetes Care 1993; 16(11): 1433-7.
14. G. Paolisso, A. D'Amore, D. Giugliano, A. Ceriello, M. Varricchio, F. D'Onofrio. Pharmacologic doses of vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetic patients. Am J Clin Nutr 1993; 57(5): 650-6.
15. A. Sharma, S. Kharb, S. N. Chugh, R. Kakkar, G. P. Singh. Evaluation of oxidative stress before and after control of glycemia and after vitamin E supplementation in diabetic patients. Metabolism 2000; 49(2): 160-2.

16. M. Boshtam, M. Rafiei, I. D. Golshadi, M. Ani, Z. Shirani, M. Rostamshirazi. Long term effects of oral vitamin E supplement in type II diabetic patients. Int J Vitam Nutr Res 2005; 75(5): 341-6.
17. A. M. de Oliveira, P. H. Rondo, L. A. Luzia, F. H. D'Abronzo, V. K. Illison. The effects of lipoic acid and alpha-tocopherol supplementation on the lipid profile and insulin sensitivity of patients with type 2 diabetes mellitus: a randomized, double-blind, placebo-controlled trial. Diabetes Res Clin Pract 2011; 92(2): 253-60.
18. N. Suksomboon, N. Poolsup, S. Sinprasert. Effects of vitamin E supplementation on glycaemic control in type 2 diabetes: systematic review of randomized controlled trials. J Clin Pharm Ther 2011; 36(1): 53-63.
19. S. Shab-Bidar, Z. Mazloum, Z. Mousavi-Shirazifard. Daily vitamin E supplementation does not improve metabolic and glycemic control in type 2 diabetic patients: a double blinded randomized controlled trial. J Diabetes 2013; 5(1): 57-8.
20. P. Torquato, D. Bartolini, D. Giusepponi, et al. Increased plasma levels of the lipoperoxyl radical-derived vitamin E metabolite alpha-tocopheryl quinone are an early indicator of lipotoxicity in fatty liver subjects. Free Radic Biol Med 2019; 131: 115-25.
21. G. Ravaglia, P. Forti, A. Lucicesare, et al. Plasma tocopherols and risk of cognitive impairment in an elderly Italian cohort. Am J Clin Nutr 2008; 87(5): 1306-13.
22. M. Casati, V. Boccardi, E. Ferri, et al. Vitamin E and Alzheimer's disease: the mediating role of cellular aging. Aging Clin Exp Res 2019.
23. G. Sharma, D. P. Muller, S. M. O'Riordan, et al. Urinary conjugated alpha-tocopheronolactone--a biomarker of oxidative stress in children with type 1 diabetes. Free Radic Biol Med 2013; 55: 54-62.
24. R. de Mutsert, M. den Heijer, T. J. Rabelink, et al. The Netherlands Epidemiology of Obesity (NEO) study: study design and data collection. Eur J Epidemiol 2013; 28(6): 513-23.
25. E. T. Cirulli, L. Guo, C. Leon Swisher, et al. Profound Perturbation of the Metabolome in Obesity Is Associated with Health Risk. Cell Metab 2019; 29(2): 488-500.e2.
26. T. Long, M. Hicks, H. C. Yu, et al. Whole-genome sequencing identifies common-to-rare variants associated with human blood metabolites. Nat Genet 2017; 49(4): 568-78.
27. G. Sharma, D. P. Muller, S. M. O'Riordan, et al. Urinary conjugated α-tocopheronolactone--a biomarker of oxidative stress in children with type 1 diabetes. Free Radic Biol Med 2013; 55: 54-62.
28. G. Sharma, D. Muller, S. O'Riordan, et al. A novel method for the direct measurement of urinary conjugated metabolites of alpha-tocopherol and its use in diabetes. Mol Nutr Food Res 2010; 54(5): 599-600.
29. D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, R. C. Turner. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28(7): 412-9.
30. T. M. Wallace, J. C. Levy, D. R. Matthews. Use and abuse of HOMA modeling. Diabetes Care 2004; 27(6): 1487-95.
31. M. Matsuda, R. A. DeFronzo. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care 1999; 22(9): 1462-70.
32. B. Singh, A. Saxena. Surrogate markers of insulin resistance: A review. World J Diabetes 2010; 1(2): 36-47.

