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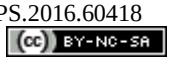
ISSN 2231-3354 

Effects of adding tocotrienol-tocopherol mixed fraction and vitamin C supplementation on coronary risk biomarkers in patients with hypercholesterolaemia with moderate coronary risk

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ABSTRACT

This study was a prospective clinical trial to investigate the effects of adding combined tocotrienol-tocopherol mixed fraction **(**TTMF) and vitamin C (TTMF+C) supplementation on coronary biomarkers in non-statin and statin treated patients with hypercholesterolaemia (HC) with moderate coronary risk. A total of 35 patients were randomised at baseline into one of two groups, (G1) TTMF+C (320mg TTMF plus 500mg vitamin C) alone daily and (G2) TTMF+C (320mg TTMF plus 500mg vitamin C) plus atorvastatin 10 mg daily. The entire supplementation were taken for 12 months. Fasting serum samples were taken at baseline, 2weeks, 3months, 6months and 12months post-randomisation and analysed for inflammatory biomarkers; high sensitivity C-reactive protein (hsCRP) and interleukin-6 (IL6). Combination of TTMF and vitamin C supplementation leads to neutral effects on lipid profiles and inflammation; with no added benefit in statin-treated HC patients with moderate coronary risk. This neutral effects may be attributed to the tocopherol composition in TTMF which could possibly attenuate any potential beneficial effects of tocotrienols. Clinical studies using pure tocotrienols in the absence of tocopherols would further confirm this.

INTRODUCTION

Hypercholesterolaemia (HC) is one of the major risk factor for the development of atherosclerosis and atherosclerosis-related complications such as coronary artery disease (CAD) and stroke (Schleicher and Friess, 2007), however the high low-density lipoprotein cholesterol (LDL-c) is a major risk factor for CAD, and it is implicated in the progression of plaque formation, endothelial dysfunction and oxidative stress in CAD (Schleicher and Friess, 2007; Stamler *et al.,* 2000; Vogel *et al.,* 1998). Atherosclerosis has been well established to be a chronic inflammatory process involving endothelial dysfunction due to risk factors such as smoking, hypertension, and diabetes; oxidation of LDL-c to form ox-LDL, engulfment of ox-LDL by macrophages to form foam cells and subsequent formation of atherosclerotic plagues (Salonen *et al.,* 2003; Brown *et al.,* 2001).There is clear evidence that the monocyte-derived macrophages, T lymphocytes and a large number of proinflammatory cytokines such as IL6 play a key role in all the phases of atherosclerosis (Salonen *et al.,* 2003; Brown *et al.,* 2001). However, C-reactive protein is an acute-phase reactant that markedly increases during an inflammatory response. C-reactive protein levels have been helpful for decades in monitoring many diseases. Another use for this old test has gained momentum as a result of observations that minor elevations of

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C-reactive protein are predictive of cardiovascular events in patients with CAD (Liao, 2002). Several strategies in the management of patients with CAD and elevated cholesterol levels have been proposed. They include (1) raising HDL-c with weight loss, exercise, diet and smoking cessation (life style modification); (2) increasing HDL-c to LDL-c ratio with statins; (3) Inhibiting LDL-c oxidation and atherogenesis with antioxidants; and 4- improving both the lipid profile and antioxidant status with a combination of a statin, and antioxidant therapy (Madamanchi *et al.*, 2005).

Statins are the most effective therapy in reducing cholesterol levels thereby inhibiting the progression of atherosclerosis and reducing the incidence of CHD (Upadhya *et al.*, 2001). Statins have been shown to improve the endothelial relaxation, an antihypertensive effect and beneficial action on cardiac function beside than cholesterol-lowering effects (Haverkate *et al.*, 1997; Upadhya *et al.*, 2001).

A variety of studies suggested that antioxidant-rich diet may reduce the risk of developing atherosclerosis or slow its progression (Hansson, 2001). In vitro studies have shown that antioxidants such as Vitamin E (tocotrienol) and vitamin C reduce lipid peroxidation and free radical damage, which are important intermediaries in the pathogenesis of atherosclerosis (Hansson, 2001; Cheung *et al.*, 2001; Collaborative Group, 2001). In addition, a low dietary intake of antioxidant vitamins was associated with greater rates of progression of atherosclerosis in observational studies (Kritchevsky *et al.*, 1995).

