Metabolic syndrome increases dietary α-tocopherol requirements as assessed using urinary and plasma vitamin E catabolites: a double-blind, crossover clinical trial[1–3]

Maret G Traber,[4] Eunice Mah,[5,6] Scott W Leonard,[4] Gerd Bobe,[4] and Richard S Bruno[5]*

[4]Linus Pauling Institute, Oregon State University, Corvallis, OR; and [5]Human Nutrition Program, The Ohio State University, Columbus, OH

ABSTRACT

Background: Vitamin E supplementation improves liver histology in patients with nonalcoholic steatohepatitis, which is a manifestation of the metabolic syndrome (MetS). We reported previously that α-tocopherol bioavailability in healthy adults is higher than in those with MetS, thereby suggesting that the latter group has increased requirements.

Objective: We hypothesized that α-tocopherol catabolites α-carboxyethyl hydroxychromanol (α-CEHC) and α-carboxymethylbutyl hydroxychromanol (α-CMBHC) are useful biomarkers of α-tocopherol status.

Design: Adults (healthy or with MetS; n = 10/group) completed a double-blind, crossover clinical trial with four 72-h interventions during which they co-ingested 15 mg hexadeuterium-labeled *RRR*-α-tocopherol (d_6-α-T) with nonfat, reduced-fat, whole, or soy milk. During each intervention, we measured α-CEHC and α-CMBHC excretions in three 8-h urine collections (0–24 h) and plasma α-tocopherol, α-CEHC, and α-CMBHC concentrations at various times ≤72 h.

Results: During the first 24 h, participants with MetS compared with healthy adults excreted 41% less α-CEHC (all values are least-squares means ± SEMs: 0.6 ± 0.1 compared with 1.0 ± 0.1 μmol/g creatinine, respectively; P = 0.002), 63% less hexadeuterium-labeled (d_6)-α-CEHC (0.04 ± 0.02 compared with 0.13 ± 0.02 μmol/g creatinine, respectively; P = 0.002), and 58% less d_6-α-CMBHC (0.017 ± 0.004 compared with 0.041 ± 0.004 μmol/g creatinine, respectively; P = 0.0009) and had 52% lower plasma d_6-α-CEHC areas under the concentration curves [area under the curve from 0 to 24 h ($AUC_{0–24h}$): 27.7 ± 7.9 compared with 58.4 ± 7.9 nmol/L × h, respectively; P = 0.01]. d_6-α-CEHC peaked before d_6-α-T in 77 of 80 paired plasma concentration curves. Urinary d_6-α-CEHC 24-h concentrations were associated with the plasma $AUC_{0–24\ h}$ of d_6-α-T (r = 0.53, P = 0.02) and d_6-α-CEHC (r = 0.72, P = 0.0003), and with urinary d_6-α-CMBHC (r = 0.88, $P < 0.0001$), and inversely with the plasma inflammation biomarkers C-reactive protein ($r = -0.70$, P = 0.0006), interleukin-10 ($r = -0.59$, P = 0.007), and interleukin-6 ($r = -0.54$, P = 0.01).

Conclusion: Urinary α-CEHC and α-CMBHC are useful biomarkers to noninvasively assess α-tocopherol adequacy, especially in populations with MetS-associated hepatic dysfunction that likely impairs α-tocopherol trafficking. This trial was registered at clinicaltrials.gov as NCT01787591. *Am J Clin Nutr* 2017;105:571–9.

Keywords: α-carboxyethyl hydroxychromanol, α-CEHC, α-carboxymethylbutyl hydroxychromanol, bioavailability, metabolic syndrome, nutrient requirements, vitamin E

INTRODUCTION

Nonalcoholic fatty liver disease is the liver manifestation of metabolic syndrome (MetS)[7] and is the primary cause of chronic liver disease in the United States, with an estimated prevalence of 80–100 million Americans (1–3). Nonalcoholic steatohepatitis (NASH) is characterized by fatty acid infiltration into hepatocytes and also by hepatic inflammation, oxidative stress, and cellular injury (4). NASH can progress to cirrhosis, hepatocellular carcinoma, and eventually death (4, 5). There are limited therapies available, but vitamin E supplementation has improved liver-function tests and histology in both children and adults with NASH (6, 7). Peroxidative damage to lipids is also elevated in patients with NASH (8, 9), thereby suggesting that requirements for vitamin E, which is a lipid-soluble antioxidant, may be higher. Notably, we have reported lower apparent α-tocopherol bioavailability in individuals with MetS than in healthy participants after receipt of an oral dose of 15 mg hexadeuterium-labeled *RRR*-α-tocopherol (d_6-α-T) (10).

For α-tocopherol, the term bioavailability (11) is defined as the extent and rate of the incorporation of α-tocopherol into the circulation and is dependent on absorption, lipoprotein incorporation, trafficking, and lipoprotein-mediated tissue uptake as

[1] Supported by DSM Nutrition, the National Dairy Council, and the NIH National Institute of Diabetes and Digestive and Kidney Diseases (grant DK081761) and the NIH National Center for Advancing Translational Sciences (grant UL1TR001070).

[2] The funders had no influence on the clinical trial design, implementation, data analysis, or interpretation regarding the conclusions presented.

[3] Supplemental Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

[6] Present address: Biofortis 211 East Lake Street, Addison, IL 60101.

*To whom correspondence should be addressed. E-mail: maret.traber@oregonstate.edu.

[7] Abbreviations used: $AUC_{0–24h}$, AUC from 0 to 24 h; d_6, hexadeuterium-labeled; d_6-α-T, hexadeuterium-labeled *RRR*-α-tocopherol; MetS, metabolic syndrome; NASH, nonalcoholic steatohepatitis; OSU, The Ohio State University; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; α-CEHC, α-carboxyethyl hydroxychromanol; α-CMBHC, α-carboxymethylbutyl hydroxychromanol; α-TTP, α-tocopherol transfer protein; γ-CEHC, γ-carboxyethyl hydroxychromanol.

Received May 16, 2016. Accepted for publication December 2, 2016.

First published online January 11, 2017; doi: 10.3945/ajcn.116.138495.

well as liver α-tocopherol catabolism (12). Thus, the time to achieve maximum plasma α-tocopherol concentrations from an oral-labeled dose is fairly long and usually observed at 9–12 h postdosing (10, 13–15). The assessment of the actual bioavailability requires comparisons of concentrations after intravenous and oral administrations. The intravenous dose allows for the evaluation of the disposition of the 100% dose entered into the circulation; in contrast, the apparent or relative α-tocopherol bioavailability can be estimated with the use of an oral dose of labeled vitamin E and comparing the responses in different groups or treatments.

