Ascorbic acid and dehydroascorbic acid as biomarkers of oxidative stress caused by smoking[1-3]

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ABSTRACT   Using a reliable, newly developed assay for ascorbic acid (reduced form) and dehydroascorbic acid (DHAA; the oxidized form) in plasma, we studied the influence of age, sex, and smoking on 219 healthy, age-stratified, and randomly selected subjects representing the Danish population. The mean ($\pm$ SD) plasma total ascorbic acid (ascorbic acid + DHAA) concentration was lower in smokers (62.8 $\pm$ 24.9 μmol/L) than in nonsmokers (74.9 $\pm$ 23.6 μmol/L) ($P < 0.001$) and the DHAA content was 1.8 $\pm$ 4.0% of the total ascorbic acid in smokers compared with 0.1 $\pm$ 3.1% in nonsmokers ($P < 0.001$). A significant inverse correlation between the DHAA fraction and the total ascorbic acid concentration was found in smokers ($P < 0.002$) but not in smokers; the slopes of the linear regressions were significantly different in the two groups ($P < 0.005$). The mean plasma concentration of total ascorbic acid was higher in females than in males ($P < 0.005$); this difference persisted in multivariate analysis when smoking was adjusted for. No age dependence could be identified. The data show that smoking results in severe oxidative stress, depletion of the ascorbic acid pool, and insufficient reduction capacity to maintain ascorbic acid in the reduced form in plasma. We suggest that the additional analysis of DHAA allows further differentiation in the assessment of oxidative stress and may provide an objective way of determining vitamin C requirements in smokers. Preliminary findings suggest that a vitamin C dose that results in a plasma concentration of $\approx$70 μmol/L or higher is required in smokers.   *Am J Clin Nutr* 1997;65:959–63.

KEY WORDS   Vitamin C, ascorbic acid, dehydroascorbic acid, oxidative stress, smoking, population study, plasma, ascorbic acid requirement, humans, Danes

INTRODUCTION

Tobacco smoke contains a large number of toxic chemicals and causes numerous effects on physiologic and biochemical functions. Long-term smoking is associated with an increased risk of several cancers, cardiovascular disease, and many other chronic diseases (1–3). The large content of gas-phase oxidants and reactive oxygen species in smoke (4–6) can explain the increased level of oxidative stress that is consistently observed in smokers (7–9).

Ascorbic acid is the most abundant and effective water-soluble antioxidant in human plasma and is believed to be of major importance for protection against diseases and degenerative processes caused by oxidative stress (10, 11). It has been known for several years that cigarette smoking is associated with a depletion of the ascorbic acid pool (2). Although smokers have a lower dietary intake of ascorbic acid than nonsmokers (12), the ascorbic acid depletion by cigarette smoking appears to occur predominantly via mechanisms independent of dietary intake, ie, as a direct result of exposure to the smoke per se (13, 14).

Both ascorbic acid and its oxidation product dehydroascorbic acid (DHAA) are known to have antiscorbutic effects. Plasma concentrations of DHAA are low and this has been related to rapid tissue uptake and activity of dehydroascorbic reductase (15). The activity of this enzyme in cells, however, appears to be dependent on both ascorbic acid and the dominant intracellular reductant glutathione (11). Detailed information on the kinetics and the interaction and cooperation among ascorbic acid, DHAA, and glutathione in vivo is not available; however, it seems possible that low plasma ascorbic acid concentrations could lead to impaired transport of DHAA, impaired reduction of DHAA back to ascorbic acid, or both. Furthermore, a combination of increased oxidative stress from tobacco smoke and low ascorbate plasma concentrations could lead to elevation of DHAA in plasma. Consequently, the ratio of DHAA to total ascorbate could be a marker of oxidative stress.

Assessment of DHAA as well as ascorbic acid itself has been subject to much speculation regarding their relevance as biomarkers of oxidative stress (16, 17). Considerable discrepancy was found between relative DHAA and ascorbic acid reference concentrations for healthy adults in previous studies (17–26), with the fraction of DHAA ranging from 0% to 20% of the total ascorbic acid concentration. More recent reports, however, agree that the amount of DHAA in plasma is 0% or a small percentage of the total ascorbic acid concentration (17, 26), as suggested by Dhariwal et al (25). Because of the technical difficulties in maintaining the in vivo ratio between DHAA and ascorbic acid ex vivo and because virtually all ascorbic acid

appears to be kept reduced in human plasma, the value of DHAA as a biomarker of oxidative stress has not been thoroughly evaluated.

We report the results of a cross-sectional population study monitoring ascorbic acid and DHAA in plasma. These data show the potential of both ascorbic acid and DHAA as biomarkers of oxidative stress and as potential means of estimating vitamin C requirements.

