

## Supplemented $\alpha$ -Tocopherol Apparently Does Not Enter the Plaque Compartment

### *In Response:*

Kontush and colleagues claim that vitamin E is not deficient in advanced atherosclerotic plaques of our patient cohort.<sup>1</sup> They suggest that our conclusion should not be based on the comparison of vitamin E/cholesterol ratios in plaque versus control plasma nor on the use of  $7\beta$ hydroxy-cholesterol/vitamin E ratios.

As we pointed out in our study aimed at understanding the striking differences of vitamin E effects in experimental and clinical studies, this is the first report on lipid peroxidation, colloquially oxidative stress, and vitamin E tenure in patients with advanced atherosclerosis at both plasma and tissue level. Lipid peroxidation and vitamin E are strictly linked, thus the simultaneous assay of their levels should show the current free radical flux *in vivo* and the related consumption of protective antioxidants. With regard to our findings, the plasma level of  $7\beta$ hydroxy-cholesterol, a marker of lipid peroxidation, is significantly higher in patients with carotid atherosclerosis compared with healthy subjects, whereas plasma vitamin E concentration is reduced by  $\approx 50\%$ . This pattern resembles the kinetics of LDL oxidation *in vitro*, which shows the rise of lipid peroxidation products after a large consumption of vitamin E carried in the LDL molecule.<sup>2,3</sup> Thus, vitamin E is unambiguously reduced in the plasma of our patients with advanced carotid atherosclerosis. On the other hand, the analysis at tissue level showed increased oxysterols in plaque compared with normal vessels, but inconsistent differences were found in vitamin E levels between plaque and normal vessels. Because there is no "normal" plaque to serve as a reference, we used normal vessels and plasma considered as compartments. In addition, we chose the ratio  $7\beta$ hydroxy-cholesterol/vitamin E to have further details of the relative amounts in these compartments, and we measured a 200-fold gradient of  $7\beta$ hydroxy-cholesterol/vitamin E ratio between plaque and plasma compartments. Therefore, any antioxidant therapy should affect this gradient. Consistent with the oxidative stress/antioxidant imbalance in patients with carotid atherosclerosis, we found that lipid peroxidation and antioxidant status in plasma were affected positively by vitamin E supplementation. The novel information which emerged from our study is that vitamin E supplementation does not affect the levels of oxysterols and vitamin E in the plaque. These findings suggest that supplemental vitamin E did not reach the carotid plaque, which might be because of the inadequate dosage and/or period of supplementation or on the involvement of unknown mechanisms. Therefore, a cautionary note should be sounded with the conclusion by Kontush that we "have consistently documented the absence of a deficit of vitamin E in human plaque tissue, even at advanced stages of atherosclerosis." In fact, the absence of a deficit implies that the "optimal" vitamin E concentration in the plaque has been well established, and that this value coincides with the value we measured. Thus, the only conclusion that can be drawn is that vitamin E is not absent in the plaque, and the amount present is not adequate to counteract the lipid peroxidation in the plaque. We believe that turnover studies *in vivo* with deuterium-labeled vitamin E might be useful to clarify the vitamin E kinetics in relation to plaque.

It is the opinion of Kontush et al that the decrease in plasma vitamin E levels reported in our population has not been observed consistently. However, it should be remembered that atherosclerosis, as a ubiquitous vessel lesion in CVD, displays different clinical presentations, ie, coronary, peripheral, and cerebral. These varied clinical manifestations of atherosclerosis are still unresolved and require further research before any conclusions can be made, including in the extreme situation of advanced carotid disease without coronary disease, or coronary multivessel involvement with normal carotids. In the population with carotid atherosclerosis reported in the study, we found a mean vitamin E/cholesterol ratio of  $3.05 \mu\text{mol}/\text{mmol}$ . In contrast, we did not find vitamin E depletion in patients undergoing primary percutaneous transluminal coronary angioplasty (PTCA), who showed a mean plasma vitamin E/cholesterol ratio of  $6.3 \mu\text{mol}/\text{mmol}$  (unpublished data). Cherubini et al<sup>4</sup>