Vitamin C is the main water soluble antioxidant in human plasma and is hypothesized to have protective role in the development of atherosclerotic heart disease (Kritchevsky *et al.*, 1995; Steinberg and Witztum, 2002).

Tocotrienols are members of the vitamin E family. An essential nutrient for the body, vitamin E is made up of four tocopherols (alpha, beta, gamma, delta) and four tocotrienols (alpha, beta, gamma, delta), (Brigelius FR and Traber MG, 1999). However, tocopherol-tocotrienol- mixed fraction (TTMF) is a vitamin E product for supplementation and comprise of all the eight compounds: alpha, beta, gamma and delta-tocotrienols and tocopherols (Food and Nutrition Board, 2000; Morris and Carson, 2003). Tocotrienol, (TCT), a class of Vitamin E analogues, is believed to modulate several mechanisms associated with atherosclerosis and coronary heart disease (CHD), (Morris and Carson, 2003). Some studies have also reported tocotrienols as having strong antioxidants activity due to their free radical scavenging abilities that is associated with lowering tumor formation, DNA and cell damage (Food and Nutrition Board, 2000; Morris and Carson , 2003).

The objectives of this clinical trial study were to investigate the effects of adding combined TTMF and vitamin C (TTMF+C) supplementation on coronary biomarkers in non-statin and statin treated patients with hypercholesterolaemia (HC) with moderate coronary risk.

MATERIALS AND METHODOLOGY

This study was a prospective study clinical trial involving HC patients in the moderate risk category. Thirty five patients categorized as moderate category according to the National Cholesterol Education Program Adult Panel Treatment III (NCEP ATP III) were recruited from the Specialists Clinics of a Teaching Institution and from the health screening programme. Written informed consent and Institutional Ethical Committee approval were obtained prior to commencement of intervention study. Patients were selected using the inclusion and exclusion criteria to determine their eligibility for the clinical trial (CT). The inclusion criteria were: Males age 30- 65 years or post menopausal women (≥ 6 months post- menopausal), HC defined as LDL-c : 3.4 - 4.9 mmol/L, Fasting Triglyceride (TG) ≤ 4.5 mmol/L, and ≥ 2 additional risk factor and Framingham 10 years coronary risk scoring < 20%. While the exclusion criteria were: CAD/Peripheral vascular disease (PVD) positive, Fasting Triglyceride ≥ 4.5 mmol/L, Severe obesity (BMI ≥ 35kg/m^2), Diabetes mellitus type 1 and 2 , Uncontrolled hypertension (DBP > 105mm Hg), Secondary hyperlipidemia (e.g. hypothyroidism, renal disease (serum creatinine >110 umol/L) and liver diseases, Pre-menopausal women and those taking oral oestrogen therapy, Regular intake of anti-oxidant and other drugs having anti oxidative properties, Chronic inflammatory disorder and limited mobility, Severe disease that shortens life expectancy (eg: malignancy), and contraindications or adverse reaction to statins. Study design: Newly diagnosed patients who fulfilled the set criteria were randomized straight away while patients who were already on lipid lowering treatment or antioxidant underwent a 4weeks washout period prior to randomization. The patients were randomised to either one of two groups according to the study design: group 1 (subjects given TTMF+C (320mg TTMF plus 500mg vitamin C per day up to 12 months) and group 2 (subjects receiving TTMF+C (320mg TTMF plus 500mg vitamin C) daily plus atorvastatin 10 mg daily up to 12 months). TTMF was supplied by Golden Hope Bioganic Sdn. Bhd., Malaysia with the following composition: alpha tocopherol (44mg), alpha tocotrienol (118.48%), beta tocotrienol (9.04%), gamma tocotrienol (117.28%), delta tocotrienol (75.20%) and palm super olein (128mg). Commercially available Vitamin C was used (Flavettes®, ECM Pharma Sdn. Bhd., Malaysia). According to the above study design, consultation had been given in baseline "BL", 2weeks time, 3 months, 6 months, and 12 months. All patients received dietary counselling within third month (3/12) of entry into study. Fasting blood samplezs were collected on every visit at BL, 2 weeks, 3 months, 6 months, 9 months, and 12 months. During each visit, 20ml of fasting blood sample were taken, and serum was separated by centrifugation at 4500 rpm for 10 minutes. The serum was aliquoted into fresh micro centrifuge tube (1.5 ml graduated camblab, micro tubes, England) and kept in -80°c freezer until analysis. Moreover, waist circumference, waist hip ratio and body mass index were measured during each visit.

