Vitamin E sequestration by liver fat in humans

Pierre-Christian Violet, … , Maret G. Traber, Mark Levine


BACKGROUND We hypothesized that obesity-associated hepato-steatosis served as a pathophysiologic chemical depot for fat-soluble vitamins and altered normal physiology. Using α-tocopherol (vitamin E) as a model vitamin, pharmacokinetics and kinetics principles were utilized to determine whether excess liver fat sequestered α-tocopherol in women with obesity-associated hepato-steatosis vs healthy controls.

METHODS Custom-synthesized deuterated α-tocopherols (d₃- and d₆-α-tocopherols) were administered to hospitalized healthy women and women with hepato-steatosis under IND guidelines. Serial samples obtained over 72 hours were analyzed by LC/MS. Fluorescent-labelled α-tocopherol was custom-synthesized for cell studies.

RESULTS In healthy subjects, 85% of intravenous d₆-α-tocopherol disappeared from the circulation within 20 minutes but reappeared within minutes and peaked at 6-8 hours. d₃- and d₆-α-Tocopherols localized to lipoproteins. Lipoprotein redistribution occurred only in vivo within 1h, indicating a key role of liver in rapid uptake and re-release into the circulation. Compared to healthy subjects, subjects with hepato-steatosis had similar d₆-α-tocopherol entry rates into liver, but reduced initial release rates (p<0.001). Similarly, pharmacokinetics parameters of AUC and Maximum Concentration (Cₘₐₓ) were reduced (AUC0-8 ,p<0.01; Cₘₐₓ p<0.02) in hepato-steatosis subjects, indicating reduced hepatic d₆-α-tocopherol output. Reduced kinetics and pharmacokinetics parameters (AUC and Cₘₐₓ) in hepato-steatosis […]

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Pierre-Christian Violet, Ph.D\textsuperscript{1,2}; Ifechukwude C. Ebenuwa, M.D.\textsuperscript{1,2}; Yu Wang, Ph.D.\textsuperscript{2}; Mahtab Niyiayi, M.D.\textsuperscript{2}; Sebastian J. Padayatty, M.B.B.S., Ph.D.\textsuperscript{2}; Brian Head MS\textsuperscript{3}; Kenneth Wilkins Ph.D.\textsuperscript{4}; Stacey Chung, Ph.D.\textsuperscript{5}; Varsha Thakur, Ph.D.\textsuperscript{3}; Lynn Ulatowski, Ph.D.\textsuperscript{2}; Jeffrey Atkinson, Ph.D.\textsuperscript{6}; Mikel Ghelfi, Ph.D.\textsuperscript{6}; Sheila Smith, R.N., B.S.N.\textsuperscript{2}; Hongbin Tu, Ph.D.\textsuperscript{2}; Gerd Bobe, Ph.D.\textsuperscript{3}; Chia-Ying Liu, M.D.\textsuperscript{7}; David W. Herion, M.D.\textsuperscript{8}; Robert D. Shamburek, M.D.\textsuperscript{9}; Danny Manor, Ph.D.\textsuperscript{5}; Maret G. Traber, Ph.D.\textsuperscript{3}; Mark Levine, M.D.\textsuperscript{2,10}.
\textsuperscript{1}Contributed equally

Affiliations:
\textsuperscript{2}Molecular and Clinical Nutrition Section, Intramural Research Program, NIDDK; National Institute of Health(NIH), Bethesda, MD 20892
\textsuperscript{3}Linus Pauling Institute, Oregon State University, Corvallis, Oregon, USA.
\textsuperscript{4}Office of the Director, NIDDK, NIH, Bethesda, MD 20892
\textsuperscript{5}Departments of Pharmacology and Nutrition, School of Medicine, Case Western Reserve University and the Case Comprehensive Cancer Center, Cleveland, Ohio 44106.
\textsuperscript{6}Department of Chemistry, Brock University, St. Catharines, Ontario L2S 3A1, Canada.
\textsuperscript{7}Radiology and Imaging Sciences, Clinical Center, NIH, Bethesda, MD 20892
\textsuperscript{8}Clinical Research Informatics, Clinical Center, NIH, Bethesda, MD 20892
\textsuperscript{9}Cardiovascular Branch, Intramural Research Program, NHLBI; NIH, Bethesda, MD 20892
\textsuperscript{10}To whom correspondence should be addressed

Corresponding author:
Mark Levine
Molecular and Clinical Nutrition Section, Digestive Disease Branch
National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)
National Institute of Health (NIH)
Building 10, room 4D52,
9000 Rockville Pike,
10 Center Drive,
Bethesda, Md 20892

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In accordance with disclosure guidelines, we report no financial relationships or conflicts. No authors have professional or financial affiliations that will be perceived to bias the presentation.
ABBREVIATIONS
AUC0-8: Area under the curve from time 0 – 8 hours
AUC0-72: Area under the curve from time 0 – 72 hours
BDP: bodipy
d6-α-tocopherol: Deuterated α-tocopherol with 6 deuterium atoms
d3-α-tocopherol: Deuterated α-tocopherol with 3 deuterium atoms
DEXA: Dual Energy X-ray Absorptiometry
HDL: high density lipoprotein
HPLC: high performance liquid chromatography
HS: obesity-associated hepato-steatosis
IND: investigational new drug application
LC/MS: liquid chromatography/mass spectrometry
LDL: low density lipoprotein
MRS: magnetic resonance spectroscopy
NASH: non-alcoholic steatohepatitis
PI: propidium iodide
TTP: α-tocopherol transfer protein
VLDL: very low density lipoprotein
Abstract:

BACKGROUND

We hypothesized that obesity-associated hepato-steatosis was a pathophysiologic chemical depot for fat-soluble vitamins and altered normal physiology. Using \( \alpha \)-tocopherol (vitamin E) as a model vitamin, pharmacokinetics and kinetics principles were utilized to determine whether excess liver fat sequestered \( \alpha \)-tocopherol in women with obesity-associated hepato-steatosis vs healthy controls.

METHODS

Custom-synthesized deuterated \( \alpha \)-tocopherols (d\(_3\)- and d\(_6\)-\( \alpha \)-tocopherols) were administered to hospitalized healthy women and women with hepato-steatosis under IND guidelines. Fluorescent-labelled \( \alpha \)-tocopherol was custom-synthesized for cell studies.

RESULTS

In healthy subjects, 85% of intravenous d\(_6\)-\( \alpha \)-tocopherol disappeared from the circulation within 20 minutes but reappeared within minutes and peaked at 3-4 hours. d\(_3\)- and d\(_6\)-\( \alpha \)-Tocopherols localized to lipoproteins. Lipoprotein redistribution occurred only in vivo within 1h, indicating a key role of liver in uptake and re-release. Compared to healthy subjects who received 2 mg, subjects with hepato-steatosis had similar d\(_6\)-\( \alpha \)-tocopherol entry rates into liver but reduced initial release rates (p<0.001). Similarly, pharmacokinetics parameters (AUC, Maximum Concentration [C\(_{\text{max}}\)]) were reduced (AUC\(_{0-\text{inf}}\),p<0.01;C\(_{\text{max}}\) p<0.02) in hepato-steatosis subjects, indicating reduced hepatic d\(_6\)-\( \alpha \)-tocopherol output. Reductions in kinetics and pharmacokinetics parameters in hepato-steatosis subjects who received 2mg were echoed by similar reductions in
healthy subjects when comparing 5 and 2 mg doses. In vitro, fluorescent-labelled α-tocopherol localized to lipid in fat-loaded hepatocytes, indicating sequestration.

CONCLUSIONS

The unique role of the liver in vitamin E physiology is dysregulated by excess liver fat. Obesity-associated hepato-steatosis may produce unrecognized hepatic vitamin E sequestration, which might subsequently drive liver disease. Our findings raise the possibility that hepato-steatosis may similarly alter hepatic physiology of other fat-soluble vitamins.