Vitamin E catabolism is a xenobiotic process that regulates vitamin E homeostasis by preferentially catabolizing the non–α-tocopherol forms (16), whereas α-tocopherol is preferentially secreted into plasma as a function of the hepatic α-tocopherol transfer protein (α-TTP) (17). Initially, cytochrome P450 4F2 ω-hydroxylates the tail of various vitamin E forms (16, 18) and the tail undergoes several rounds of β oxidation, which ultimately result in the formation of carboxyethyl hydroxychromanol with carboxymethylbutyl hydroxychromanol as its immediate precursor (**Figure 1**) (19). Hypothetically, α-carboxyethyl hydroxychromanol (α-CEHC) is synthesized endogenously when the quantity of hepatic α-tocopherol exceeds the capacity of α-TTP to facilitate α-tocopherol secretion from the liver into the circulation. Indeed, urinary α-CEHC excretion has been correlated with increasing amounts of both dietary and plasma α-tocopherol concentrations in healthy participants (20). However, to our knowledge, it is unknown whether urinary α-CEHC excretion reflects α-tocopherol intake from a single meal or whether its changes reflect long-term vitamin E status.

In the current study, we hypothesized that plasma concentrations and urinary excretions of α-CEHC and its precursor catabolite α-carboxymethylbutyl hydroxychromanol (α-CMBHC) would be lower in participants with MetS than in healthy adults (10), thereby reflecting whole-body α-tocopherol status. In addition, urinary α-CEHC and α-CMBHC would serve as more-sensitive biomarkers of α-tocopherol status than would plasma α-tocopherol. The urine and plasma samples were collected and analyzed as part of a clinical trial (clinicaltrials.gov; NCT01787591) as previously reported (10).

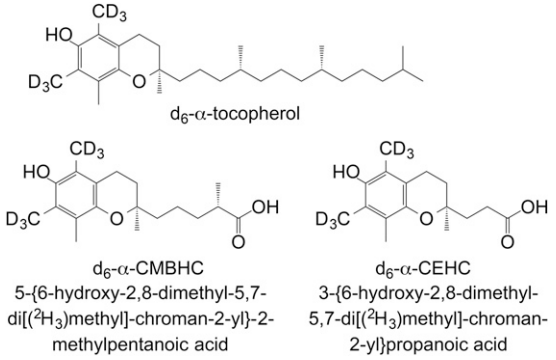

FIGURE 1 Chemical structures of d_6-α-tocopherol, d_6-α-CEHC, and d_6-α-CMBHC are shown with the locations of deuterium atoms indicated as Ds. d_6-α-CEHC, hexadeuterium-labeled α-carboxyethyl hydroxychromanol; d_6-α-CMBHC, hexadeuterium-labeled α-carboxymethylbutyl hydroxychromanol; d_6-α-tocopherol, hexadeuterium-labeled *RRR*-α-tocopherol.

METHODS

Materials

All HPLC-grade solvents and most chemicals were from Fisher Scientific. α-CEHC, γ-carboxyethyl hydroxychromanol (γ-CEHC), and α-CMBHC standards were obtained from Cayman Chemical. Ascorbic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and β-glucuronidase (type H-1; ≥300,000 U/g β-glucuronidase activity and ≥10,000 U/g sulfatase activity) were obtained from Sigma-Aldrich. d_6-α-T was kindly provided by DSM Nutritional Products.

Participants and study design

The Institutional Review Board at The Ohio State University (OSU) approved the protocol for this clinical trial; the Oregon State University Institutional Review Board deferred to OSU. Participants were recruited from the Columbus, Ohio, area, and all aspects of the clinical trial were performed at OSU General Clinical Research Center from July 2013 to May 2014. Complete details of the clinical trial have been reported previously (10). In this double-blind, crossover clinical trial, sex- and age-matched healthy participants and those with MetS (n = 5 women and 5 men/group; age range: 24–40 y) completed 4 milk interventions (nonfat, reduced-fat, whole, or soy milk) with each 72-h milk intervention separated by washout period ≥2 wk (**Supplemental Figure 1**). The milk-intervention order was determined by simple randomization (computer-generated random numbers).

MetS was defined by the presence of ≥3 established risk factors (21). Participants were also required to be weight stable (±2 kg during the past 3 mo); be non–dietary supplement users >2 mo; have not used medications that are known to affect lipid metabolism; be nonsmokers; consume <3 alcoholic drinks/d; have performed <5 h of aerobic activity/wk; have no history of gastrointestinal disorders or lactose intolerance; and be willing to follow a prescribed eucaloric diet that contained 5 mg α-tocopherol/d, as was consistent with median intakes of Americans (22), and vitamin C at intakes that met the sex-specific Recommended Daily Allowance (23) for the 3 d preceding and during the first day of each milk intervention.

After an overnight fast, participants ingested, at 0 h, encapsulated d_6-α-T (15 mg) with 240 mL nonfat milk, reduced-fat milk, whole milk, or soy milk as described previously (10). Urine was collected in 8-h intervals during the first 24 h of each milk intervention. Blood samples were collected before (0 h) and at 3, 6, 9, 12, 24, 36, 48, and 72 h after co-ingestion of test beverages and d_6-α-T. Plasma α-tocopherol (10) and its catabolites α-CEHC and α-CMHBC were assessed from blood that was collected into evacuated tubes containing sodium heparin. Urine and plasma samples were frozen and shipped on dry ice by overnight freight to the Linus Pauling Institute where they were kept frozen until analyzed for vitamin E catabolites.

Dietary intakes

Participants were provided meals that contained either 2000 or 2500 kcal on the basis of a best estimate of individuals' energy requirements with the use of the Harris-Benedict equation (24). Participants were instructed by a registered dietitian to consume all provided foods and beverages, which provided 5 mg α-tocopherol

regardless of the energy content. These menus were used for the 3 d preceding and the first day of each milk intervention. After an overnight fast, participants ingested the test milk with encapsulated d_6-α-T (15 mg) at ~0700 (0 h), had lunch immediately after the 6-h blood draw, and had dinner immediately after the 12-h blood draw. Participants were given a snack to consume anytime between lunch and dinner (no control of the timing). Study personnel recorded food and beverage intakes during the first 12 h of each intervention. Participants were asked to complete a food diary for the 3 d immediately preceding each intervention. A review of food records by a dietitian indicated that participants only consumed the provided foods as we reported previously (10).

