

Similar Reduction of Cholesterol-Adjusted Vitamin E Serum Levels in Simple Steatosis and Non-Alcoholic Steatohepatitis

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OBJECTIVES: Reduced vitamin E levels have been reported in patients with non-alcoholic steatohepatitis (NASH), but no conclusive data on patients with simple steatosis (SS) are available. Aim of this study was to investigate the association between serum vitamin E levels and SS.

METHODS: A cohort of 312 patients with cardio-metabolic risk factors was screened for liver steatosis by ultrasonography (US). We reasonably classified as SS patients with US-fatty liver, normal liver function tests (LFTs) and with Cytokeratin 18 < 246 mIU/ml. Liver biopsy was performed in 41 patients with US-fatty liver and persistent elevation of LFTs (> 6 months). Serum cholesterol-adjusted vitamin E (Vit E/chol) levels were measured.

RESULTS: Mean age was 53.9 ± 12.5 years and 38.4% were women. Non-alcoholic fatty liver disease (NAFLD) was detected at US in 244 patients; of those 39 had biopsy-proven NASH and 2 borderline NASH. Vit E/chol was reduced in both SS (3.4 ± 2.0 , $P < 0.001$), and NASH (3.5 ± 2.1 , $P = 0.006$) compared with non-NAFLD patients (4.8 ± 2.0 $\mu\text{mol}/\text{mmol}$ chol). No difference was found between SS and NASH ($P = 0.785$). After excluding patients with NASH, a multivariable logistic regression analysis found that Vit E/chol (odds ratio (OR): 0.716, 95% confidence interval (CI) 0.602–0.851, $P < 0.001$), alanine aminotransferase (ALT, OR: 1.093, 95% CI 1.029–1.161, $P = 0.004$), body mass index (OR: 1.162, 95% CI 1.055–1.279, $P = 0.002$) and metabolic syndrome (OR: 5.725, 95% CI 2.247–14.591, $P < 0.001$) were factors independently associated with the presence of SS.

CONCLUSIONS: Reduced vitamin E serum levels are associated with SS, with a similar reduction between patients with SS and NASH, compared with non-NAFLD patients. Our findings suggest that the potential benefit of vitamin E supplementation should be investigated also in patients with SS.

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Subject Category: Liver

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most frequent and emerging liver disease in the general population.¹ The management of NAFLD has become a major clinical challenge to healthcare practitioners.² The term NAFLD includes different liver clinical conditions ranging from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH), eventually progressing to cirrhosis and hepatocellular carcinoma.³ Mechanisms underlying progression from SS to NASH or cirrhosis remain poorly understood.

Oxidative stress seems to be one of the factors responsible for the occurrence of liver damage and for the progression to cirrhosis.^{4,5} Several markers of oxidative stress have been measured so far in NAFLD patients with mixed results.⁶ Particular attention have been focused on vitamin E, which is an important lipid-soluble vitamin exerting an antioxidant effect acting as free radical scavenger,⁷ and protecting cell

membranes and lipoproteins from lipid peroxidation. Vitamin E is considered a good index of the antioxidant status, as it has been shown to be inversely correlated with markers of oxidative stress.^{8,9}

In the context of NAFLD, vitamin E has been found to be reduced in patients with NASH, and findings from the PIVENS trial¹⁰ suggested that vitamin E supplementation was effective in improving liver histology in patients with NASH. Nevertheless, the association between vitamin E levels and the severity of NASH was not confirmed by other studies.^{6,11–13}

One particular aspect of this problem concerns whether a reduction in the levels of vitamin E may also occur in patients with SS, i.e., before the onset of NASH. So far, no study has explored this issue.

The aim of our study was to examine the association between the serum levels of vitamin E and the presence of SS in a cohort of patients with cardio-metabolic risk factors.