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INTRODUCTION

The global obesity epidemic has sobering consequences to upend human health (1-3). Especially concerning is obesity-associated hepato-steatosis (HS), or non-alcoholic fatty liver. Non-alcoholic fatty liver is already the most common precursor of chronic liver disease in the Americas and Western Europe (4-6). Specifically, non-alcoholic fatty liver precedes non-alcoholic steatohepatitis (NASH) and ultimately cirrhosis and hepatocellular carcinoma. The penetrance of this spectrum of liver diseases is increasing rapidly and concurrently with obesity and diabetes, with staggering consequences to morbidity, mortality, and health care costs. For NASH treatment, some patients had reduced inflammation and hepatocyte injury with pharmacologic doses of vitamin E (α-tocopherol), but fibrosis leading to cirrhosis was not improved (7,8). For NASH prevention, the only current strategy is maintenance of ideal body weight (9,10). Nevertheless, prevention strategies are gold standards to avert the need for treatment, disease consequences, and huge societal cost.

Although indirect, there are several clues that α-tocopherol, distinct from its role in partially treating disease, could prevent NASH. First, the liver participates in α-tocopherol physiology. Dietary α-tocopherol is trafficked out of the liver on lipoproteins, with involvement of the hepatocyte-localized α-tocopherol transfer protein (TTP) (11,12). Second, because α-tocopherol is fat soluble, excess fat in hepatocytes could serve as an unintended chemical sink. By sequestering α-tocopherol so that it is biologically less available or unavailable, liver fat could cause a local deficiency of the vitamin within liver, over years leading to progression of liver disease: from non-alcoholic fatty liver, to non-alcoholic fatty liver disease, and finally
NASH/hepatocellular carcinoma. Third, use of α-tocopherol in treatment of NASH (13,14) hints at an underlying local nutritional deficiency of α-tocopherol in liver.

With this background, we hypothesized that liver fat accompanying obesity could act as an unintended chemical depot for the lipophilic α-tocopherol. If correct, then release of α-tocopherol from the liver could be attenuated, and its physiological efficacy reduced. Essential pre-requisites for testing these concepts are development and use of new approaches to characterize α-tocopherol physiology in both healthy people, and in people with obesity-associated hepato-steatosis, without evidence of hepatitis. We utilized specially synthesized deuterated α-tocopherol preparations as investigational new drug (IND) products for simultaneous oral and intravenous administration in humans (15-18). These materials were used to perform kinetics and pharmacokinetics studies in healthy women and those with HS. Deuterated α-tocopherols doses were selected to be similar to amounts in foods. Pre-clinical studies were performed in animals, and in cultured hepatocytes using fluorescent labelled α-tocopherol (19,20).
RESULTS

Plasma concentrations of d6-, d3-α-tocopherol and redistribution into lipoproteins in healthy women.

To characterize vitamin E trafficking in healthy subjects (demographics in Table 1, Figure 1), we utilized two custom-synthesized deuterated α-tocopherol preparations: an oral solution of d3-α-tocopherol, and an intravenous small-particle (particles<0.5nm) sterile emulsion with d6-α-tocopherol. Both were administered to subjects under the auspices of an approved US-FDA Investigational New Drug Application (held by ML). Deuterated tocopherols at a dose of 5mg, an amount found in an avocado, were administered simultaneously orally and intravenously at time 0 to healthy hospitalized women (Figure 2A). Deuterated α-tocopherols were measured in plasma for up to 72 hours (Figure 2B). Nearly 85% of intravenous d6-α-tocopherol disappeared from the circulation within 20 minutes, with reappearance peaking at 3 hours and remaining steady until 8 h (Figure 2C). Reappearance of oral d3-α-tocopherol was slower, presumably due to the kinetics of intestinal absorption and lymphatic transport to the thoracic duct (21-23). To evaluate the trafficking of tocopherols in lipoprotein particles, we calculated %enrichment, which describes the percentage of deuterated α-tocopherols compared to total α-tocopherols on lipoproteins. Deuterated α-tocopherols were localized to lipoproteins (VLDL, HDL, LDL), with a shift of the vitamin to HDL and LDL over the time-course (Figure 2D, E). Approximately 100% of plasma d6- and d3-α-tocopherols were recovered on lipoproteins, implicating liver involvement (Figure 2F). When blood samples were incubated ex-vivo with d6-α-tocopherol, this shift to lipoproteins (LDL and HDL) was absent (Figure 2G). These data show that d6-α-tocopherol was translocated within minutes from plasma to the liver of healthy subjects, followed by reappearance (release) into the circulation and redistribution on lipoproteins.
To evaluate whether α-tocopherol pharmacokinetics parameters other than C\textsubscript{max} and AUC are influenced by administered doses of deuterated α-tocopherol (dose effect), we performed similar studies using a lower dose (2mg) of deuterated α-tocopherol (half an avocado) in healthy subjects. We compared d\textsubscript{6}-α-tocopherol pharmacokinetics parameters between 2mg vs 5mg doses (Figure 2H, Supp.Table 1). As expected, results revealed reduced C\textsubscript{max} and AUC\textsubscript{0-72} for 2mg dose (56% and 60% reduction respectively of those during the 5 mg dosing) in healthy subjects. All other pharmacokinetic parameters were similar (Supp.Table 1). We also compared d\textsubscript{6}-α-tocopherol initial release kinetics over the first 4 hours, when release was linear (initial rate conditions, Figure 2H inset) between matched healthy subjects who received 2 and 5mg during different hospitalizations (see methods), and found reduced release kinetics with the 2mg dose (5mg, 0.195µM.h\textsuperscript{-1};2mg, 0.083µM.h\textsuperscript{-1} [2.4fold-decrease]). Taken together, these findings underscore the essential role of the liver in α-tocopherol physiology, with similar pharmacokinetics with both 5mg and 2mg doses, excepting at the lower dose reduced initial release, C\textsubscript{max}, and AUC.

**Influence of liver fat on d\textsubscript{6}-α-tocopherol plasma concentrations: pharmacokinetics and kinetics in 10 healthy and 6 hepatic-steatosis (HS) subjects**

With this foundation indicating the essential role of the liver in rapidly taking up intravenously administered d\textsubscript{6}-α-tocopherol and subsequently releasing it into plasma, we hypothesized that excess liver fat would sequester fat-soluble α-tocopherol and attenuate its release from the liver to the circulation similarly to acquiring a smaller dose of vitamin E delivered to the liver. We tested our hypothesis by comparing pharmacokinetics and kinetics changes in healthy and HS subjects. Given similar pharmacokinetics of 2mg and 5mg doses
(Supp.Table 1), we chose the lower 2mg dose because a lower dose could provide a more sensitive means to probe liver fat effects, and this is an amount found in many serving sizes of vitamin E rich foods. Additionally, we used intravenous d<sub>6</sub>-α-tocopherol to bypass confounding effects of gastrointestinal absorption with orally administered d<sub>3</sub>-α-tocopherol.

**Effect of liver fat on pharmacokinetics parameters of d<sub>6</sub>-α-tocopherol in healthy vs HS subjects:**

Plasma d<sub>6</sub>-α-tocopherol concentrations were measured after intravenous administration of 2 mg d<sub>6</sub>-α-tocopherol (Figure 3A). To evaluate possible differences in α-tocopherol liver physiology between healthy and HS subjects, we calculated and compared pharmacokinetics parameters for d<sub>6</sub>-α-tocopherol in the two groups (Table 2). HS subjects had a 30% reduction in d<sub>6</sub>-α-tocopherol C<sub>max</sub> vs healthy subjects (0.34 ± 0.04 µM vs 0.48 ± 0.02 µM, P<0.05). HS subjects also had a 30% reduction in d<sub>6</sub>-α-tocopherol AUC<sub>0-8</sub> (time to C<sub>max</sub>) and 25% reduction in AUC<sub>0-72</sub> (Table 2). There were no significant differences in the other d<sub>6</sub>-α-tocopherol parameters (T<sub>max</sub>, K<sub>e</sub> and half-life) between HS and healthy groups (Table 2). Together, these findings show altered pharmacokinetics (reduced C<sub>max</sub> and AUC) in HS subjects in a similar pattern observed with 5mg vs 2mg (Figure 2H, Supp. Table 1).