reported a significant reduction of plasma vitamin E/cholesterol ratio in elderly patients with moderate carotid atherosclerosis compared with the control population ( $3.5$  versus  $4.8 \mu\text{mol}/\text{mol}$ ,  $P < 0.001$ ). Iannuzzi et al<sup>5</sup> found that the ratio of plasma vitamin E to plasma cholesterol was inversely related to the presence of plaque at the carotid bifurcation, with a significant linear trend. These data could be relevant at the clinical level because vitamin E could represent a new noninvasive marker of carotid atherosclerosis. However, this possibility is limited by some uncertainty regarding the reference value of plasma vitamin E in healthy subjects, which, as reported in the table by Kontush, varies from  $2.3$  to  $6.3 \mu\text{mol}/\text{mmol}$  cholesterol. One possible explanation accounting for the wide range of plasma vitamin E concentration in healthy subjects could be a selection bias concerning control subjects. In the study of coronary heart disease reported in Kontush's table (ref. 15), the lipid-adjusted value of vitamin E was  $2.8 \mu\text{mol}/\text{mmol}$  in controls. This study<sup>6</sup> should not be considered in the evaluation of the normal range of vitamin E because hyperlipidemia, a major risk factor for atherosclerosis, was included in the enrollment of healthy subjects. Thus, studies specifically addressed to the current areas of uncertainty surrounding the reference plasma concentration of vitamin E are needed.

We agree with Kontush et al that vitamin E, like any other antioxidant, can act as a prooxidant under favorable conditions. Stocker provided evidence that vitamin E can induce LDL oxidation *in vitro*,<sup>7</sup> and we used ascorbic acid as a recycler in a Fenton reaction to obtain a fast LDL oxidation for radiolabeling.<sup>8</sup>

However, these data cannot be readily transferred to the complex matrices of the living organism. The prooxidative effect of vitamin E is mechanistically possible, but the antioxidant action of vitamin E is supported by decades of articles and books based on the chemistry of its chain-breaking antioxidant properties. Although the results of several antioxidant supplementation trials have not been conclusive about its benefits, it has been well established that supplemental vitamin E is able to reduce markers of lipid peroxidation *in vivo*,<sup>9-11</sup> but there is no evidence of a hypothetical enhanced lipid peroxidation *in vivo* after vitamin E supplementation. In addition, several experimental reports have demonstrated the inhibition of atherosclerosis in animal models, but, to the best of our knowledge, there is no data available that shows an acceleration of atherosclerosis by vitamin E in animal models. Going from the bench to the bedside, if vitamin E worked as a prooxidant *in vivo*, we could introduce innovative therapeutic strategies aimed at reducing vitamin E content in the body. Are there substantial data to allow that?

The matter of the source and the species of free radicals that are responsible for LDL oxidation *in vivo* is speculative. The pathway of hypochlorite and peroxynitrite *in vivo* are intimately connected with the superoxide/hydroxyl radical system generated by membrane oxidases of phagocytes.

With regard to the conclusion of the letter, we agree with Kontush that HDL-associated proteins might be involved in atherosclerosis, but we do not readily understand the role of HDL-associated proteins in the context of our study, which is based on the analysis of an antioxidant. It is still too early to introduce HDL-associated proteins into the class of antioxidants, and the authors themselves state that the "precise mechanism of action deserves detailed investigation." The inhibition of oxidative stress *per se* does not necessarily imply an antioxidant action. To clarify this concept, if we had a new drug designed to block the assembly of NADPH oxidase we should obtain an inhibition of the superoxide release by neutrophils; in this case the drug should be classified as an enzyme inhibitor, not as an antioxidant. We suggest restricting the term "antioxidant" to molecules that have shown relevant kinetic constants in intercepting free radicals.

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