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METHODS

The study included 312 consecutive outpatients presenting with cardio-metabolic risk factors, referring to the Day Service of Internal Medicine at the Umberto I—University Hospital in Rome from February 2011 to September 2013. At baseline, anthropometric data including waist circumference, height and weight were recorded and body mass index (BMI) was calculated for each patient. Routine clinical and biochemical evaluation and ultrasonography (US) examination for liver steatosis were obtained for all patients.

Inclusion criteria were: US evidence of fatty liver (see below), no history of current or past excessive alcohol drinking as defined by an average daily consumption of alcohol > 20 g; negative tests for the presence of hepatitis B surface antigen and antibody to hepatitis C virus. Exclusion criteria were: presence of cirrhosis and other chronic liver diseases, current supplementation with antioxidants or vitamins.

Cardiovascular and metabolic risk factors were defined as follows: arterial hypertension as repeated elevated blood pressure values ($\geq 140/\geq 90$ mm Hg) or taking antihypertensive drugs;¹⁴ diabetes as the presence of symptoms of diabetes and a casual plasma glucose ≥ 200 mg/dl (11.1 mmol/l), or fasting plasma glucose ≥ 126 mg/dl (7.0 mmol/l), or presence of anti-diabetic treatment.¹⁵ Metabolic syndrome (MetS) was classified according to the modified criteria of the Adult Treatment Panel III Expert Panel of the National Cholesterol Education Program.¹⁶

All patients provided written informed consent. The study protocol was approved by the local ethical board of Sapienza University of Rome and was carried out according to the principles of the Declaration of Helsinki.¹⁷

Blood Collection and laboratory analyses. After overnight, fasting and supine rest for at least 10 min, blood was withdrawn from the antecubital vein. Serum was divided into aliquots and stored at -80°C until assays were performed.

Patients underwent routine laboratory analyses including aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), fasting blood glucose, and lipid profile (total cholesterol, low density lipoprotein, high-density lipoprotein, and triglycerides).

Vitamin E. Serum levels of vitamin E (α -tocopherol) were measured by High-Performance Liquid Chromatography.¹⁸ Serum samples (100 μl) were supplemented with tocopheryl acetate (internal standard), deproteinized by the addition of ethanol, and extracted with hexane. Phase separation was achieved by centrifugation. The collected upper phase was evaporated and analyzed using an Agilent 1200 Infinity series High-Performance Liquid Chromatography system equipped with an Eclipse Plus C18 column (4.6 \times 100 mm). Vitamin E serum levels were expressed as ratio between serum α -tocopherol concentration ($\mu\text{mol/l}$) and serum total cholesterol concentration (mmol/l) (Vit E/chol, $\mu\text{mol}/\text{mmol}$), which better expresses the circulating levels of vitamin E.¹⁹

Cytokeratin 18-M30 (CK-18). Serum levels of CK-18 were measured as marker of liver damage with a commercial

immunoassay (Tema Ricerca, Italy) and expressed as mIU/ml, as previously reported.⁵ Intra-assay and inter-assay coefficients were 6 and 7%, respectively.

Assessment of liver steatosis. We performed liver US to assess the presence of steatosis. All US were performed by the same operator who was blinded to laboratory values using a GE VividS6 (GE Healthcare, Wauwatosa, WI) apparatus equipped with a convex 3.5 MHz probe. Liver steatosis was defined according to Hamaguchi criteria based on the presence of abnormally intense, high-level echoes arising from the hepatic parenchyma, liver–kidney difference in echo amplitude, echo penetration into deep portion of the liver and clarity of liver blood vessel structure.^{20,21} Steatosis was assessed semi-quantitatively on a scale of 0–6, defining 4 classes of liver steatosis degree: 0 absent; 1,2 mild; 3,4 moderate; and 5,6 severe.