**Effect of liver fat on kinetics parameters of d<sub>6</sub>-α-tocopherol in healthy vs HS subjects**

Based on the reduced d<sub>6</sub>-α-tocopherol C<sub>max</sub> and AUC suggesting decreased tocopherol output from the liver, we separately characterized d<sub>6</sub>-α-tocopherol release kinetics in healthy and HS subjects over the first 4 hours, when release was linear (initial rate conditions, Figure 3B). While the rate of removal of d<sub>6</sub>-α-tocopherol (disappearance) from plasma into the liver was similar in healthy and HS subjects (p>0.49 ; Supp.Figure1), the rate of d<sub>6</sub>-α-tocopherol
release from the liver into the circulation (reappearance) was reduced in HS vs healthy 2mg subjects (0.057 vs 0.083 µM/h, P<0.001) (Figure 3B, C). Upon quantitation of liver fat (measured by MRS, see supplementary methods), values of liver fat above 5% were associated with a reduced rate of d6-α-tocopherol release (Figure 3D, R²=0.62). If body fat was also involved in d6-α-tocopherol sequestration, then body fat (determined by DEXA (Dual Energy X-ray Absorptiometry)) should similarly be related to the rate of d6-α-tocopherol reappearance in plasma. We performed separate analyses on the HS cohort and the healthy cohort. No association was apparent in either Healthy (R²= 0.076, Figure 3E) or HS groups (R²=0.007, Figure 3F). These data suggest that reduced tocopherol liver output in HS subjects is associated with reduced release kinetics.

*Fat ‘traps’ vitamin E in vitro and in vivo.*

We investigated in vitro and in animals whether fat increased α-tocopherol content in liver. When oleic acid fat-loaded hepatocytes were incubated with fluorescent-labelled α-tocopherol, they contained more tocopherol and lost less over time as assessed by flow cytometry and microscopy (p<0.01, Figure 4A-C). Using oleic acid-loaded cells compared to controls, fluorescent-labelled α-tocopherol was co-localized to the fat droplet (Figure 4D, Supp.Figure 2). Consistent with these observations, livers of mice fed high fat diet had more α-tocopherol compared with controls, with content of α-tocopherol in diet held constant (p< 0.01, Figure 4E). These findings show that when hepatocytes are induced to accumulate fat intracellularly, the resulting lipid droplets sequester vitamin E. Taken together, these findings further support our hypothesis that excess liver fat sequesters α-tocopherol, reducing output from the liver.
Plasma d₆- and d₃-α-tocopherol concentrations and redistribution into lipoproteins in 10 healthy vs 6 HS women: dual-isotope analyses

Just as d₆-α-tocopherol administered intravenously revealed liver physiology for α-tocopherol, d₃-α-tocopherol administered orally could be used to additionally evaluate intestinal physiology for α-tocopherol. We compared pharmacokinetics and lipoprotein physiology in healthy and HS subjects utilizing 2mg dosing with both oral d₃-α-tocopherol and intravenous d₆-α-tocopherol (dual-isotope analyses).

\textit{d₃-α-Tocopherol pharmacokinetics in healthy vs HS subjects}

\textit{d₃-α-Tocopherol concentrations were measured in the same plasma samples as d₆-α-tocopherol over 72 hours in healthy and HS subjects (Figure 5A). We compared bioavailability of d₃-α-tocopherol (AUC₀₋₇₂, relative bioavailability) and fractional absorption (AUC d₃/d₆, true bioavailability) in healthy and HS subjects (Table.2). There was a 20\% reduction in relative bioavailability in HS subjects compared to healthy subjects (8.6 vs 10.7 µM*h, AUC₀₋₇₂, Table 2). The fractional absorption was higher in HS subjects (67\%) compared to healthy subjects (60.6\%). Despite not achieving statistical significance, these differences raised the possibility that additional, extra-hepatic factors might modulate α-tocopherol pharmacokinetics.}

\textit{Effects of liver and body fat on d₃- and d₆-α-tocopherol lipoprotein redistribution in healthy vs HS subjects}

Based on previous findings (Figure 2) that α-tocopherol is localized to lipoproteins (VLDL, LDL, HDL) with liver-mediated α-tocopherol lipoprotein redistribution, we calculated
d_6- and d_3-\(\alpha\)-tocopherol %enrichment over 8h (T_{\text{max}}) in healthy vs HS subjects. Because lipoprotein production is increased in subjects with fatty liver disease(24-27), we predicted that \(\alpha\)-tocopherol %enrichment would be reduced in the HS subjects. We compared the AUC_{0-8} for d_6- and d_3-\(\alpha\)-tocopherol %enrichments in healthy vs HS patients (see methods) and found significant reductions in both d_6- and d_3-\(\alpha\)-tocopherol %enrichments (p< 0.05: Figure 5B; Table 3 and Supp.Figure 3). Reduced \(\alpha\)-tocopherol %enrichments in HS subjects’ lipoproteins is consistent with the notion that liver fat sequesters vitamin E, thereby altering \(\alpha\)-tocopherol physiology.

Increased VLDL production reflects increased lipoprotein production in fatty liver disease(24,26). If only liver fat and no other body fat pools modulated d_3-\(\alpha\)-tocopherol, then the degree of reduction in %enrichment for VLDL fractions should be similar for both the oral d_3-\(\alpha\)-tocopherol and intravenous d_6-\(\alpha\)-tocopherol in HS subjects. Conversely if another fat pool, such as intestinal fat, modulated primarily d_3-\(\alpha\)-tocopherol, then HS subjects might have a greater reduction of d_3-\(\alpha\)-tocopherol in VLDL compared to d_6-\(\alpha\)-tocopherol. The data showed that the reduction in % lipoprotein enrichment at 8h in HS (vs healthy subjects) for the d_3 oral dose (Figure 5D, p<0.01) was 1.3-fold higher that the reduction in % lipoprotein enrichment for the d_6 IV dose (Figure 5E, p<0.05), implying that another fat pool could be involved.

We probed these findings in HS subjects by calculating d_3-\(\alpha\)-tocopherol bioavailability (AUC_{0-72}) as a function of body fat, with comparison to healthy subjects. d_3-\(\alpha\)-Tocopherol bioavailability was negatively correlated with body fat in HS subjects (R^2=0.71, Figure 5F), but not in healthy subjects (R^2=0.05, Figure 5G). Because d_3-\(\alpha\)-tocopherol accounts for intestinal absorption, while d_6-\(\alpha\)-tocopherol does not, these findings imply that there is an additional role of intestinal fat in modulating \(\alpha\)-tocopherol physiology.
DISCUSSION

Administration of intravenous d6-α-tocopherol revealed the roles of the liver in α-tocopherol physiology in healthy women, and its contribution to the vitamin’s pathophysiology in women with HS. In healthy women, ~85% of d6-α-tocopherol administered intravenously was removed from the circulation within 20 minutes, followed by reappearance (release) in plasma and redistribution on lipoproteins. Redistribution was absent when blood was incubated with deuterated tocopherol ex vivo, indicating a role of the liver in α-tocopherol physiology in humans. Use of both kinetics and pharmacokinetics approaches to characterize d6-α-tocopherol revealed profound differences between healthy and HS women. For pharmacokinetics, HS subjects had significantly reduced Cmax and AUC compared to 2mg healthy controls, consistent with reduced liver output of vitamin E despite administration of similar doses. Consistent with these data, a similar pattern of reduced Cmax and AUC was observed in 5mg vs 2mg healthy subjects (Supp.Table 1). Combined with our cell and animal data, the altered pharmacokinetics strongly indicate that liver fat sequesters α-tocopherol and attenuates its release, independent of the α-tocopherol transfer protein. For kinetics, initial d6-α-tocopherol removal from the circulation (disappearance rate) was as rapid in women with HS as in healthy women, but the initial release kinetics of d6-α-tocopherol reappearance in plasma were reduced by 30% (p<0.001; Figure 3B). A similar pattern of reduced release kinetics was seen when comparing 5mg vs 2mg in healthy subjects (Supp.Figure 4). These data support the hypothesis that excess liver fat sequesters tocopherol and results in pathophysiology that is analogous to a reduced vitamin E dose in healthy subjects. Since triglyceride release rates are 2-3 fold higher in subjects with non-alcoholic fatty liver disease or metabolic syndrome (24,26-28) our findings likely underestimate the actual sequestration of α-tocopherol in livers of HS subjects. That d6-α-
tocopherol sequestration is only associated with liver fat and is not affected by other fat stores (Figure 3D, E) provides additional support of our hypothesis. Only with increasing liver fat was there a highly-correlated inverse relationship to d₆-α-tocopherol release.