Measurements of plasma and urinary carboxyethyl hydroxychromanols

The methodologies that we used required hydrolysis such that unconjugated α-CEHC, γ-CEHC, and α-CMBHC forms were analyzed (25). Plasma samples (100 μL) were mixed with 1% ascorbic acid (100 μL) and β-glucuronidase (100 μL of 10 mg/L in 10 mmol/L acetate buffer; pH 6.8), incubated for 1 h at 37°C, and subsequently cooled to room temperature. Urine samples (100 μL) were mixed with 500 μL 6N HCl (26), incubated for 1 h at 37°C, and subsequently cooled to room temperature. Plasma or urine unconjugated catabolites were extracted with 4 mL diethyl ether, and an aliquot of the ether fraction was collected and dried under nitrogen. Samples were resuspended in 1:1 (volume:volume) water:methanol containing trolox (as an internal standard) for injection into the liquid chromatography–tandem mass spectrometer (API3000; SCIEX), which was equipped with a turbo ion-spray source that was set to negative mode. Multiple-reaction monitoring of the following transitions was used: α-CEHC (277 → 162), hexadeuterium-labeled (d_6)-α-CEHC (283 → 168), γ-CEHC (263 → 149), α-CMBHC (319 → 162), d_6-α-CMBHC (325 → 168), and trolox (249 → 162). Analyte concentrations were calculated from the standard curves that were generated from peak areas of authentic nondeuterated compounds with correction for the recovery of the internal standard trolox. The limit of detection for carboxyethyl hydroxychromanol was <20 fmol injected (0.001 μM). The limit of quantitation for carboxyethyl hydroxychromanol was 40 fmol injected (signal-to-noise ratio = ~6). Urinary creatinine was measured with the use of a kit (#55A) from Sigma-Aldrich.

Statistical analysis

Data were analyzed with the use of Statistical Analysis System software (SAS, version 9.4; SAS Institute). Baseline data of healthy participants and those with MetS were compared with the use of Student's independent *t* test with the assumption of equal variances (**Table 1**).

Urine samples that were collected during the first three 8-h intervals of each milk intervention were used for the statistical analysis of labeled and unlabeled vitamin E catabolites and creatinine excretion. To determine total 24-h urinary excretions of labeled and unlabeled vitamin E catabolites and creatinine, we summed the molar quantities that were excreted in the three 8-h urine collections, and each catabolite was divided by the amount of urinary creatinine (grams) that was excreted to report data expressed as μmol/g creatinine. To compare catabolite excretions of healthy participants with those of subjects with MetS, we analyzed the 24-h urine data with the use of the SAS PROC MIXED procedure (SAS Institute) as previously described (27, 28). Fixed effects included health status (healthy or with MetS), the order of the milk intervention (first, second, third, or fourth), the milk intervention (whole, reduced fat, skim, or soy milk), and sex (male or female). The variance-covariance structure of repeated measures within participants was modeled with the use of a compound symmetry matrix (i.e., with equal variances and equal covariances). Approximations of Kenward and Roger (28) were used to obtain the correct df. To determine group-dependent and group-independent time effects, we included the three 8-h collection intervals (0–8, 8–16, and 16–24 h) and the interactions between 8-h collection intervals with health status, the milk intervention, sex, and the order of the milk intervention into the statistical model. Other 2-way interactions were assessed for significance but were not included in the final statistical model because of a lack of significance. The variance-covariance structure of repeated measures within participants was modeled with the use of a Kronecker product that consisted of a compound symmetry matrix for the intervention order and an unstructured variance-covariance matrix for the collection interval. When statistically significant interactions of health status by

TABLE 1
Participant characteristics according to health status[1]

	Healthy (n = 10)	MetS (n = 10)	P
Age, y	30.3 ± 1.3	32.8 ± 1.6	0.240
BMI, kg/m^2	22.6 ± 0.7	37.7 ± 3.0	0.001
Waist circumference, cm	75 ± 2	116 ± 7	0.001
Systolic blood pressure, mm Hg	119 ± 3	128 ± 7	0.260
Diastolic blood pressure, mm Hg	74.1 ± 2.5	79.0 ± 3.2	0.242
Glucose, mmol/L	4.95 ± 0.11	5.97 ± 0.24	0.001
Insulin, mU/L	4.0 ± 1.0	10.3 ± 1.7	0.010
HOMA-IR	0.91 ± 0.24	2.76 ± 0.54	0.008
HDL cholesterol, mmol/L	1.45 ± 0.08	1.07 ± 0.09	0.004
LDL cholesterol, mmol/L	2.14 ± 0.17	3.12 ± 0.36	0.024
Cholesterol, mmol/L	4.02 ± 0.15	4.98 ± 0.38	0.032
Triglyceride, mmol/L	0.93 ± 0.11	1.73 ± 0.24	0.008
Total lipid,[2] mmol/L	4.95 ± 0.23	6.70 ± 0.48	0.004
Alanine aminotransferase, U/L	12.3 ± 2.0	14.3 ± 2.4	0.528
Aspartate aminotransferase, U/L	12.5 ± 2.7	10.9 ± 0.9	0.576
Oxidized LDL, U/L	51.8 ± 3.5	69.1 ± 4.2	0.005
Vitamin C, μmol/L	72.6 ± 4.5	53.0 ± 4.9	0.008
Uric acid, μmol/L	305 ± 18	352 ± 27	0.168
α-Tocopherol, μmol/L	22.2 ± 1.2	23.9 ± 0.9	0.282
α-Tocopherol, μmol/mmol cholesterol	5.57 ± 0.31	5.06 ± 0.43	0.351
α-Tocopherol, μmol/mmol lipid	4.54 ± 0.27	3.71 ± 0.27	0.042
γ-Tocopherol, μmol/L	2.27 ± 0.11	3.70 ± 0.42	0.004
γ-Tocopherol, μmol/mmol cholesterol	0.57 ± 0.03	0.81 ± 0.14	0.113
γ-Tocopherol, μmol/mmol lipid	0.47 ± 0.03	0.59 ± 0.10	0.241
C-reactive protein, mg/L	2.24 ± 0.12	3.68 ± 0.42	0.014
TNF-α, pg/mL	9.1 ± 0.6	10.6 ± 0.6	0.086
IL-10, pg/mL	2.28 ± 0.10	2.82 ± 0.17	0.014
IL-6, pg/mL	0.73 ± 0.17	2.13 ± 0.50	0.016

[1] All values are means ± SEMs. Group differences were analyzed with the use of Student's independent *t* test. MetS, metabolic syndrome. Reproduced from reference 10 with permission.
[2] Calculated as the sum of total cholesterol and triglyceride.

collection period were observed, Tukey's multiple comparison tests were applied to assess significance within and across health-status groups.

Plasma samples that were obtained from blood collected at 0, 3, 6, 9, 12, and 24 h during each of the milk interventions were used for the statistical analysis of labeled and unlabeled α-CEHCs and α-CMBHCs; plasma d_6-α-T data were from Mah et al. (10). Areas under the plasma 0–24-h concentration curves [i.e., AUC from 0 to 24 h (AUC_{0-24h})] were calculated with the use of the trapezoidal rule. The same statistical model as described previously for the summed 24-h urinary data was used to compare the AUC_{0-24h} of healthy participants with that of subjects with MetS. To determine time effects that were dependent or independent of the group, we included sample-collection times (0 h was included only for unlabeled α-CEHC concentrations; labeled and unlabeled α-CEHC concentrations included 3, 6, 9, 12, and 24 h) and the interactions between collection times and health status, milk intervention, and sex in the statistical model. Other 2-way interactions were assessed for significance but were not included into the final statistical model because no significant effects were detected.