Biochemical Analysis

Serum samples were analyzed for biochemical tests – fasting serum lipids (FSL) and fasting plasma glucose (FPG) by an automated analyser Cobas Integra 400 PLUS (Roche Diagnostics, Switzerland). Biomarkers of Inflammation were analysed as follows:

1. High sensitivity C-reactive protein (hsCRP), was analysed by immunoturbidimetry method on an automated analyser Cobas Integra 400 PLUS (Roche diagnostic, Switzerland).
2. Interleukin-6 (IL6) was analyzed by immunoturbidimetry method on the automated immunoassay system Immulite 1000 analyser (Medical System, Genova, Italy).
3. Tumor Necrosis Factor – alpha (TNF-α) was analyzed by immunoturbidimetry method on the automated immunoassay system Immulite 1000 analyser (Medical System, Genova, Italy).

Statistical analysis

All data were analyzed by SPSS 12.0.1 programme (Chicago, USA). The level of the significance of the result was taken at $p<0.05$. Kolmogorov Smirnov test was done to determine the distribution of data. Parametric and parametric statistical analysis were used for normal and non-normal distribution of data respectively. Student's t-test or Mann-Whitney test was used for variables with normal or non-normal distribution respectively for between group differences. Within group pre and post treatment differences for each variable were analysed by paired t-test or Wilcoxon matched-test for those variables with normal distribution and non-normal distribution respectively. Normality was determined using Kolmogrov-Smirnov test. P-value of <0.05 was taken as significant. The statistical analyses was performed on the Statistical Package for Social Sciences (SPSS version 12.0.1) software.

RESULTS

The baseline characteristics of our HC patients in the moderate risk category, from groups 1 (G1) and group 2 (G2) are shown in table 1. The data showed that subjects in both groups were matched for all baseline characteristics except for LDL-c ($p<0.05$). TC showed reduction trends for G1, (figure 1). Statistically there were significant reductions in TC in G1 at 2 weeks and 3 months ($p<0.0001$), 6 months ($p<0.0001$) and 12 months ($p<0.0001$) compared to baseline. There were no significant differences in TC levels for G2 (figure1).

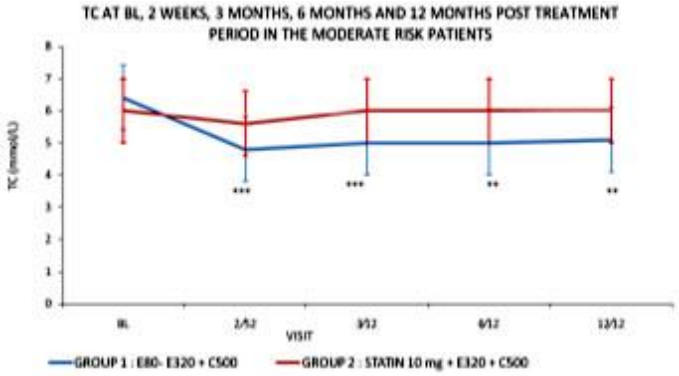

Fig. 1: Total cholesterol concentration according to visits., **p<0.001, ***p<0.0001 compared to Baseline., Data are expressed as Mean ± SEM., BL= baseline, 2/52 = 2 weeks, 3/12 = 3 months, 6/12 = 6 months, 12/12 = 12 months.

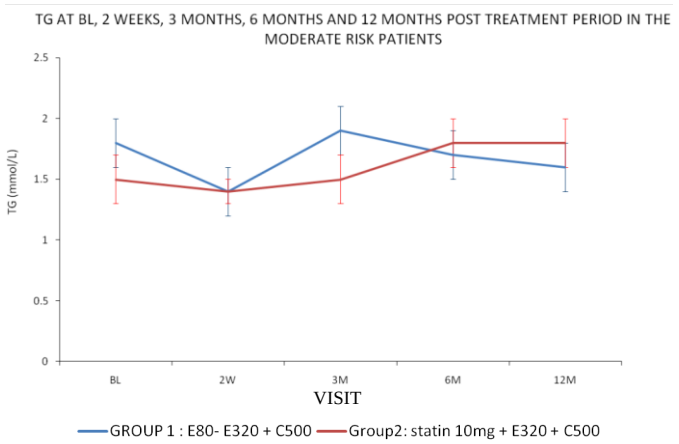

Fig. 2: Total triglyceride concentration according to visits. Data are expressed as Mean ± SEM

Table 1: Baseline characteristics of HC patients in moderate risk category.