Administration of oral d₃-α-tocopherol also revealed new physiology and pathophysiology, but findings with d₃-α-tocopherol are more complex for several reasons. Several hours lapsed before d₃-α-tocopherol appeared in plasma. α-Tocopherol is first absorbed in intestine, undergoes lymphatic transport to the thoracic duct, and enters the general circulation prior to uptake by the liver and subsequent re-release (21-23). Consistent with other studies using only oral tocopherol (29), our findings revealed reduced d₃-α-tocopherol bioavailability in HS vs healthy subjects, although the findings did not achieve statistical significance. Our data also revealed a negative correlation between d₃-α-tocopherol bioavailability and %body fat (DEXA) but not liver fat. Taken together, our findings suggest that a body fat compartment, possibly intestinal fat, additionally modulates d₃-α-tocopherol. The lack of statistical significance in d₃-α-tocopherol bioavailability for healthy vs HS subjects may be explained by the variability in percent body fat in our cohort (33-57%) as well as effects of subsequent food ingestion on α-tocopherol trafficking (18,29). Future studies focusing on subjects with similar percentage of body fat, with and without hepato-steatosis, as well as controlled fasting and post-prandial studies can address these possibilities.

To further evaluate the effect of body (i.e. intestinal) fat on d₃-α-tocopherol physiology, we compared fractional absorption in HS vs healthy subjects, using the dual-isotope technique (18). Given the additional contribution that intestinal fat could have to modulate d₃-α-tocopherol but not d₆-α-tocopherol, we had anticipated a reduction in the d₃:d₆ AUC ratio, that would indicate reduced fractional absorption in the HS group. Surprisingly, our findings revealed higher
fractional absorption in HS vs healthy subjects, albeit a statistically insignificant difference. These findings for fractional absorption in the HS group are likely due to relative changes in d3-α-tocopherol vis-à-vis changes in d6-α-tocopherol, resulting from varying degrees of modulation by intestinal and liver fat. These findings also reveal limitations in the use of fractional absorption to estimate α-tocopherol bioavailability in subjects who are obese or have hepato-steatosis. The findings also showed that both d3- and d6-α-tocopherol release on lipoproteins was reduced in women with HS compared to healthy subjects, and that there were reductions in %enrichment of d3- and d6-α-tocopherol in several lipoprotein fractions in HS subjects, consistent with increased lipoprotein production associated with hepato-steatosis (24,26,28).

The approximately 1.3-fold reduction in d3- vs d6 %enrichment in HS subjects was consistent with an added role of a body fat compartment (i.e. intestinal fat) in d3-α-tocopherol modulation. Sequestration of vitamin E in other body locations with excessive fat pools associated with obesity, such as an intestinal fat pool, might further reduce vitamin E delivery to the liver. Downstream potential consequences of extrahepatic fat sequestration sites include reduction in bio-available intrahepatic vitamin E and reduced antioxidant capacity. To test consequences of excess body fat pools, future studies can investigate d6-α-T and d3-α-T kinetics in obese patients who do not have hepato-steatosis.

Subjects in this study were selected based on percentage hepato-steatosis, and specifically chosen to have less than 2% or more than 7% steatosis, so as to ‘bookend’ potential effects of liver fat. Women were purposely selected to have steatosis but not hepatitis, based on normal-range serial liver function testing, and FIB-4 score (30). Although liver biopsy is the gold standard to diagnose hepatitis, we were unable to justify the risk of biopsy given our inclusion
criteria, and absence of patient-specific benefit that could be expected from biopsy vs. risk of harm.

It is unknown whether sequestered vitamin E in humans is functional, quiescent or utilized by increased anti-oxidant requirements. In liver, it is possible that sequestered vitamin E might be utilized to quench oxidants generated within excess fat. Nevertheless, due to sequestration of vitamin E, there may be less of it available to other cellular compartments. As a consequence of anti-oxidant crosstalk, discussed in the next paragraph, less tocopherol availability could increase risk of oxidant damage in many hepato-cellular compartments.

Based on its established activity as an anti-oxidant (electron donor) (31-34), α-tocopherol may be utilized for quenching oxidants that are generated during hepatic cell metabolism, especially as an outcome of lipid oxidation (35,36). Hydrophobicity of vitamin E confines its direct antioxidant activity to the lipid milieu of biological membranes, distinct from the redox status in water soluble compartments (37). The cellular NAD(P)H / GSH antioxidant system is reserved mostly for soluble targets and for reduction of peroxidative end-products - lipid hydroperoxides. Loss of glutathione dependent reductases (for instance the selenium dependent glutathione peroxidase 4, or GPx4) substantially raises oxidative events and stress to cells. Much of this can be avoided by lipid soluble antioxidants such as α-tocopherol, which limit production of lipid peroxides by terminating the radical chain reaction (34). Additionally, there is in vitro evidence that interaction, or cross-talk, occurs between water- and lipid-soluble antioxidant molecules at the membrane–cytosol interface (38,39), with support from clinical data (40). Thus, via direct and indirect actions, vitamin E can have antioxidant activity in multiple cell compartments. Although other α-tocopherol functions in liver are poorly characterized, α-tocopherol could influence membrane transport of nutrients into hepatocytes; intermediary
metabolism of lipids; activities of membrane-associated enzymes in sugar catabolic pathways; or lipid export (41,42).

What is the clinical relevance of α-tocopherol sequestration and decreased hepatic release? The data suggest that when obesity is accompanied by liver fat, meal-derived α-tocopherol as well as lipoprotein-derived α-tocopherol could be diverted to a liver fat depot, perhaps with diminished local availability. Especially after food ingestion, fat in hepatocytes might generate excess oxidants (5,25,43), precisely when there would be decreased availability of α-tocopherol for oxidant quenching, perhaps leading to chronic oxidant-induced liver damage (i.e. progression to inflammation, hepatocyte injury, and irreversible cirrhosis). In an obese individual, this chain of events is repeated over thousands of meals leading to further exacerbation of NASH. Overall, it is possible that excess food intake may inadvertently create a local vitamin deficiency because of unintended fat deposition in liver: famine from feast (44,45) (Figure 6).

The famine from feast concept opens unexpected experimental and treatment doors. For NASH, the concept supplies a reason why only some patients respond to treatment with vitamin E (α-tocopherol) (7,8). By the time hepatitis is clinically apparent, correcting local chronic vitamin E deficiency may be too late, with irreversible fibrosis already present. Although there are clues (46), it is unknown whether liver fat affects disposition and metabolism of the three other fat-soluble vitamins in humans, and vitamin E experiments here may serve as a paradigm. Rodents have a different lipoprotein profile than humans (47-49), and serial sampling to detect rapid hepatic uptake and release in rodents is not possible with current technology. Famine from feast derives from nutrition concepts advanced more than 70 years ago, concepts that disease could increase vitamin requirements (50,51). Knowledge and tools did not exist then to explore
how nutritional needs are changed by disease but do now. As exemplified by vitamin C and data presented here, characterization of normal physiology and pharmacokinetics of vitamins in humans may unveil pathophysiology plus unexpected use in disease treatment (44,52-54). From the data here, it is possible that a fat-induced vitamin deficiency may be correctable: increased vitamin E ingestion could restore a normal hepatic release pattern. A path already exists for vitamin supplementation to prevent disease. Folate addition to flour is estimated to be responsible for a 30% decrease of neural tube defects in the United States (55). The data here raise the intriguing possibility that timely vitamin E supplementation might attenuate progression of hepatosteatosis to NASH, perhaps by correcting fat-induced, localized, hepatic vitamin E deficiency prior to inflammation, hepatitis, and fibrosis. We believe that studies to advance these possibilities should proceed expeditiously.
METHODS

Material

Bodipy (BDP) α-tocopherol was synthesized as described(19). Propidium Iodide (PI), cell culture media, and reagents for cell culture were purchased from Life Technologies (Frederick, MD, USA). HepG2, MCARH7777 (Rat hepatoma; ATCC CRL-1601) cells were purchased from ATCC. The Huh7.5 (Hepatocellular carcinoma) cell line was provided by Francis Chisari (The Scripps Research Institute, La Jolla, CA). Cells expressing TTP were previously described(12). Immortalized human hepatocytes (IHH) were a generous gift from R Ray, St. Louis University, St. Louis MO USA. All chemicals were purchased from Sigma Aldrich (St Louis, MO, USA), solvents were purchased from fisher scientific (Waltham, MA, USA).