To compare collection times at which plasma d_6-α-CEHC concentrations increased before the increase in plasma d_6-α-T, each milk-intervention comparison was assigned a value; the outcome was equal to 1 if d_6-α-CEHC increased before d_6-α-T in all 4 paired curves/participant, and the outcome was equal to zero if it did not. A binomial test was used to assess significance.

Associations between urinary 24-h d_6-α-CEHC (medians of 4 milk interventions), the plasma 24-h AUC of d_6-α-T or d_6-α-CEHC (medians of 4 milk interventions), and baseline characteristics obtained at the beginning of the clinical trial were calculated with the use of Spearman correlation coefficients.

The order of milk interventions did not affect the outcomes that were investigated in this study. Only outcomes that were pertinent to our a priori hypotheses are discussed in Results. Data are reported as least-squares means ± SEMs (except for data shown in Table 1, which are reported as means ± SEMs); all statistical tests were 2-sided, and significance was set as $P \leq 0.05$.

RESULTS

Participants and α-tocopherol pharmacokinetics

Complete details of the plasma d_6-α-T pharmacokinetics studies in these participants were reported previously and showed that, compared with healthy adults, subjects with MetS had lower plasma d_6-α-T bioavailability irrespective of milk-fat consumption (10). Baseline evaluations showed that adults with MetS had greater ($P \leq 0.05$) waist circumference, higher fasting glucose and triglycerides, and lower HDL cholesterol than did healthy adults, whereas systolic and diastolic blood pressures were not different by health status (Table 1). Notably, plasma unlabeled α-tocopherol concentrations in the 2 health-status groups were not significantly different between groups, but plasma γ-tocopherol concentrations were greater in subjects with MetS ($P = 0.004$) (Table 1).

Urinary unlabeled and labeled vitamin E catabolites

During the first 24 h of the milk interventions, participants with MetS, compared with healthy participants, excreted 22% less γ-CEHC (1.7 ± 0.1 compared with 2.2 ± 0.1 μmol/g creatinine, respectively; $P = 0.01$), 41% less α-CEHC (0.6 ± 0.1 compared with 1.0 ± 0.1 μmol/g, respectively; $P = 0.002$), 63% less d_6-α-CEHC (0.04 ± 0.02 compared with 0.13 ± 0.02 μmol/g, respectively; $P = 0.002$), and 58% less d_6-α-CMBHC (0.017 ± 0.004 compared with 0.041 ± 0.004 μmol/g, respectively; $P = 0.0009$). The 24-h urinary d_6-α-CEHC excretion was ~3 times that of d_6-α-CMBHC and one-tenth of that of unlabeled α-CEHC. Differences in α-CEHC excretion occurred while participants were provided a standardized eucaloric diet that contained 5 mg α-tocopherol/d for the 3 d that preceded each intervention plus 15 mg d_6-α-T with breakfast on the first day; there were no differences in α-CEHC excretion between milk interventions on the basis of health status.

Because the urinary 24-h creatinine excretion was greater in participants with MetS than in the healthy participants (1.96 ± 0.10 compared with 1.53 ± 0.10 g, respectively; $P = 0.008$), we assessed whether the observed differences in vitamin E catabolite excretion were confounded as a result of the greater creatinine excretion in participants with MetS. Without correction for urinary creatinine excretion, vitamin E catabolite excretion (micromoles) remained significantly lower in participants with MetS than in healthy participants (**Supplemental Figure 2**).

To determine the time intervals at which the 2 health groups differed in vitamin E catabolite excretion, we included the three 8-h collection intervals (0–8, 8–16, and 16–24 h) in the statistical model. Participants with MetS compared with healthy participants excreted less urinary γ-CEHC ($P = 0.01$) and less α-CEHC ($P = 0.002$); interval-dependent changes in urinary catabolite excretion were observed for γ-CEHC ($P = 0.02$), α-CEHC ($P = 0.02$), d_6-α-CEHC ($P < 0.0001$), and d_6-α-CMBHC ($P < 0.0001$) (**Figure 2**). No significant interactions between the health-status group and the interval-collection period were shown for either γ-CEHC ($P = 0.65$) or α-CEHC ($P = 0.16$). Significant interactions between the health-status group and collection period were detected for d_6-α-CEHC ($P = 0.04$) and d_6-α-CMBHC ($P = 0.0006$). To evaluate time-dependent group differences, the following 2 orthogonal contrasts for the interaction term were constructed: *1*) a comparison of the linear effect of the collection period (the third collection period compared with the first collection period) between health-status groups and *2*) a comparison of the quadratic effect of the collection period (the second collection period compared with the mean of the first and third collection periods) between health-status groups. Significant group differences were observed for the quadratic comparison such that the response curve in healthy participants was more quadratic than was the response in participants with MetS (d_6-α-CEHC: $P = 0.04$; d_6-α-CMBHC: $P = 0.0002$).

Men compared with women excreted less α-CEHC (0.67 ± 0.08 compared with 0.99 ± 0.08 μmol/g creatinine, respectively; $P = 0.01$), α-CMBHC (0.23 ± 0.03 compared with 0.30 ± 0.03 μmol/g creatinine, respectively; $P = 0.06$), and γ-CEHC (1.75 ± 0.13 compared with 2.25 ± 0.13 μmol/g creatinine, respectively; $P = 0.01$) over the first 24 h. Because the urinary 24-h creatinine excretion was greater in men than in women (2.10 ± 0.10 compared with 1.40 ± 0.10 g; $P = 0.0001$), we assessed whether the observed differences in vitamin E catabolite excretion were confounded as a result of the greater creatinine excretion in men and in participants with MetS. Without correction for urinary creatinine excretion, neither unlabeled nor