Parameters	G1 (n= 21)	G2 (n=14)	All (n=35)	*P* value
[a]Age (years)	51.3 ± 9.0	53.2 ± 8.4	52.1 ± 8.7	NS
[b]Gender (Male/Female)(%)	16/5 (76.2:23.8)	11/3 (78.6:21.4)	27/8 (77.1: 22.9)	NS
[a]Waist circumference (cm)	90.4 ± 10.5	89.3 ± 8.9	89.9 ± 9.7	NS
[a]Waist Hip Ratio	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	NS
[a]BodyMass Index (kg/m2)	27.7 ± 2.9	27.3 ± 3.2	27.5 ± 3.0	NS
[b]Current smoker (%)	23.8	21.4	22.9	NS
[a]Systolic BP (mm Hg)	131.0 ± 14.8	134.1± 18.5	132.2 ± 16.1	NS
[a]Diastolic BP (mm Hg)	78.1 ± 8.2	81.4 ± 17.6	79.4 ±10.0	NS
TC (mmol/L)	6.1 ± 0.5	6.4 ± 0.9	6.2 ± 0.8	NS
TG (mmol/L)	1.7 ± 0.9	1.6 ± 0.7	1.7 ± 0.8	NS
LDL-c (mmol/L)	3.9 ± 0.4	4.5 ± 0.9	4.2 ± 0.7	<0.05
HDL-c (mmol/L)	1.3 ± 0.3	1.2 ± 0.2	1.2 ± 0.3	NS

Data are expressed as [a] mean ± SD or [b] as proportion (%)., BP: blood pressure, TC: Total Cholesterol, TG: Triglycerides, LDL-c: low density lipoprotein cholesterol, HDL-c: high density lipoprotein cholesterol, NS: Not significant.

Meanwhile, figure 2 shows there were no significant differences in TG levels between visits 2 weeks, 3 months, 6 months, and 12 months compare to baseline, in both G1 and G2. Same with results of HDL-c and LDL-c (G2) as showed in figure 3, 4 respectively, there were no significant differences in HDL-c levels between visits 2 weeks, 3 months, 6 months and 12 months compare to baseline G2, while in G1 there were significant differences in LDL-c concentration level between visit 2 weeks, 3 months, 6 months and 12 months compare to baseline, which p<0.0001 for group 2(figure 4).

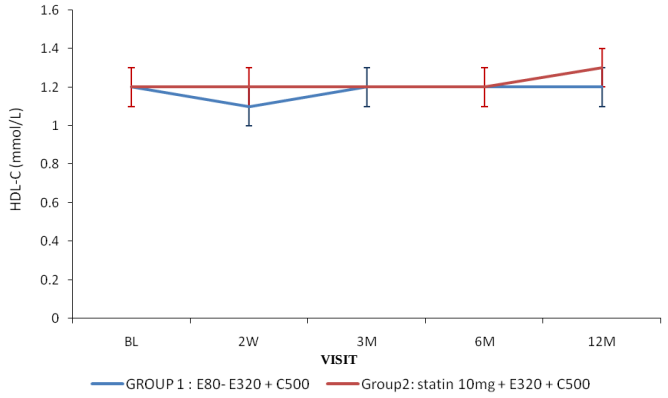

Fig. 3: HDL-c Concentration at BL, 2 weeks, 3 months, 6 months and 12 months post treatment in moderate risk patients.

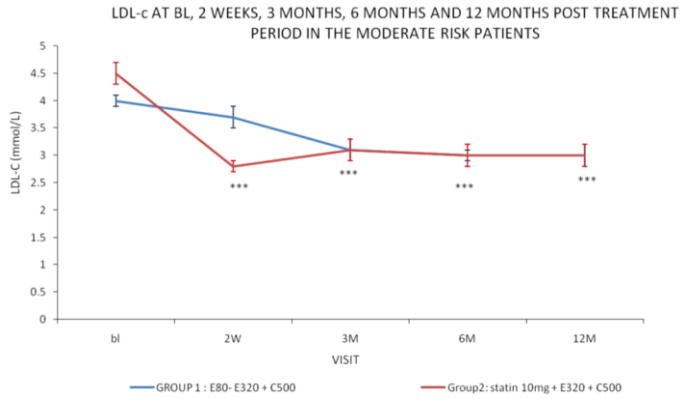

Fig. 4: Low density lipoprotein-cholesterol concentration level. ***p<0.0001 compared to Baseline