We reasonably classified as SS patients with US evidence of liver steatosis and normal liver function tests (LFTs, ALT ≤ 35 U/L and gamma-glutamyl transpeptidase ≤ 36 U/L) and with a value of CK-18 < 246 mIU/ml. This value for the diagnosis of NASH was taken from a multicentre validation study previously published by Feldstein *et al.*²² where the value of 246 mIU/ml showed the best combination of sensitivity (75%) and specificity (81%).

Liver histology. Percutaneous US-guided liver biopsy was performed in NAFLD patients with persistent elevation of liver enzymes (> 6 months). Liver biopsy was conducted under conscious sedation using a 16-gauge Klatskin needle. Liver fragments were fixed in buffered formalin for 2–4 h and embedded in paraffin with a melting point of $55\text{--}57^{\circ}\text{C}$. Three- to four- μm sections were cut and stained with hematoxylin and eosin and Masson's trichrome stains. A single pathologist blinded to identity, history, and biochemistry of patients analyzed all the slides. A minimum biopsy specimen length of 15 mm or at least the presence of 10 complete portal tracts was required. Liver biopsy specimens were classified as to "definite NASH" (with a NAFLD activity score (NAS) ≥ 5),²³ "borderline NASH"²⁴ and "not NASH".

Statistical analysis. Categorical variables were reported as counts (percentage); continuous variables were expressed as mean \pm s.d. or median and interquartile range. Normal distribution of parameters was assessed by Kolmogorov–Smirnov test. Student unpaired *t*-test and Pearson correlation analysis were used for normally distributed continuous variables. Appropriate nonparametric tests (Mann–Whitney *U*-test and Spearman rank correlation test) were employed for all the other variables. Group comparisons were performed using Fisher's *F*-test (Analysis of variance) or Kruskal–Wallis test when needed.

After excluding patients with NASH, logistic regression analysis was performed to evaluate the association between SS and Vit E/chol, and after adjustment for MetS, age, female gender, smoking, GGT, ALT, AST, statins, and BMI.

Variables were entered with a forced procedure method (one step) and we used the composite variable of MetS instead of its single components (which were excluded from the model).

All tests were two-tailed and a statistical significance was set at a *P* value of <0.05. Analyses were performed using computer software packages (IBM SPSS Statistics v20.0, Armonk, NY).

RESULTS

Clinical and biochemical characteristics are listed in Table 1. In the whole population, mean age was 53.9 ± 12.5 years and 38.4% were women; 59.2% of patients were affected by MetS. A diagnosis of US liver steatosis was made in 262 (84.0%) of the 312 screened patients. Based on CK-18 values, we excluded 18 patients with US liver steatosis who had normal LFTs but high values of CK-18 (≥ 246 mIU/ml). Thus, 244 patients with steatosis were used for the analysis. Of these, 22.2% had mild, 41.9% moderate and 35.5% severe steatosis at US.

In 41 of the 244 NAFLD patients, we observed a persistent elevation of LFTs. These patients, 22 males and 19 females, underwent liver biopsy, which revealed in 39 cases the presence of NASH and in 2 borderline NASH.

A significant difference between patients with and without SS was observed for BMI, MetS, and diabetes (Table 1). SS patients had significantly higher mean values of fasting plasma glucose, and triglycerides, while high-density lipoprotein cholesterol values were significantly reduced (Table 1). Concerning serum markers of liver damage ALT and GGT were significantly higher in patients with SS compared with those without (Table 1).

Moreover, a progressive higher value of CK-18 was detected from patients without fatty liver, to those with SS and NASH [100.0 (80.0–125.0), 176.0 (145.0–190.0), 266.0 (250.0–284.7) mIU/ml, respectively, $P < 0.001$ NAFLD vs. SS, $P < 0.001$ SS vs. NASH].

Patients with NASH were younger, less affected by arterial hypertension, with a higher prevalence of MetS and a

significant elevation of LFTs (Table 1) compared with NAFLD patients.