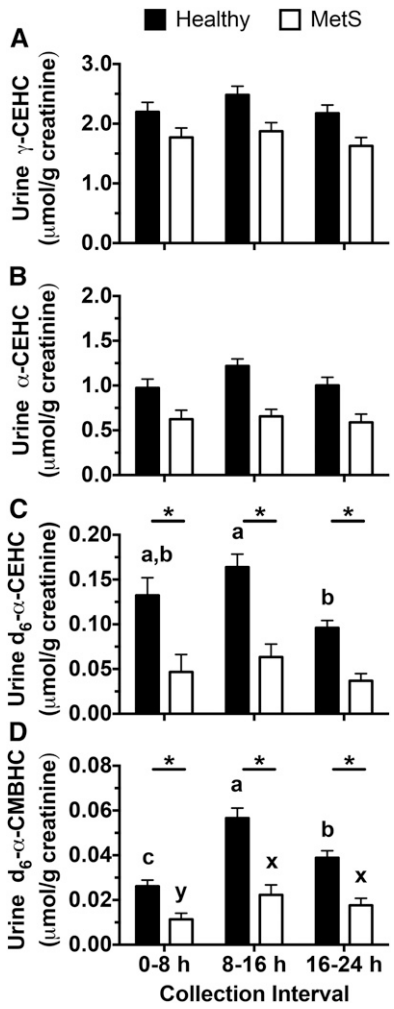

FIGURE 2 Least-squares mean ± SEM urinary vitamin E catabolite excretion in healthy participants and subjects with MetS. Unlabeled and labeled vitamin E catabolite excretions (μmol/g creatinine, n = 10/group) in healthy participants and subjects with MetS were measured with the use of liquid chromatography–tandem mass spectrometry in urine samples that were collected during the first three 8-h collection intervals of each milk intervention. (A) Differences in γ-CEHC concentrations were observed between health-status groups (P = 0.004) and between collection periods (P = 0.02); no interaction was observed (P = 0.65). (B) Differences in α-CEHC concentrations were observed between health-status groups (P = 0.0002) and between collection periods (P = 0.02); no interaction was observed (P = 0.16). (C) Differences in d_6-α-CEHC concentrations were observed between health-status groups and collection periods (both $P < 0.0001$); an interaction of health status by collection interval was observed (P = 0.04). (D) Differences in d_6-α-CMBHC concentrations were observed between health status and collection periods (both $P < 0.0001$); an interaction of health status by collection interval was observed (P = 0.0006). For panels C and D, to evaluate the time-dependent group differences, the following 2 orthogonal contrasts for the interaction term were constructed: *1*) a comparison of the linear effect of the collection period (the third collection period compared with the first collection period) between health-status groups and *2*) a comparison of the quadratic effect of the collection period (the second collection period compared with the mean of the first and third collection period) between health-status groups. Significant group differences were observed for the quadratic comparison such that the response curve in healthy participants was more quadratic than the response in participants with MetS (d_6-α-CEHC: P = 0.04; d_6-α-CMBHC: P = 0.0002). Tukey's multiple comparison test was used within and across health-status groups; significant differences between health status in each collection interval are shown with an asterisk above the collection interval; significant differences across collection intervals within health-status groups are shown by different letters (healthy: a, b, and c; MetS: x and y) ($P \leq 0.05$). d_6-α-CEHC, hexadeuterium-labeled α-carboxyethyl hydroxychromanol; d_6-α-CMBHC, hexadeuterium-labeled α-carboxymethylbutyl hydroxychromanol; MetS, metabolic syndrome; α-CEHC, α-carboxyethyl hydroxychromanol; γ-CEHC, γ-carboxyethyl hydroxychromanol.

labeled vitamin E catabolite excretion values micromoles) were significantly different between men and women (data not shown).

Milk fat and vitamin E catabolite excretion

In the parent clinical trial (10), the fat content of the milk that was co-ingested with the d_6-α-T had no significant impact on plasma d_6-α-T pharmacokinetic variables. In this evaluation of urinary vitamin E catabolites, the fat-content of the milk did not significantly affect the 24-h urinary excretion of γ-CEHC (P = 0.52), α-CEHC (P = 0.35), or d_6-α-CEHC (P = 0.25), but it did affect d_6-α-CMBHC (P = 0.04) excretion with significantly higher d_6-α-CMBHC excreted during the fat-free–milk intervention (0.035 ± 0.004 μmol/g creatinine) than during the reduced-fat–milk intervention (0.028 ± 0.004 μmol/g creatinine; P = 0.04), soy-milk intervention (0.028 ± 0.004 μmol/g creatinine; P = 0.05) or whole-milk intervention (0.026 ± 0.004 μmol/g creatinine; P = 0.008). When we compared the effects of the milk intervention within urinary collection periods, in the first 8-h collection period, participants who consumed fat-free milk excreted more urinary d_6-α-CEHC (0.14 ± 0.07 μmol/g creatinine) than they did after the consumption of reduced-fat mile (0.09 μmol/g creatinine; P = 0.05), soy milk (0.06 μmol/g creatinine; P = 0.006), or whole milk (0.07 μmol/g creatinine; P = 0.006). Similar results were shown for urinary d_6-α-CMBHC excretion when participants who consumed fat-free milk were compared with participants who consumed reduced-fat milk (P = 0.006), soy milk (P = 0.05), or whole milk (P = 0.004). Significances of interactions of the collection period with the milk intervention were shown for d_6-α-CEHC (P = 0.002) and d_6-α-CMBHC (P = 0.08).

Plasma α-CEHC

We also measured plasma unlabeled and labeled α-CEHCs and α-CMBHCs during each of the milk interventions for ≤72 h. Plasma unlabeled and labeled α-CEHC concentrations for the first 24 h are reported herein and remained detectable up to 72 h (data not shown); unlabeled and labeled α-CMBHC concentrations were too low and variable to be considered reliable (data not shown). Plasma d_6-α-CEHC was detectable in all participants by 3 h during each milk intervention except for in 1 participant with MetS after consumption of skim milk.

Over the 4 milk interventions, the $AUC_{0–24h}$ of plasma unlabeled α-CEHC concentrations in participants with MetS was 33% lower than in healthy participants, but this difference did not reach significance between the 2 groups (268 ± 48 compared with 401 ± 48 nmol/L × h, respectively; P = 0.07). The $AUC_{0–24h}$ of plasma d_6-α-CEHC concentrations was 52% lower in participants with MetS than in healthy participants (27.7 ± 7.9 compared with 58.4 ± 7.9 nmol/L × h, respectively; P = 0.01). No significant effects of sex, milk intervention, or intervention order were observed on plasma unlabeled or labeled α-CEHC $AUC_{0–24h}$.

Healthy participants compared with subjects with MetS had higher plasma unlabeled ($P = 0.009$) (**Figure 3**A) and d_6-α-CEHC ($P = 0.006$) (Figure 3B) concentrations. Collection time-dependent changes were also observed for unlabeled ($P = 0.05$; Figure 3A) and d_6-α-CEHC ($P < 0.0001$; Figure 3B) concentrations. No significant interaction of health status by time was observed either for unlabeled ($P = 0.12$) or d_6-α-CEHC ($P = 0.23$) concentrations.

Comparisons of plasma α-CEHC– and α-tocopherol–concentration time curves

Perhaps the most remarkable finding in this study concerned the comparisons of healthy participants with subjects with MetS with regard to comparisons of plasma d_6-α-CEHC with plasma d_6-α-T concentrations [reported previously in Mah et al. (10)] (**Figure 4**). Plasma d_6-α-CEHC concentrations increased before the increase in plasma d_6-α-T in both groups ($P < 0.0001$). Plasma d_6-α-T concentrations in healthy subjects did not differ significantly from those in participants with MetS until 9 h (10), which was after the plasma d_6-α-CEHC peak at 6 h.