Clinical research trial

Patient screening and enrollment

Clinical research to study α-tocopherol pharmacokinetics in women was approved by the Institutional Review Board, NIDDK/NIAMS, NIH, and conducted in accordance with NIH guidelines (protocol 09-DK-0097). This trial, a nonrandomized nonblinded cohort and crossover interventions was registered at clinicaltrials.gov as NCT00862433. Healthy women (aged 18-40y) with normal physical exams, screening laboratory studies and liver fat $<2\%$ based on magnetic resonance spectroscopy (MRS) and without serious or chronic medical illnesses were eligible. Women with hepat-steatosis (aged 18–55), had liver fat $\geq 7\%$ (measured by MRS); had no other causes of chronic liver disease such as autoimmune, alcoholic or infectious hepatitis;
and had no other uncontrolled medical illness (blood pressure < 160/90 mm Hg, hematocrit > 30%, HgbA1c < 8.5%, absence of significant microvascular or macrovascular diabetic complications) were eligible. Exclusion criteria included active tobacco use; alcohol abuse (estimated average alcohol consumption > 20 g/d in the 6 months prior to enrollment or binge-drinking behavior); recent illicit drug use; food allergy to soy, egg, milk protein (casein), or wheat/gluten; coagulopathy; unwillingness to use effective contraceptive methods for the duration of the study. All women studied had serial normal liver function tests, normal platelet counts, and FIB-4 scores < 0.92 (30) (Table.1).

We screened 39 subjects and enrolled 10 healthy and 6 HS subjects who met inclusion criteria. Among the healthy group, we used 2 different doses (5mg and 2mg) of deuterated tocopherols. All 10 healthy subjects participated in the 2mg intervention, while 6 out of the 10 also participated in the 5mg intervention. $d_6$- and $d_3$-α-tocopherol half-lives were < 40 hours (18), and the washout period used was > 1400 hours (>2 months; > 30 x half-life) between interventions. All HS subjects received the 2mg intervention.

**Deuterated α-tocopherol administration and sampling in humans**

d$_3$-α-Tocopherol and d$_6$-α-tocopherol were synthesized as described (15,16,20) under good manufacturing practices (GMP). d$_3$-α-Tocopherol for oral administration was diluted in tocopherol-stripped soybean oil at a concentration of 50mg/ml. Stripping is a commercial process in which natural oils are distilled at 250°C under vacuum to remove volatile phenols such as vitamin E. While the detailed protocol is proprietary, examples of the process’s efficiency are documented (56,57). For the d$_6$-α-tocopherol, no additives were added, and was prepared for intravenous administration as a sterile lipid emulsion under GMP as described (17).
The oral and intravenous preparations were administered to patients under an Investigational New Drug Application (IND) (held by ML).

Enrolled subjects were admitted to the NIH Clinical Research Center as inpatients for 4-5 days (from 20xx to 20xx). Subjects were fasted overnight from day 0 to day 1. On day 1 early AM intravenous cannulae were inserted into both arms (see Figure 2A for protocol sampling scheme). For the morning meal, all subjects received the same custom-prepared liquid meal containing 40% fat. When 2/3 of the liquid meal was consumed, d3-α-tocopherol (the oral vitamin E dose at either 2 or 5 mg) was placed on the subject’s tongue and the remainder of the liquid meal was consumed within 5 minutes. At that time, the intravenous d6-α-tocopherol dose (the same as the oral dose) was administered in the opposite arm of the sampling arm with the infusion end defined as time 0. Blood samples were obtained as shown in protocol sampling scheme (Figure 2A). Samples were placed on ice at the bedside prior to plasma isolation by centrifugation, aliquoting into cryovials, flash freezing in dry ice, and storage at -80º C until analyses.

**Dietary and nutritional monitoring**

All consumed food was prepared by the NIH nutrition staff using tocopherol-stripped vegetable oil. Diet composition. Subjects meals, snacks and beverages contained minimal amounts of vitamin E (on average, <6 mg α-T per day and ~12 mg γ-T per day). Diets were composed of 55% carbohydrate, 15% protein and 30% fat. Food items rich in vitamin E were excluded (such as wheat germ oil, almonds, sunflower seeds, snack chips and spinach). To avoid confounding results with variable vitamin C status (31,58) ascorbic acid stores were saturated in all subjects by prescribing one gram oral vitamin C daily for seven days two weeks prior to admission,
followed by 250 mg daily beginning one week prior to admission and continuing for the duration of the subject’s participation in the study.

**Imaging to quantitate liver and body fat**

Liver fat was determined by Magnetic Resonance Spectroscopy (MRS), performed on 3T MRI scanners (Achieva, Philips, Amsterdam, The Netherlands). Body fat was determined by DEXA whole body composition analyses (Hologic Discovery QDR, Hologic, Marlborough, MA, USA).

**Analytical Procedures and statistics**

**Lipoprotein isolation**

Plasma samples were obtained after infusion of d₆-α-tocopherol at times 0, 0.17, 1, 2, 4, and 8 hours. Lipoproteins (VLDL, LDL, HDL) were separated using discontinuous salt density-gradient ultracentrifugation as described (59). For lipoprotein isolation using *ex vivo* blood samples: 30 minutes prior to administration of the d₆- and d₃-α-tocopherols to subjects, four 10 ml blood samples from each subject were withdrawn directly into sodium heparin tubes. To produce a final concentration of 7 µM, 5.5 µl of d₆-α-tocopherol intravenous injection solution (concentration: 5.4 µg/µl) was added *ex vivo* to each 10 ml blood sample, followed by incubation at 37°C for indicated times of 0, 2, 10, and 60 minutes. At the end of incubation, samples were processed for lipoprotein separation by ultracentrifugation as described (59).

**α-Tocopherol analyses**
Labeled d3-, d6-, d9-\(\alpha\)-tocopherol (d9 as an internal standard) and unlabeled d0-\(\alpha\)-tocopherol were analyzed using high-performance liquid chromatography/mass spectrometry (LC/MS) in plasma and lipoproteins as described\(^{(18,60,61)}\). Sample were thawed at room temperature immediately prior to use, and saponified at 70˚C with alcoholic KOH + 1% ascorbic acid. Samples were then cooled, antioxidants (1% ascorbic acid in water and 25 \(\mu\)g butylated hydroxytoluene in ethanol) and Internal Standards (IS) [d9-\(\alpha\)-tocopherol, Isotec Labs; the isotopic distribution was 0% d0-\(\alpha\)-tocopherol, 0% d3-\(\alpha\)-tocopherol, 0% d6-\(\alpha\)-tocopherol, 0.6% d7-\(\alpha\)-tocopherol, 11.0% d8- \(\alpha\)-tocopherol and 88.4% d9- \(\alpha\)-tocopherol; >99% deuterium-labeled \(\alpha\)-tocopherol \((62)\)] added, and samples extracted with hexane. The extracts were dried under nitrogen, resuspended in 1:1 ethanol:methanol, and then injected either into an LC-MS (Waters 2695 Separations Module and a Micromass ZQ2000) or an LC-MS/MS (either an Applied Biosystems/MDS Sciex API 3000, Foster City, CA, or a Waters XEVO TQD, Milford, MA). For single-ion recording detection (negative mode), mass-to-charge ratio (m/z) data were obtained for \(\alpha\)-tocopherol (d0- \(\alpha\)-tocopherol, m/z 429; d3- \(\alpha\)-tocopherol, m/z 432; d6- \(\alpha\)-tocopherol, m/z 435 and d9-\(\alpha\)-tocopherol, m/z 438) and d0- \(\alpha\)-tocopherol (415). For MS/MS detection (negative mode), analytes were detected using multiple reaction monitoring (MRM) of transitions: d0-\(\alpha\)-tocopherol, m/z 429/163; d3- \(\alpha\)-tocopherol, m/z 432/166; d6-\(\alpha\)-tocopherol, m/z 435/169 and d9-\(\alpha\)-tocopherol, m/z 438/172; and d0-\(\alpha\)-tocopherol, m/z 415/149.