Vitamin E levels. To establish normal values of Vit E/cholesterol we included a control group of 20 healthy subjects (HSs, 13 men and 7 women) from the same geographic area. HSs had normal weight and no alterations in lipid profile or LFTs and were not taking any drug or supplement. Differently from the group of non-NAFLD patients, HSs had no cardio-metabolic risk factors. Mean value of serum Vit E/cholesterol in HSs was 5.3 ± 1.5 $\mu\text{mol}/\text{mmol}$ (Figure 1). Subjects with SS showed significantly lower mean values of serum Vit E/cholesterol when

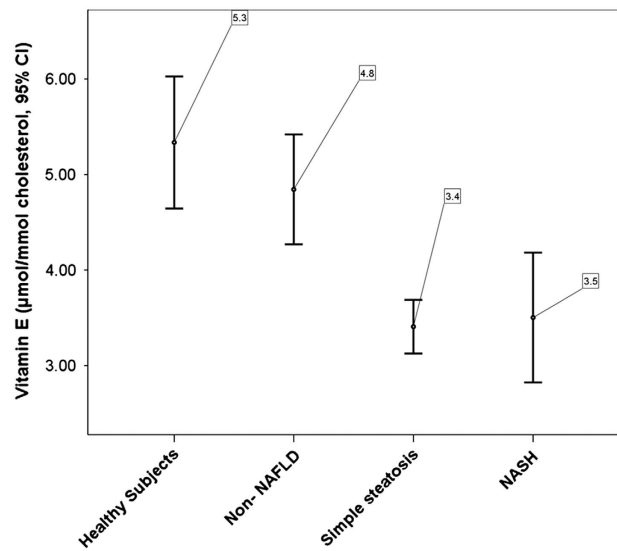


Figure 1 Mean values of vitamin E/cholesterol.

Table 1 Clinical and serological characteristics of patients

	non-NAFLD (n = 50)	Simple steatosis (n = 203)	<i>P</i> value ^a	NASH/borderline NASH (n = 41)	<i>P</i> value ^a
Age (years)	55.0 ± 13.5	55.1 ± 12.1	0.996	47.0 ± 11.5	0.007
Women (%)	36.0	37.4	0.850	46.3	0.392
Smoking (%)	28.0	20.7	0.260	34.1	0.649
Body mass index (kg/m ²)	26.7 ± 3.6	31.5 ± 5.7	<0.001	30.0 ± 4.6	0.126
Arterial hypertension (treated) (%)	68.0	65.5	0.868	39.0	0.007
Diabetes (%)	16.0	30.5	0.026	26.8	0.300
Statins (%)	38.0	35.0	0.742	29.3	0.505
Metabolic syndrome (%)	25.0	69.0	<0.001	53.7	0.005
Fasting plasma glucose (mg/dl)	96.7 ± 22.5	109.3 ± 36.2	0.017	101.7 ± 34.7	0.491
Total cholesterol (mg/dl)	202.6 ± 44.0	200.6 ± 39.4	0.749	192.7 ± 34.7	0.693
LDL-c (mg/dl)	123.2 ± 39.4	119.4 ± 34.0	0.478	114.0 ± 30.1	0.628
HDL-c (mg/dl)	55.7 ± 16.8	48.5 ± 13.7	0.010	49.1 ± 22.4	0.139
Triglycerides (mg/dl)	117.5 ± 66.2	169.9 ± 125.3	0.008	150.0 ± 99.6	0.503
Gamma-glutamyl transpeptidase ^b (U/l)	21.0 (14.0–28.0)	23.0 (17.0–34.0)	0.032	52.0 (34.0–138.5)	<0.001
Alanine aminotransferase (U/l)	20.0 ± 8.1	26.9 ± 9.4	0.031	78.8 ± 39.3	<0.001
Aspartate aminotransferase (U/l)	19.5 ± 5.9	21.4 ± 7.8	0.315	47.9 ± 27.6	<0.001
CK-18 M30 ^b (mIU/ml)	100.0 (80.0–125.0) (min 60.0–max 150)	176.0 (145.0–190.0) (min 51.0–max 245.0)	<0.001	266.0 (250.0–284.7) (min 247–max 308.0)	<0.001

CK-18, cytokeratin 18; HDL-c, high-density lipoprotein; LDL-c, low density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.
^avs. non-NAFLD patients.