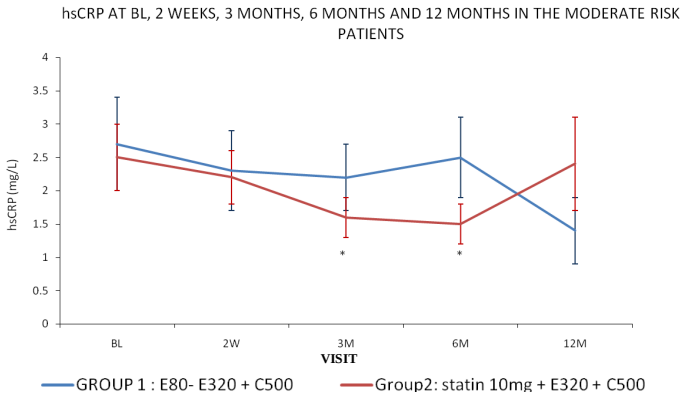

Fig. 5 High sensitivity C-reactive protein (hsCRP) concentration level. *p<0.05 compared to Baseline

Figure 5 shows the absolute value of hsCRP, there were significant differences between 3 months and 6 months this compare to baseline in G 2. Meanwhile figures 6 and 7 show the absolute value for IL6, and TNF-α respectively. There were no significant differences of percentage change (delta 2 weeks, 3monhs, 6 months and 12 months) between the two treatment groups.

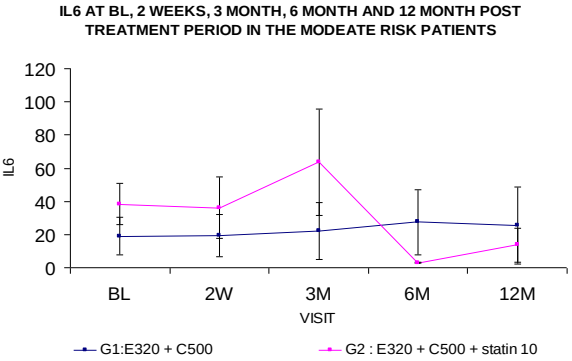

Fig. 6. Interleukin-6 (IL6) concentration level (pg/mL). ***** p<0.05 compared to baseline.

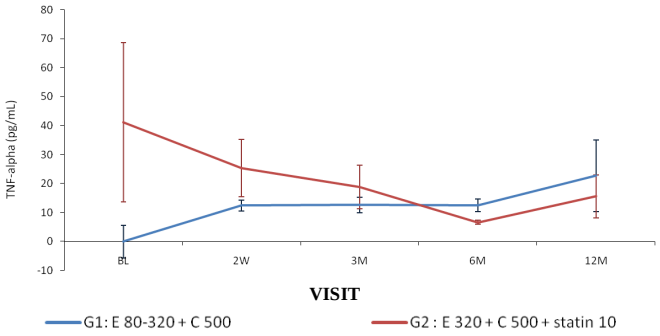

Fig. 7: Tumor necrosis factor-alpha (TNF-α) concentration level at BL, 2 weeks, 3 months, 6 months and 12 months in the moderate risk patients.

DISCUSSION

Two antioxidants have been used in this study; Vitamin C which is a potent scavenger of superoxide radicals and TTMF which is the major peroxyl radical scavenger in biological lipid phases and both their antioxidant action has been ascribed to their ability to chemically act as a lipid-based free radical chain-breaking molecules, thereby inhibiting lipid peroxidation and protecting the organism against oxidative damage. Indeed, the interaction between vitamins C and E in the antioxidant defense of biochemical systems is well established because vitamin C can reduce tocopheroxyl radicals directly or indirectly and thus support the antioxidant activity of vitamin E (Dhalla *et al.*, 2000).