**Pharmacokinetics analyses.**

Plasma \(\alpha\)-tocopherol pharmacokinetics parameters were calculated for all subjects using concentration-time data following administration of d6- and d3-\(\alpha\)-tocopherols. Area under the Curve (AUCs) of plasma and lipoprotein d6-\(\alpha\)-tocopherols were calculated by using the
trapezoidal rule. Commercially available software (Stata, www.stata.com) was used to estimate pharmacokinetics parameters (Table.2). To specifically evaluate the effect of liver fat on α-tocopherol physiology, and to bypass confounding effects associated with gastro-intestinal absorption, we compared pharmacokinetics parameters from intravenously administered d₆-α-tocopherol: Cₘₐₓ (maximal concentration), Tₘₐₓ (time of maximal concentration) and AUC₀₋₈ₕ (AUC up to Tₘₐₓ). To account for intestinal fat that could potentially modulate α-tocopherol physiology, we calculated similar pharmacokinetics parameters for the orally administered d₃-α-tocopherol in both healthy and HS groups. Bioavailability for healthy and HS subjects was calculated as d₃-α-tocopherol AUC₀₋₇₂. α-Tocopherol fractional absorption (absolute bioavailability) was calculated using ratios of d₃-α-tocopherol to d₆-α-tocopherol AUCs plasma α-tocopherol concentration-time (0-72h or 0-8h) curves multiplied by 100% based on the dual isotope method(18). Pharmacokinetics calculations were separately calculated for d₃- and d₆-α-tocopherol enrichment in lipoprotein fractions.

Kinetics analyses

d₆-α-Tocopherol initial release kinetics were calculated over the first 4 hours when release was linear. Calculation of d₃-α-tocopherol release kinetics was not possible due to insufficient sampling time points over the relevant time period, due to constraints on patient blood volume withdrawal limits.

Lipoprotein % enrichment calculations

\[
(1) \quad d₃αt_{(sample \ corrected)} = \frac{d₃αt_{(baseline \ area \ count)}}{d₆αt_{(baseline \ area \ count)}} \times d₆αt_{(baseline \ area \ count)}
\]
\[(2)\quad d_6\alpha t_{(\text{sample corrected})} = \frac{d_6\alpha t_{(\text{baseline area count})}}{d_0\alpha t_{(\text{baseline area count})}} \times d_0\alpha t_{(\text{sample})}\]

\[(3)\quad \text{total } \alpha t = d_3\alpha t_{(\text{sample corrected})} + d_6\alpha t_{(\text{sample corrected})} + d_0\alpha t_{(\text{sample})}\]

\[(4)\quad d_3\alpha t \%\text{enrichment} = 100 \times \frac{d_3\alpha t_{(\text{sample corrected})}}{\text{total } \alpha t}\]

\[(5)\quad d_6\alpha t \%\text{enrichment} = 100 \times \frac{d_6\alpha t_{(\text{sample corrected})}}{\text{total } \alpha t}\]

[Abbreviations for formulae as follows: \(d_3\alpha t\), \(d_3\)-\(\alpha\)-tocopherol; \(d_6\alpha t\), \(d_6\)-\(\alpha\)-tocopherol; \(d_0\alpha t\), unlabeled \(\alpha\)-tocopherol]

**Cell and Animal Studies**

**BDP \(\alpha\)-tocopherol experiments**

Cells were loaded with 100\(\mu\text{M}\) of oleic acid (Avanti polar lipid) for 12 to 14h followed by incubation with BDP \(\alpha\)-tocopherol. After 2h incubation cells were washed with culture media and then analyzed by flow cytometry (T=0) or incubated with culture media with 1\% FBS for 30h and then analyzed. After incubation cells were collected using trypsin, washed with PBS, stained with PI for 20 min, and analyzed by flow cytometry (ADP Cyan, Beckman coulter), using FL-1 detector (Em: 488, Ex: 530/40) for BPD \(\alpha\)-tocopherol and FL-3 detector (Em: 488, Ex: 613/20) for PI. Only PI negative cells (live cells) were used for BDP \(\alpha\)-tocopherol detection. The same quantity of cells was used in all measurements (40 000 PI negative cells). The mean of
the total FL-1 fluorescence spectra was used to compare the difference in BDP α-tocopherol quantity in different samples. For microscopy experiments with BDP α-tocopherol, α-tocopherol transfer protein expression was induced in McARH7777 cells with doxycycline for a total of 48h incubation(7). After 24h of induction, cells were loaded with 0 µM or 100 µM oleic acid for 24h. Cells were then loaded with BDP α-tocopherol as above and lipid droplets were stained with 100nM Nile Red. Cells were washed with phosphate buffered saline and fixed with 4% paraformaldehyde for 15min; coverslips were mounted on slides with Slow Fade and sealed. Images were taken using confocal microscopy [Zeiss LSM510Meta Confocal microscope; 100x objective (488 Argon laser, 543 HeNe)] or fluorescence microscopy (EVOS Cell Imaging Systems, Life technologies).

**Animal experiments.**

Animal procedures were approved by the Animal Care and Use Committees at NIH and Case Western Reserve University. C57bl/6 mice, purchased from Jackson Laboratories (Bar Harbor, ME) were fed with normal chow or High Fat chow (high fat/Sucrose diet, 45% sucrose and 55% fructose (wt/vol)) with normal α-tocopherol levels (Harlan Teklad customs diet TD.130142; 150 mg/Kg diet of α-tocopherol acetate). After 16 weeks on diet, mice were starved overnight and sacrificed. To process samples, 30mg of mouse liver was homogenized (Turrax blender) into 100μL of cold PBS and diluted 1:1 using 100μL of ice-cold water. Ice-cold methanol 300μL was added to the sample which was vortexed for 1min, followed by addition of 600μL ice-cold hexane. Each sample was vortexed again for 1min, and 400μl of the upper phase were transferred to a new tube followed by drying (speedVac, ThermoFisher). The dried material was re-suspended into 50μL of ethanol (200 proof) + 0.5% butylated hydroxytoluene (wt/vol). 10μL
was used for HPLC analysis, and α-tocopherol concentrations were normalized using liver weight.

**Cells and animal α-Tocopherol analyses**

In cells and animal tissues, α-tocopherol was analyzed by HPLC with coulometric electrochemical detection (39, 40), but with various modifications. Instrumentation: temperature-controlled autosampler and dual-piston pump (Waters Chromatography, Milford, MA, USA); coulometric electrochemical detector (Coulochem III, ESA-Dionex, Chelmsford, MA, USA). Detector settings: electrode 2, 600 mV; electrode 1, -400 mV. Mobile phase contained 30mM lithium acetate (final concentration) dissolved in 95% methanol/water, flow rate 1 ml/min, injection volume 10 μl. The column was silica-based 5 μm, 4.6 mm × 15 cm C-8 (Eclipse XDB-C8, Agilent technologies; Santa Clara, CA, USA). The column was conditioned with mobile phase at 1 ml/min for 24–36 h prior to running standards and samples and washed once monthly with 30% Methanol/water for 24 h, 1 ml/min. Guard Cartridges Bioadvantage Basic C8 5μm were essential for optimum performance (Thomson Instruments, Clear Brook, VA, USA) and were replaced after injection of 150–200 biological samples to avoid online sample oxidation.