^bData are expressed as median and interquartile range.

Table 2 Multiple logistic regression analysis of determinants of simple steatosis

	OR	95% CI		P value
Vitamin E/cholesterol	0.716	0.602	0.851	<0.001
Statins	0.721	0.297	1.752	0.471
Smoking	0.651	0.255	1.664	0.370
Age	0.978	0.944	1.012	0.203
Female	0.955	0.401	2.275	0.917
Body mass index	1.162	1.055	1.279	0.002
Aspartate aminotransferase	1.001	0.938	1.068	0.985
Alanine aminotransferase	1.093	1.029	1.161	0.004
Gamma-glutamyl transpeptidase	0.995	0.975	1.016	0.654
Metabolic syndrome	5.725	2.247	14.591	<0.001
Constant	0.058			0.123

CI, confidence interval; OR, odds ratio.

compared with HSs ($P < 0.001$) and to non-NAFLD patients ($P < 0.001$) (Figure 1). Similarly, reduced Vit E/chol values were obtained in the subgroup of 41 NASH/borderline NASH patients ($P = 0.006$ vs. NAFLD). No difference was found between patients with SS and those with NASH/borderline NASH ($P = 0.785$). Vit E/chol and CK-18 were inversely correlated (Rs: -0.185 , $P = 0.004$).

Determinants of simple steatosis. To assess the association between SS and Vit E/chol, we performed a multivariable logistic regression analysis, after excluding the 41 patients with NASH/borderline NASH. We found that Vit E/chol (odds ratio (OR): 0.716, $P < 0.001$), ALT (OR: 1.093, $P = 0.004$), BMI (OR: 1.162, $P = 0.002$) and MetS (OR: 5.725, $P < 0.001$) were factors independently associated with the presence of SS (Table 2).

DISCUSSION

We provide evidence that reduced serum vitamin E levels are independently associated with the presence of SS in a cohort of patients with cardio-metabolic risk factors.

Clinical studies so far have analyzed serum vitamin E levels of patients with NASH in comparison with HSs,^{6,11–13} and no data on patients with SS are available.

We reasonably classified as SS patients with presence of fatty liver at US, normal LFTs and low CK-18 (< 246 mIU/ml). Although this classification is not completely accurate, it reflects common clinical practice, which does not allow to perform liver biopsy in all patients with US evidence of liver steatosis and normal liver biomarkers. In fact, liver biopsy should be considered only for NAFLD patients at increased risk to have NASH, and who would most benefit from histologic diagnosis to guide therapeutic approach.¹⁰ Moreover, in asymptomatic patients with hepatic steatosis detected on imaging and normal liver biochemistry, a liver biopsy cannot be recommended.¹⁰

A peculiarity of our study is also in the presence of a control group of patients with cardio-metabolic risk factors but without liver steatosis at US. This allows a better analysis of the association between the presence of NAFLD and the reduction of vitamin E levels. In fact, NAFLD is strongly associated with MetS,^{25,26} and it could be possible

that the reduced Vit E/chol in NAFLD mirrors the coexistence of MetS.

We found that serum vitamin E levels, along with ALT, BMI, and MetS, were factors associated with the presence of SS. The independent association between SS and Vit E/chol suggests that other mechanisms than MetS may account for the inverse association between liver steatosis and Vit E/chol.

An interesting finding of our study is the similar reduction of Vit E/chol levels in patients with SS and in those with biopsy-proven NASH, compared with NAFLD patients.