33. M. Looman, E. J. Feskens, M. de Rijk, et al. Development and evaluation of the Dutch Healthy Diet index 2015. Public Health Nutr 2017; 20(13): 2289-99.
34. D. I. Thurnham, J. A. Davies, B. J. Crump, R. D. Situnayake, M. Davis. The use of different lipids to express serum tocopherol: lipid ratios for the measurement of vitamin E status. Ann Clin Biochem 1986; 23 (Pt 5): 514-20.
35. L. Schmölz, M. Birringer, S. Lorkowski, M. Wallert. Complexity of vitamin E metabolism. World J Biol Chem 2016; 7(1): 14-43.
36. S. E. Kahn, R. L. Hull, K. M. Utzschneider. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006; 444(7121): 840-6.
37. L. Marseglia, S. Manti, G. D'Angelo, et al. Oxidative stress in obesity: a critical component in human diseases. Int J Mol Sci 2014; 16(1): 378-400.
38. M. D. Tobin, N. A. Sheehan, K. J. Scurrah, P. R. Burton. Adjusting for treatment effects in studies of quantitative traits: antihypertensive therapy and systolic blood pressure. Stat Med 2005; 24(19): 2911-35.
39. B. Schloerke, J. Crowley, D. Cook, et al. Ggally: extension to ggplot2. (R package version 1.4). 2018.
40. T. M. Therneau, T. Lumley. A Package for Survival Analysis in R. 2015.
41. N. V. Dimitrov, C. Meyer, D. Gilliland, M. Ruppenthal, W. Chenoweth, W. Malone. Plasma tocopherol concentrations in response to supplemental vitamin E. Am J Clin Nutr 1991; 53(3): 723-9.
42. K. M. Lebold, A. Ang, M. G. Traber, L. Arab. Urinary α-carboxyethyl hydroxychroman can be used as a predictor of α-tocopherol adequacy, as demonstrated in the Energetics Study. Am J Clin Nutr 2012; 96(4): 801-9.
43. A. Ascherio, M. J. Stampfer, G. A. Colditz, E. B. Rimm, L. Litin, W. C. Willett. Correlations of vitamin A and E intakes with the plasma concentrations of carotenoids and tocopherols among American men and women. J Nutr 1992; 122(9): 1792-801.
44. M. G. Traber, E. Mah, S. W. Leonard, G. Bobe, R. S. Bruno. Metabolic syndrome increases dietary alpha-tocopherol requirements as assessed using urinary and plasma vitamin E catabolites: a double-blind, crossover clinical trial. Am J Clin Nutr 2017; 105(3): 571-9.
45. G. Bobe, T. J. Cobb, S. W. Leonard, et al. Increased static and decreased capacity oxidation-reduction potentials in plasma are predictive of metabolic syndrome. Redox Biol 2017; 12: 121-8.
46. E. Mah, T. N. Sapper, C. Chitchumroonchokchai, et al. alpha-Tocopherol bioavailability is lower in adults with metabolic syndrome regardless of dairy fat co-ingestion: a randomized, double-blind, crossover trial. Am J Clin Nutr 2015; 102(5): 1070-80.
47. S. A. Pope, P. T. Clayton, D. P. Muller. A new method for the analysis of urinary vitamin E metabolites and the tentative identification of a novel group of compounds. Arch Biochem Biophys 2000; 381(1): 8-15.
48. J. K. Lodge, M. G. Traber, A. Elsner, R. Brigelius-Flohé. A rapid method for the extraction and determination of vitamin E metabolites in human urine. J Lipid Res 2000; 41(1): 148-54.
49. C. S. Gross, A. T. Milhorat, E. J. Simon. The metabolism of vitamin E. I. The absorption and excretion of d-alpha-tocopheryl-5-methyl-C14-succinate. J Biol Chem 1956; 221(2): 797-805.

50. A. Eisengart, A. T. Milhorat, E. J. Simon, L. Sundheim. The metabolism of vitamin E. II. Purification and characterization of urinary metabolites of alpha-tocopherol. J Biol Chem 1956; 221(2): 807-17.

Supplementary materials

Supplementary Table 1 Comparison of main characteristics between included and excluded participants

Supplementary Table 2 Associations between urinary α-tocopherol metabolites and measures of glucose homeostasis in general population

Supplementary Table 3 Associations between urinary α-tocopherol enzymatic-to-oxidized metabolite ratios and measures of glucose homeostasis in general population

Supplementary Table 4 Associations between circulatory α-tocopherol metabolites and measures of glucose homeostasis in participants without vitamin E supplement use

Supplementary Table 5 Associations between circulatory and urinary metabolites and measures of glucose homeostasis in all participants in censored normal regression (Model 3)

Supplementary Figure 1 Flowchart of participants exclusion

Supplementary Figure 2 Intercorrelation of α-tocopherol metabolites in blood and urine in participants without vitamin E supplement use

Supplementary Figure 3 Association between urinary metabolites and (A) HOMA-B, (B) HOMA-IR, (C) Insulinogenic Index, and (D) Matsuda Index in participants without vitamin E supplement use

Supplementary Figure 4 Association between urinary metabolite ratios and (A) HOMA-B, (B) HOMA-IR, (C) Insulinogenic Index, and (D) Matsuda Index in participants without vitamin E supplement use

The Supplementary materials for this article can be found online at:
https://doi.org/10.1016/j.clnu.2021.01.039