**Statistics**

Statistical significance (p<0.05) was analyzed as follows: two-way ANOVA (with adjustment for *post hoc* multiple comparisons) for the data in figures 3B; Student's *t* test (Unpaired, Two-tailed) was used for the data in Table 2 and in figures 3C, 4A-C, 4E, 5B-D; for the data of within-subject changes in $C_{\text{max}}$ and AUC$_{0-72}$ following administration of IV d$_6$ α-
tocopherol to 10 healthy subjects at 2mg and 6 healthy subjects at 5mg, as shown in supplementary table 1 (a Wald-type F-test of contrasts formed from regression coefficients in a mixed-effects model with Kenward-Roger approximation for the statistic’s denominator degrees of freedom (63)). P values for all figures: *p<0.05; **p<0.01; ***p<0.001. N ≥ 3 for all in vitro experiments; for mouse experiment, n=5 per group; for all clinical experiments, n indicated in figure legends, always ≥ 6 subjects. The major statistical endpoints (primary objective) of the clinical study with healthy subjects and those with hepato-steatosis were mean differences in the C\text{max} and AUC\text{0-8} (at time to C\text{max} or T\text{max}) of d\text{6-α}-tocopherol. The study was originally designed to show a statistically significant (P ≤ 0.05) difference in C\text{max} values of 50% or greater between the two groups with at least 90% power (18). Those design-stage power calculations were based on a standard deviation of 1.0 and indicated a minimum of 7 subjects per group; upon reviewing interim data the estimated standard deviations were consistent with values much smaller than 1.0 for the primary objective’s endpoint. Thus, enrollment concluded after enrolling 6 subjects in the hepato-steatosis group and 10 subjects in the healthy control group, and calculations were inverted to state power in terms of a detectable difference in C\text{max} values for a range of standard deviation values while targeting a conventionally acceptable level of power (minimum 80%, up to 90%), assuming such differences would be deemed statistically significant (P ≤ 0.05); see Supplementary Table 1.

**Study Approval**

This study was reviewed and approved by Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases and National Institute of Arthritis and
Musculoskeletal and Skin Diseases. Signed informed consent documents were obtained from each participant included in the study.

For animal study, experimental protocols were approved by the OACU of National Institute of Health and National Institute of Diabetes and Digestive and Kidney Diseases at Bethesda, MD.
Author Contributions:
M.G., and C-Y.L.; Data Curation, P-C.V., I.C.E., B.H, G.B., and M.T.; Writing – Original Draft,
I.C.E., P-C.V. and M.L.; Writing – Review & Editing, I.C.E., P-C.V., M.T., D.M., R.S. and
M.L.; Funding Acquisition, M.T. and M.L.; Supervision, M.L. Formal Analysis, K.W.

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Center Nutrition Staff, NIH; Scott Leonard, MS for technical assistance; Grants DK053213-13 to
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instrumentation. Funders had no input into study outcomes.
Reference List


Figure 1: Recruitment scheme.

The recruitment scheme is shown for this clinical intervention (NCT00862433), conducted at the NIH Clinical Research Center. Data from all participants are included in the analyses. In green, 6 healthy women of the 10 healthy women who received 2mg α-tocopherol also received after a period of washout 5mg α-tocopherol. IV, intravenous; PO, per oral administration.
Figure 2

(A) Protocol sampling scheme. (B) $d_6$-$\alpha$-tocopherol 5mg (●) was administered intravenously and $d_3$-$\alpha$-tocopherol 5mg (○) was administered orally at time zero in 6 subjects, plasma samples obtained over 72h. (C) Plasma samples in (B), magnified time interval -2 to 8h. (D) $d_6$-$\alpha$-Tocopherol %enrichment (▲ HDL; ■ LDL; ● VLDL) in lipoproteins following administration of 5mg at time zero in 6 subjects. (E) $d_3$-$\alpha$-tocopherol %enrichment (▲ HDL; ■ LDL; ● VLDL) in lipoproteins following administration of 5mg at time zero in 6 subjects. Because HDL and LDL points were superimposed, for clarity HDL and LDL curves were slightly offset on the Y axis. (F) % of recovery of $d_3$-$\alpha$-tocopherol (●) and $d_6$-$\alpha$-tocopherol (■) in all lipoproteins compared to plasma concentrations in 6 subjects. (G) Distribution of 7µM of $d_6$-$\alpha$-tocopherol in lipoproteins over time ex-vivo (▲ HDL; ■ LDL; ● VLDL). (H) $d_6$-$\alpha$-Tocopherol 5mg (●) and 2mg (○) was administered intravenously at time zero in 6 subjects, plasma samples obtained over 72h; inset: initial rates of $d_6$-$\alpha$-tocopherol reappearance for these 2 doses. SL; slope: 0.195µM.h$^{-1}$ for 5mg and 0.083 µM.h$^{-1}$ for 2mg dose. Data represented as mean ± SEM. For all panel n=6 for HS and n=10 for Healthy cohorts.
Figure 3: Influence of liver fat on d6-α-tocopherol plasma concentrations: pharmacokinetics and kinetics in 10 healthy and 6 hepato-steatosis (HS) subjects.

(A) d6-α-tocopherol 2mg administered IV in healthy (○) or HS subjects (●) over 72h. (B-D) Initial rates of d6-α-tocopherol reappearance matched to patient status (C), to % liver fat by MRI (D). (E-F) Initial rates of d6-α-tocopherol reappearance to % body fat by DEXA in Healthy (E) and HS (F) subjects. For all panels n=6 for HS and n=10 for Healthy cohorts. (A,B) Data represented as mean ± SEM. (C) Data represented as median.
Figure 4. Fat traps vitamin E in vitro and in vivo.

(A-C) BDP-α-tocopherol quantification by flow cytometry 30h after incubation using Huh7.5, HepG2C2A, or IHH cells without/with 100µM oleic acid. (D) confocal microscopy of McARH7777 cells without/with 100µM oleic acid (Nile red staining) incubated 2h with 10µM BDP-α-tocopherol (green); (A-C), n=6, representative experiment of 3 independent repeats. (E) mouse liver α-tocopherol (HPLC analyses) using animals fed normal chow/high fat normal vitamin E chow (HFNE); n=5. For all figures: * = p<0.05; ** = p<0.01; *** = p<0.001, Student's t test (Unpaired, Two-tailed). Data represented as mean ± SD.
Figure 5: d_6-, d_3- α-Tocopherol plasma concentrations and redistribution into lipoproteins in 10 healthy vs 6 HS women: dual isotope analyses.

(A) d_3-α-tocopherol 2mg administered IV in healthy (○) or HS subjects (○) over 72h, data represented as mean ± SD. (B-C) area under the curve (AUC) of (B) d_3-α-tocopherol %enrichment and (C) d_6-α-tocopherol %enrichment for VLDL, LDL and HDL from 0 to 8 hours following administration of 2mg d_3- or d_6-α-tocopherol in healthy and HS Subjects. (D-E) d_3-α-Tocopherol %enrichment (D) and d_6-α-tocopherol %enrichment (E) in VLDL in healthy (■) and HS (■) at time 8 hours, data represented as Median. * = p<0.05; ** = p<0.01; *** = p<0.001, Student's t test (Unpaired, Two-tailed). (F,G) AUC_{0-72h} of d_3-α-tocopherol matched to % body fat by DEXA for HS (F) and healthy (G) subjects. For all panel n=6 for HS and n=10 for Healthy cohorts.
Figure 6: Proposed vitamin E (α-tocopherol) physiology in healthy subjects and pathophysiology in subjects with hepato-steatosis.

Left (Healthy subjects): Following oral ingestion, α-tocopherol is transported (via chylomicrons to the thoracic duct and general circulation) to hepatocytes on lipoproteins. Internalized α-tocopherol is specifically recognized and transported through hepatocytes by Tocopherol Transfer Protein (TTP), with release into the Space of Disse and lipoprotein capture, shown as VLDL. Biologically available vitamin E quenches reactive oxygen species (ROS) generated by normal hepatocyte metabolism.