This comparable reduction of vitamin E is intriguing, but of difficult pathophysiologic explanation, given the difference in the natural history of SS and NASH. Our findings suggest that patients with NAFLD may have an early enhanced systemic oxidative stress, which leads to a reduction in natural antioxidant pool, including vitamin E. However, in the present study we cannot establish whether the imbalance of the oxidant status may directly contribute to the progression from SS to NASH.

Moreover, differently from previous studies, we expressed values of vitamin E after adjustment for total serum cholesterol. This is important, since the plasma concentration of fat-soluble vitamins, such as vitamin E, varies with the amount of plasmatic lipids, and thus requires lipid standardization, especially in conditions likely to raise serum cholesterol.¹⁹

The high percentage of NASH at liver biopsy is only partially surprising. In fact, the reported rate of biopsy-proven NASH is highly variable, and can reach 40% in unselected patients without known liver disease, as reported in a Greek study on 600 autopsies.¹ In our study, patients who underwent liver biopsy had all persistent elevation of LFTs for at least 6 months, and more than 50% suffered from MetS with a mean BMI of 30 kg/m²; all these conditions are strictly associated with the presence of NASH.^{27,28} This finding supports the high prevalence of NASH in high-risk patients with cardio-metabolic disorders.

Our study has a number of limitations. We detected fatty liver by US, a qualitative method, inadequate to quantify $< 30\%$ liver fat content. Nevertheless, US is safe and inexpensive and uses standardized criteria to define liver steatosis.²¹ Moreover, the separation of SS from NASH is difficult with non-invasive methods, and the presence of NASH is underestimated given the possibility to perform liver biopsy only in patients with persistent abnormalities in LFTs. Serum CK-18 levels were measured to reduce this potential classification bias. However, even if the cutoff of CK-18 used in our study for the discrimination of patients with SS from those with NASH derives from a multicentre study, it is not currently recommended for the non-invasive diagnosis of NASH.

The study may have some implications. Considering that low levels of vitamin E are associated with increased cardiovascular risk,^{29–32} our findings may further support the role of NAFLD as cardiovascular risk factor. In this context, statins could be a promising therapeutic approach, as they increase serum vitamin E,^{8,33,34} and improve liver steatosis.³⁵

In conclusion, we found that patients with SS and those with biopsy-proven NASH have a similar reduction of serum levels of Vit E/chol compared with non-NAFLD patients, suggesting

that oxidative stress imbalance could occur in the early stages of fatty liver disease.

CONFLICT OF INTEREST

Guarantor of the article: Francesco Angelico, MD.

Specific author contributions: Pastori Daniele: study concept and design. Analysis and interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted. Baratta Francesco: study concept and design. Acquisition, analysis and interpretation of data. Approved the final draft submitted. Carnevale Roberto: study concept and design. Acquisition, analysis and interpretation of data. Approved the final draft submitted. Cangemi Roberto: interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted. Del Ben Maria: acquisition, analysis and interpretation of data. Critical revision of the manuscript. Approved the final draft submitted. Polimeni Licia: acquisition, analysis and interpretation of data. Critical revision of the manuscript. Approved the final draft submitted. Labbadia Giancarlo: acquisition, analysis and interpretation of data. Critical revision of the manuscript. Approved the final draft submitted. Nocella Cristina: interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted. Scardella Laura: interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted. Pani Arianna: Interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted. Pignatelli Pasquale: interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted. Francesco Violi: interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted. Francesco Angelico: interpretation of data. Drafting of the manuscript. Critical revision of the manuscript. Approved the final draft submitted.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Vitamin E serum levels are reduced in patients with biopsy-proven NASH.
- ✓ Vitamin E supplementation is currently recommended for non-diabetic patients with NASH.

WHAT IS NEW HERE

- ✓ This is the first report on the association between reduced serum Vitamin E levels and the presence of simple steatosis.
- ✓ Patients with simple steatosis and NASH showed a similar reduction of serum cholesterol-adjusted levels of Vitamin E.

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