The vascular effect of antioxidants has been observed *in vitro* and in animal models of atherosclerosis (Chen *et al.*, 2001; Wu *et al.*, 2006). However, data on long-term vascular impact of antioxidant supplementation in humans are limited and controversial. Moreover several prospective randomized controlled clinical trials such as Cambridge Heart Antioxidant Study, Secondary Prevention with Antioxidants of Cardiovascular disease

in end-stage renal disease study and cholesterol lowering atherosclerosis study reported that the administration of antioxidants reduced the risk of cardiovascular disease (Stephens *et al.,* 1996; Boaz *et al.,* 2000). Nevertheless, subsequent large interventional studies do not support a benefit from antioxidant supplementation (Brown *et al.,* 2001; Yusuf *et al.,* 2000; Collaborative Group, 2001). These clinical trials have demonstrated that vitamin E alone or in combination with vitamin C has no effect on the risk of death or prevention of cardiovascular disease. Moreover, a dose-response meta-analysis has shown that high-dosage vitamin E supplementation was associated with a small but statistically significant increased risk for mortality (Miller *et al.,* 2005). The lack of benefit seen in these clinical trials does not disprove the central role of oxidative stress in atherosclerosis and justify investigating the overall clinical impact of antioxidant treatment.

HC patients can be treated by drug such as statin which will inhibit the synthesis of cholesterol. Marked reductions in elevated cholesterol levels have been shown to improve endothelial function in HC adults (Leung *et al.,* 1993). Moreover, many studies showed that the combination of cholesterol-lowering and antioxidant therapies is to be synergistic in improving endothelial function (Neunteufl *et al*., 1998; LIPID Study Group, 1998).

According to our study, statistically there were significant reductions of TC from period of 2 weeks and 3 months, $p<0.0001$, 6 months and 12 months, $p< 0.001$ when compare to baseline for group 1 (Fig. 1). As for group 2 there were no significant differences for TC when compare to baseline, the graph did not show any reduction. Actually one would expect a reduction in G2 rather than G1 due to statin treatment but anyhow this was with little effect on the final study results. Beside that there were no significant differences in triglyceride (TG) levels between visits 2 weeks, 3 months, 6 months and 12 months compared to baseline (Fig 2).

Current study showed no significant differences of HDL-c levels among all visits when compared to baseline (Fig.3), while LDL-c concentration level between visit 2 weeks ($p<0.0001$), 3 months ($p<0.0001$), 6 months ($p<0.0001$) and 12 months ($p<0.0001$) compare to baseline for G2 also there were significant differences (Fig 4). This suggests that statin treatment has an effect on lowering the LDL level and vitamin E and C combination has neutral effects on LDL. Elevated blood levels of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) have been shown to be risk factors for coronary heart disease (CHD) incidence and total mortality in middle-aged individuals in many prospective studies (Stamler *et al.,* 2000).

Current study showed the hsCRP levels were significant differences between 3 months and 6 months compared to baseline in G2 ($p<0.05$) (Fig.5). Several groups of researchers have proved that statins have a wide range of biologic effects in addition to lipid lowering, including reductions in the levels of C-reactive protein (CRP), a phenomenon commonly termed a "pleiotropic effect" , (Topol, 2004). They have also showed that CRP levels were 30 to 40 percent lower after intensive statin therapy (Topol, 2004). In the present study, we did not observe significant changes in inflammatory markers such as IL-6 and TNF-α in subject received antioxidant supplementation. These findings emphasize a previously published data which have shown that pathophysiologic mechanism of antioxidants action is independent and even subsequent large interventional studies do not support a benefit from antioxidant supplementation (Yusuf *et al.,* 2000, Miller *et al.,* 2005), so that the precise mechanism for antioxidant action on the vasculature remains to be elucidated.

Our study has several limitations. First, the present study contains relatively small number of participants and larger studies are required to establish the beneficial vascular effect of antioxidant supplementation. Second, we did not measure plasma levels of the antioxidants which would have added the information regarding treatment compliance and would have elucidated the pathophysiology for vascular action of antioxidants. Furthermore, since the present study has focused on HC patients in moderate risk category, the application of our findings to other patient populations remains uncertain.

CONCLUSIONS

This study showed that the TTMF+C (320mg TTMF plus 500mg vitamin C combination in statin-treated moderate risk patients does not significantly improve the lipid and inflammatory status and leads to neutral effects on the markers of inflammation, lipids, and thrombogenic markers in HC patients in moderate risk category.

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COMPETING INTERESTS

The authors declare that they have no conflict interest.

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