Right (Subjects with hepato-steatosis): Following oral ingestion, some α-tocopherol might be sequestered in intestinal fat (i.e. extrahepatic fat) with a reduction in availability. Remaining available vitamin E is transported to hepatocytes on lipoproteins. Internalized α-tocopherol is diverted into liver fat, acting as a chemical sink, resulting in decreased vitamin E availability within the hepatocyte and a functional local hepatocyte deficiency. Additionally, vitamin E local release into the Space of Disse may be reduced. Less local vitamin E could lead to unquenched reactive oxygen species (ROS) that can damage hepatocytes directly and/or activate pericytes and Kupffer cells, over time producing inflammation, hepatitis, and fibrosis (cirrhosis).
### Table 1: Baseline characteristics

<table>
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<th>(normal Range)</th>
<th>Healthy 5mg intervention</th>
<th>Healthy 2mg intervention</th>
<th>HS 2mg intervention</th>
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<tr>
<td>Number of subject</td>
<td>6</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>24 (21.3-26.1)</td>
<td>24.5 (19.9-26.3)</td>
<td>42.5 (32.9-61.3)</td>
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<tr>
<td>% Liver Fat₁ (MRS)</td>
<td>0.5 (0.3-0.9)</td>
<td>0.6 (0.3-1.1)</td>
<td>12.1 (7.2-23.9)</td>
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<td>% Body Fat₂ (DEXA)</td>
<td>33.9 (26.6-39.4)</td>
<td>32.3 (26.6-39.4)</td>
<td>44.3 (32.9-56.5)</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>26 (19-30)</td>
<td>26 (19-35)</td>
<td>41 (33-53)</td>
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<tr>
<td>AST³ (U/L)</td>
<td>17 (12-27)</td>
<td>17 (14-27)</td>
<td>17 (12-24)</td>
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<tr>
<td>ALT³ (U/L)</td>
<td>11 (8-25)</td>
<td>13 (9-28)</td>
<td>20 (11-37)</td>
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<tr>
<td>Platelet (10^9/L)</td>
<td>244 (218-283)</td>
<td>231 (146-277)</td>
<td>276.5 (221-377)</td>
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<td>FIB-4 Score</td>
<td>0.58 (0.33-0.73)</td>
<td>0.63 (0.3-0.87)</td>
<td>0.60 (0.27-0.91)</td>
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<tr>
<td>TC (mg/dL)</td>
<td>150 (129-181)</td>
<td>150 (133-181)</td>
<td>165 (142-227)</td>
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<tr>
<td>HDL (mg/dL)</td>
<td>62 (49-74)</td>
<td>56 (41-81)</td>
<td>47 (34-68)</td>
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<td>LDL (mg/dL)</td>
<td>73 (58-124)</td>
<td>81 (58-111)</td>
<td>94 (80-145)</td>
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<tr>
<td>TG (mg/dL)</td>
<td>46 (31-96)</td>
<td>70 (37-138)</td>
<td>114.5 (72-181)</td>
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</table>

Demographics of healthy subjects and subjects with hepato-steatosis (HS) (n=6). Intervention groups included the following participants: 5mg healthy (n = 6), 2mg healthy (n = 10) and 2mg HS (n = 6). The medians were calculated from the baseline data of the individuals participating in that group.

1. Liver fat was determined by magnetic resonance spectroscopy.
2. Body fat was determined by dual-energy X-ray absorptiometry whole-body composition analyses (Hologic Discovery QDR; Hologic).
3. Three values for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were obtained 1-4 months prior to and during study. The overall mean for each enzyme is shown. Fibrosis-4 (FIB-4) score was calculated as follows: age ([year] x AST [U/L]) / ((PLT [10⁹/L]) x (ALT [U/L])^(1/2)). Values < 1.45 have a negative predictive value of 90% to exclude advanced fibrosis (30). Data are shown as mean (range).

Normal ranges: BMI (18-26.9), AST (0-32U/L), ALT (0-33U/L), Platelet (173.10⁹-369.10⁹/L), FIB-4 score (<1.45), TC (0-150mg/dL), HDL (40-59mg/dL), LDL (0-129mg/dL), TG (0-149mg/dL). Table Abbreviations: BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; FIB-4, fibrosis-4; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglyceride; U/L, unit per liter.
Table 2. Pharmacokinetic parameters derived from the plasma following oral d3- and IV d6-α-tocopherol administration to healthy (n=10) or HS (n=6) women

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IV d6 α-tocopherol</th>
<th>PO d3 α-tocopherol</th>
<th>p-values</th>
<th>p-values</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Healthy</td>
<td>HS</td>
<td>0.024 ± 0.001</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>Elimination Rate (Ke)</td>
<td>0.020 ± 0.002</td>
<td>0.019 ± 0.002</td>
<td>0.251</td>
<td>0.187</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>Healthy</td>
<td>30.0 ± 1.4</td>
<td>31.5 ± 1.3</td>
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<tr>
<td></td>
<td>HS</td>
<td>36.9 ± 4.4</td>
<td>39.0 ± 5.2</td>
<td>0.210</td>
</tr>
<tr>
<td>Cmax (µM)</td>
<td>Healthy</td>
<td>0.48 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>0.34 ± 0.04</td>
<td>0.20 ± 0.02</td>
<td>0.017</td>
</tr>
<tr>
<td>Tmax (h)(^1)</td>
<td>Healthy</td>
<td>7.7 ± 1.1</td>
<td>11.4 ± 0.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>7.5 ± 0.7</td>
<td>8.5 ± 0.67</td>
<td>0.011</td>
</tr>
<tr>
<td>Fractional absorption, 0-72h(^2)</td>
<td>Healthy</td>
<td>60.6% ± 5.2%</td>
<td>67.5% ± 8.3%</td>
<td>0.537</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>67.5% ± 8.3%</td>
<td>67.5% ± 8.3%</td>
<td>0.537</td>
</tr>
<tr>
<td>AUC(_{0-72}) (µM-h)(^3)</td>
<td>Healthy</td>
<td>17.5 ± 0.95</td>
<td>10.7 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>13.2 ± 1.84</td>
<td>8.6 ± 1.0</td>
<td>0.212</td>
</tr>
<tr>
<td>AUC(_{0-8}) (µM-h)(^3)</td>
<td>Healthy</td>
<td>2.86 ± 0.14</td>
<td>1.19 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>1.96 ± 0.23</td>
<td>0.80 ± 0.09</td>
<td>0.109</td>
</tr>
</tbody>
</table>

(1) C\(_{max}\): Maximal concentration post-nadir for the IV dose
(1) T\(_{max}\): Time of maximum concentration post-nadir for the IV dose.
(2) From the dual isotope method (% absorption = [oral AUC\(_{0-72h}\)/[IV AUC\(_{0-72h}\)] x 100).
(3) AUC\(_{0-72}\) is calculated from the plasma concentrations from 0 to 72 h. AUC\(_{0-8h}\) is calculated from the plasma concentration from 0 to 8h corresponding to the time of C\(_{max}\) for d6-α-tocopherol. C\(_{max}\), maximal concentration; T\(_{max}\), time of maximum concentration, Ke; Constant of elimination.
Table 3. AUC_{0-8} derived from lipoprotein percent d3- and percent d6-α-tocopherol enrichment

<table>
<thead>
<tr>
<th>Lipoprotein Parameters</th>
<th>d6-α-tocopherol</th>
<th>d3-α-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL % enrichment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>24.4 ± 4.9</td>
<td>11.0 ± 2.3</td>
</tr>
<tr>
<td>(AUC (%*h)^1)</td>
<td>9.8 ± 1.1</td>
<td>4.1 ±0.6</td>
</tr>
<tr>
<td>HS</td>
<td>0.016</td>
<td>0.017</td>
</tr>
<tr>
<td>LDL % enrichment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>22.3 ± 4.4</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td>(AUC (%*h)^1)</td>
<td>9.6 ± 1.0</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>HS</td>
<td>0.019</td>
<td>0.027</td>
</tr>
<tr>
<td>HDL % enrichment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>22.2 ± 4.7</td>
<td>6.0 ± 1.3</td>
</tr>
<tr>
<td>(AUC (%*h)^1)</td>
<td>9.9 ± 1.1</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>HS</td>
<td>0.029</td>
<td>0.051</td>
</tr>
</tbody>
</table>

AUC_{0-8} derived from lipoprotein percent d3- and percent d6-α-tocopherol enrichment. AUC_{0-8} is calculated from the lipoprotein percent enrichment, (Mean ± SEM) as described in Methods following 2mg IV d6-α-tocopherol administration in 10 healthy subjects and 6 HS subjects. Statistic: Student's t test (Unpaired, Two-tailed).