Both the $AUC_{0–24h}$ of plasma d_6-α-T concentrations ($r = 0.53$, $P = 0.02$) (**Figure 5**A) and the $AUC_{0–24h}$ of plasma d_6-α-CEHC concentrations ($r = 0.72$, $P = 0.0003$) (Figure 5B) were highly correlated with 24-h urinary d_6-α-CEHC excretion. However,

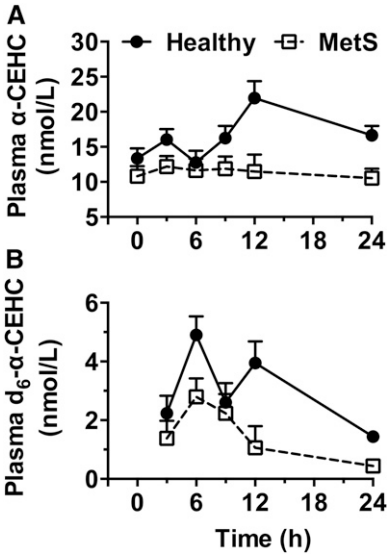

FIGURE 3 Time courses of least-squares mean ± SEM plasma unlabeled and d_6-α-CEHC concentrations in healthy participants and subjects with MetS ($n = 10$/group; in some cases, the error bar is smaller than the symbol). Plasma unlabeled and labeled vitamin E catabolite concentrations from healthy participants and subjects with MetS were measured with the use of liquid chromatography–tandem mass spectrometry at the indicated collection times for the first 24 h of each of the 4 milk interventions. (A) Differences in plasma α-CEHC concentrations were observed between health-status groups ($P = 0.009$) and collection time points ($P = 0.05$). No significant interaction of health-status group by time was observed ($P = 0.12$). Plasma α-CEHC concentrations changed over time in healthy subjects ($P = 0.005$) but not in participants with MetS ($P = 0.85$). (B) Differences in plasma d_6-α-CEHC concentrations were observed between health-status groups ($P = 0.006$) and collection time points ($P < 0.0001$). No significant interaction of health-status group by time was observed ($P = 0.23$). Plasma d_6-α-CEHC changed over time in both healthy subjects ($P < 0.0001$) and in participants with MetS ($P = 0.001$). d_6-α-CEHC, hexadeuterium-labeled α-carboxyethyl hydroxychromanol; MetS, metabolic syndrome; α-CEHC, α-carboxyethyl hydroxychromanol.

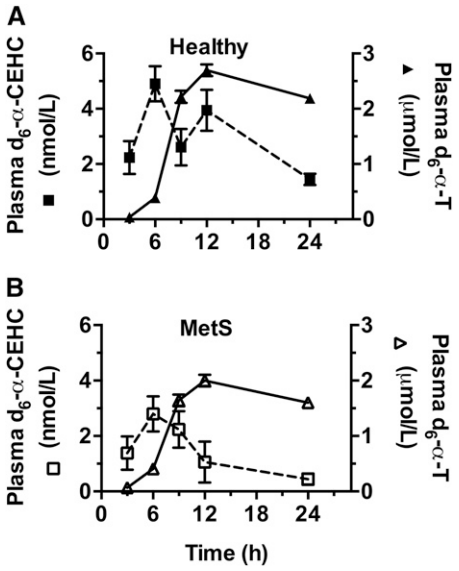

FIGURE 4 Least-squares mean ± SEM maximum plasma d_6-α-CEHC concentrations preceded maximum α-T concentrations in healthy participants and in subjects with MetS. Time courses are shown of plasma d_6-α-T [data were derived from Mah et al. (10)] and of d_6-α-CEHC (Figure 3) in healthy participants (A) and in subjects with MetS (B) who ingested 15 mg encapsulated d_6-α-T during the milk-intervention study as are concentrations of plasma d_6-α-CEHC and d_6-α-T, which were calculated from individual values during each of the 4 interventions for each subject ($n = 10$/health-status group). Plasma d_6-α-CEHC maximum concentrations increased before increases in plasma d_6-α-T in both groups. In 77 of 80 curves, the plasma d_6-α-CEHC peak preceded the d_6-α-T peak, which occurred at 12 h; only 1 of 80 curves (of a participant with MetS who consumed skim milk) showed a different time of the maximum plasma d_6-α-T concentration, which was later at 24 h (both $P < 0.0001$; each milk-intervention comparison was assigned a value; the outcome was equal to 1 if d_6-α-CEHC increased before d_6-α-T in all 4 paired curves/participant, and the outcome was zero if it did not (binomial test). d_6, hexadeuterium-labeled; d_6-α-CEHC, hexadeuterium-labeled α-carboxyethyl hydroxychromanol; MetS, metabolic syndrome; α-T, α-tocopherol.

the $AUC_{0–24h}$ of plasma d_6-α-T, was not correlated with the $AUC_{0–24h}$ of plasma d_6-α-CEHC ($r = 0.31$, $P = 0.19$), which was likely due to their dramatically different patterns of plasma concentrations over time (Figure 4). Summed 24-h urinary d_6-α-CEHC and d_6-α-CMBHC excretions were also highly correlated ($r = 0.88$, $P < 0.0001$; $n = 20$, participant medians each from 4 milk interventions) as we might have expected from their precursor-product relation.

Correlations between labeled vitamin E catabolites and other biomarkers

Vitamin E catabolites are likely made when vitamin E status is sufficient or in excess of needs. Thus, metabolic conditions that are associated with increased oxidative or inflammatory status, such as MetS (29), would be predicted to limit vitamin E catabolism. In agreement, summed 24-h urinary d_6-α-CEHC excretions were associated ($n = 20$ participant medians each from 4 interventions) with circulating concentrations of C-reactive protein ($r = -0.70$, $P = 0.0006$), IL-10 ($r = -0.59$, $P = 0.007$), IL-6 ($r = -0.54$, $P = 0.01$), insulin ($r = -0.51$, $P = 0.03$), γ-tocopherol ($r = -0.66$, $P = 0.002$), HDL cholesterol ($r = 0.45$, $P = 0.05$), diastolic blood pressure ($r = -0.51$, $P = 0.02$), waist circumference ($r = -0.60$, $P = 0.005$), and BMI ($r = -0.62$,

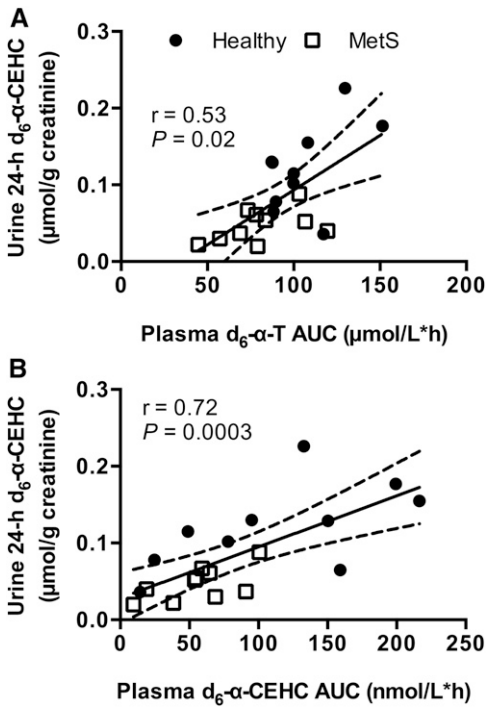

FIGURE 5 Median correlations between urinary 24-h d_6-α-CEHC excretion and plasma d_6-α-T AUC or plasma d_6-α-CEHC AUC, which were calculated from individual values during each of the 4 interventions for each subject (n = 10/health-status group). (A) Plasma d_6-α-T AUC (μmol/L $\times$ h; r = 0.53, P = 0.02) was correlated with 24-h urinary d_6-α-CEHC (μmol/g creatinine; Y = 0.00142 $\times$ X − 0.049). (B) Plasma d_6-α-CEHC AUC (nmol/L $\times$ h; r = 0.72, P = 0.0003) was correlated with 24-h urinary d_6-α-CEHC/creatinine (Y = 0.00066 $\times$ X + 0.028). d_6-α-CEHC, hexadeuterium-labeled α-carboxyethyl hydroxychromanol; d_6-α-T, hexadeuterium-labeled *RRR*-α-tocopherol; MetS, metabolic syndrome.

P = 0.003) at the beginning of the clinical trial. Summed 24-h urinary d_6-α-CMBHC excretions were also correlated with circulating concentrations of C-reactive protein (r = −0.55, P = 0.01), γ-tocopherol (r = −0.74, P = 0.0003), HDL cholesterol (r = 0.44, P = 0.05), waist circumference (r = −0.47, P = 0.04) and BMI (r = −0.47, P = 0.04) at the beginning of the clinical trial.

DISCUSSION

Participants with MetS, compared with healthy adults, excreted 30–50% less unlabeled α-CEHC, α-CMBHC and γ-CEHC in urine during the first 24 h of the 4 milk interventions after 3 d of controlled dietary vitamin E intakes that preceding the interventions. Participants with MetS also excreted significantly less urinary d_6-α-CEHC and d_6-α-CMBHC over the first 24 h after the administration of d_6-α-T (15 mg). Irrespective of whether urinary catabolite excretion data were normalized for creatinine excretion, participants with MetS, compared with healthy participants, excreted lower amounts of urinary vitamin E catabolites. Moreover, plasma concentrations of d_6-α-CEHC were also lower in participants with MetS than in healthy participants. Overall, we concluded that participants with MetS had decreased vitamin E catabolism.

Why did participants with MetS catabolize less α-tocopherol to α-CEHC? We suggest that these participants had lower vitamin E status, despite the similarity in plasma α-tocopherol concentrations ($\sim$20–25 μmol/L), compared with that of healthy participants. As we reported previously (10), plasma d_6-α-T concentrations during the 4 milk interventions were significantly lower, and turnover was significantly slower, in participants with MetS than in healthy participants. Moreover, participants with MetS had increased biomarkers of oxidative stress and inflammation, thereby suggesting that they had increased requirements for α-tocopherol (Table 1). Lower urinary d_6-α-CEHC excretions were associated with higher plasma concentrations of C-reactive protein, IL-10, IL-6, and insulin, lower plasma concentrations of HDL cholesterol, and higher diastolic blood pressure, waist circumference, and BMI, which suggested that our observations concerning vitamin E catabolism were related to the heightened inflammation and impaired cardiometabolic health in participants with MetS. In addition, participants with MetS had lower plasma ascorbic acid concentrations despite having eaten similar amounts of vitamin C as were consumed by healthy participants during the 3 d before the pharmacokinetic studies (10). Taken together, these observations support the contention that individuals with MetS have lower α-CEHC excretion because of lower vitamin E status as a result of increased oxidative and inflammatory stressors.

In participants with MetS, higher circulating lipid concentrations and slower α-tocopherol turnover artificially elevated plasma α-tocopherol concentrations, which led to the erroneous impression that these subjects had adequate vitamin E status. In contrast, we show that plasma and urine d_6-α-CEHC concentrations in participants with MetS were diminished. If there is less α-tocopherol available in the liver, less α-tocopherol can be secreted into the plasma, and less α-tocopherol is available for hepatic catabolism. However, in the absence of having liver samples available for analysis, it was unclear whether the lower plasma and urinary catabolite concentrations that were observed in participants with MetS resulted from less available α-tocopherol as a result of increased oxidative or peroxidative damage, lower habitual vitamin E intakes, impaired absorption, increased fecal excretion, or impaired hepatic lipoprotein secretion or clearance (**Figure 6**).

In the current study, urine α-CEHC concentrations were shown to reflect long-term vitamin E status. Urinary d_6-α-CEHC only represented $\sim$10% of the total urinary α-CEHC excreted daily, even though the d_6-α-T dose was 3 times higher than daily α-tocopherol intakes. In each of the 8-h urine-collection intervals, unlabeled α-CEHC was excreted at nearly equal concentrations (Figure 2B), which suggested that there was continuous α-tocopherol catabolism and α-CEHC excretion. However, an examination of the time course of urinary d_6-α-CEHC excretion by healthy participants showed that urinary α-CEHC concentration in part represented the recent (e.g., past 24 h) vitamin E intake on the basis of the observation that urinary d_6-α-CEHC peaked between 8 and 16 h (Figure 2C). In healthy participants, plasma d_6-α-CEHC concentrations also increased during the first 12 h, but by 24 h, the concentrations were diminished (Figure 3B) and remained just at detection concentrations up to 72 h. These data are also consistent with our previous observation in healthy participants that there was rapid α-tocopherol flux in and out of the liver with the entire plasma α-tocopherol pool replaced daily (32). Taken together, these findings suggest that a majority of the excreted α-CEHC is derived from the turnover of whole-body α-tocopherol stores.

Perhaps one of the most-surprising findings was the difference in the patterns of plasma unlabeled and d_6-α-CEHC concentrations that was observed between the 2 health-status groups (Figure 3), as was the relation between d_6-α-CEHC and d_6-α-T concentrations

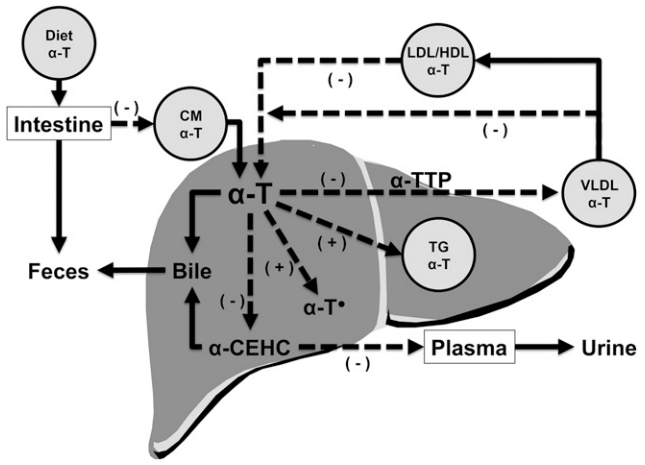

FIGURE 6 Hypothetical scheme of α-T trafficking and hepatic catabolism and disposition. MetS-associated inflammation and oxidative damage may decrease (−) or increase (+) pathways, which are indicated by dotted lines, to decrease vitamin E status. A portion of the dietary α-T is absorbed, and the remainder is excreted in the feces. Enterocytes secrete chylomicrons are enriched with α-T and, to a lesser extent, are enriched in individuals with MetS (10), possibly as a result of enterocytes trapping α-T in lipid droplets, which occurs in obese individuals (30). The liver takes up the α-T–containing chylomicron remnants. Hepatic α-T has several possible fates. α-TTP can facilitate α-T's transfer to VLDL, which is enriched with α-T to a lesser extent in MetS (10). Secreted VLDL is metabolized to LDL and HDL in the circulation (31); all mechanisms for lipid delivery to tissues can transport α-T to extrahepatic tissues and facilitate its return to the liver (12). Plasma α-T disappearance is slower, and α-T enrichment in both LDL and HDL occurs to a lesser extent, in adults with MetS (10). Hepatic α-T can be excreted in bile, but whether this pathway is affected by MetS is unknown. However, data from the current study indicate that MetS limits the hepatic metabolism of α-T to α-CEHC, which can be secreted in bile for fecal excretion or secreted into plasma, transported to the kidney, and excreted in urine, as reviewed by Traber (12). Alternatively, hepatic α-T can be sequestered in steatotic hepatocytes, which is consistent with the observation that MetS patients are frequently afflicted by nonalcoholic fatty liver disease (2). Hepatic α-T can also be oxidized to the α-T$^{\bullet}$, which is consistent with increased inflammation and oxidative stress in MetS. Overall, the limited α-T bioavailability that was observed in the participants with MetS in the current study reduced plasma and urinary α-CEHC concentrations, possibly by increasing α-T oxidation, increasing α-T, or α-CEHC excretion in feces or trapping α-T in lipid droplets in the steatotic liver or enterocyte. CM, chylomicron; MetS, metabolic syndrome; TG, triacylglyceride; α-CEHC, α-carboxyethyl hydroxychromanol; α-T, α-tocopherol; α-T$^{\bullet}$, α-tocopheroxyl radical; α-TTP, α-tocopherol transfer protein.

(Figure 4). In healthy participants, excursions in plasma unlabeled α-CEHC concentrations appeared to occur in response to meals that were provided at 6 and 12 h of each milk intervention, whereas in participants with MetS, plasma α-CEHC concentrations appeared to be unaffected by meal intake (Figure 3A). In both groups, absorbed d_6-α-T was catabolized to d_6-α-CEHC, which was detectable in plasma by 3 h and peaked at 6 h in healthy adults, with the d_6-α-CEHC peak preceding the d_6-α-T peak at 12 h in both groups (Figure 4). However, healthy participants had distinct peaks in plasma d_6-α-CEHC at 6 and 12 h, whereas plasma d_6-α-CEHC concentrations in participants with MetS increased slightly between 3 and 9 h and decreased at 12 h (Figure 3B). A recent study has shown that liver α-tocopherol must move through various hepatic compartments before α-TTP can facilitate its transfer and secretion into lipoproteins, and this process is dependent on the presence of α-tocopherol (33). Potentially, as the delivery of newly absorbed α-tocopherol and fat from the meal increases, both α-tocopherol secretion and lipoprotein secretion into plasma are stimulated and peak at roughly 12 h. In participants with MetS, liver α-tocopherol and α-CEHC trafficking may be altered, thereby resulting in greater α-tocopherol and α-CEHC biliary excretion into feces. One of the limitations of this clinical trial was that we did not measure fecal vitamin E catabolites, which have been estimated in rats to be 83% of the administered dose of radiolabeled α-tocopherol (34). Alternatively, the livers in participants with MetS were steatotic, and hepatic fat may have prevented normal α-tocopherol and α-CEHC trafficking (Figure 6).

We previously suggested that a cutoff of excreted urinary α-CEHC >1.39 μmol/g creatinine was associated with more-than-adequate vitamin E intakes (20). In the current study, only healthy women had least-squares mean $\pm$ SEM urinary α-CEHC concentrations (1.26 ± 0.11 μmol/g creatinine) that approached this cutoff, whereas healthy men (0.87 ± 0.11 μmol/g creatinine), women with MetS (0.77 ± 0.11 μmol/g creatinine), and men with MetS (0.48 ± 0.11 μmol/g creatinine) excreted less (health status, $P = 0.0002$; sex, $P = 0.003$, health status $\times$ sex, $P = 0.64$; $n = 5$/sex in each health status group). Given that the sample set from which we derived the cutoff was predominantly diet-conscious women (20), we expected that our current participants, who were nonusers of dietary supplements, would fall below this cutoff, which is a finding that is consistent with the 92% of men and 98% of women in the United States who fail to meet the Estimated Average Requirement for α-tocopherol (22).

In conclusion, our findings show that participants with MetS have lower vitamin E status on the basis of their reduced concentrations of urinary excretions of both labeled and unlabeled vitamin E catabolites (Figure 2). Comparisons of the temporal appearance of d_6-α-T and d_6-α-CEHC in plasma show extraordinarily rapid vitamin E catabolism because the peak in d_6-α-CEHC concentrations precedes that of the parent d_6-α-T (Figure 4). These findings emphasize our lack of information concerning α-tocopherol's absorption, liver trafficking, and disposition. The quantitative nature of our investigations shows that relatively low amounts (15 mg) of administered orally d_6-α-T are catabolized and show limited urinary d_6-α-CEHC excretion. These findings highlight the importance of measuring biliary and fecal excretion of both α-tocopherol and its catabolites, which are samples that were not collected in the present study. The serious nature of the consequences of MetS, especially NASH and its sequelae, emphasize the critical importance of future studies of vitamin E status in persons with MetS.

The authors' responsibilities were as follows—MGT, EM, and RSB: designed the research; MGT, GB, and RSB: analyzed the data; MGT and RSB: had primary responsibility for the final content of the manuscript; EM, SWL, and RSB: conducted the research; GB: served as a biostatistician on the project and performed the statistical analysis; and all authors: wrote the manuscript and read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

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