SUBJECTS AND METHODS

Study population

The study was conducted in accordance with the Declaration of Helsinki. The study cohort consisted of 219 people (110 women and 109 men) who agreed to participate after age-stratified selection at random from the population of Funen, Denmark, by means of the Danish Central Personal Registry (CPR). Twenty men and 20 women were selected in 10-y age strata from 20 y to 90 y. No inclusion or exclusion criteria were used except that they had to be able to read and understand a questionnaire and the informed-consent form. When necessary, subjects were visited in their homes for questioning and blood sampling. It proved impossible to attain 20 subjects in the age strata > 60 y by random identification; therefore, < 20 subjects were allowed in these groups (**Figure 1**). One-half of the participants were from rural areas and one-half were from urban areas. The overall rate of participation in the age groups from 20 to 79 y was close to 40%. On the basis of analysis of questionnaires including occupation, lifestyle, diet, and health, the study cohort was representative of the general Danish population. Forty-three percent were active smokers, 23% had stopped smoking > 6 mo before the study (except for 2 who were recent quitters), and 32% had never smoked. Among the smokers, 13 subjects were excluded from the present data because they were not everyday-smokers and the rest were almost exclusively heavy cigarette smokers; a total of 206 subjects were included in the study.

Ascorbic acid and dehydroascorbic acid analysis

Blood samples were immediately cooled on ice, centrifuged (5 min, 4000 × g, 4 °C), and the plasma fractions obtained were stabilized with an equal volume of 10% *meta*-phosphoric acid. The precipitates were removed by centrifugation (5 min, 4000 × g, 4 °C) and the samples were kept at −80 °C for < 6 mo until analyzed. The stability of the plasma samples has been thoroughly tested under these conditions (17).

Ascorbic acid and total ascorbic acid were measured by using HPLC with coulometric detection as described previously (17). Ascorbic acid peak areas were corrected for a 1.12% difference in relative response compared with the total ascorbic acid measurements, and DHAA concentrations were calculated by subtraction. The assay between-day CV was < 8%. Because of the variability of measurement and the use of a subtraction method for the calculation of DHAA concentrations, negative values resulted; we included these values.

Statistical analysis

Data were analyzed by using STATISTICA 5.0 (StatSoft, Tulsa, OK). Multifactorial analysis of variance and analysis of covariance were used to estimate the effects of sex, smoking,
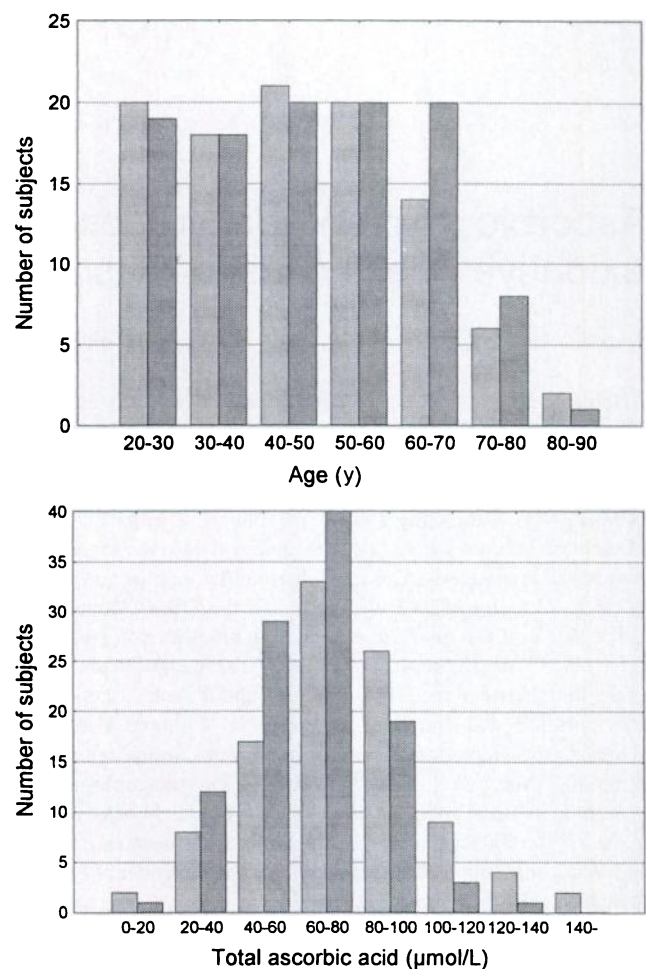

FIGURE 1. Distribution of age and total ascorbic acid concentrations in plasma in women (lighter bars) and men (darker bars) in the study population.

and age on ascorbic acid, total ascorbic acid, and DHAA. Linear regression was done by the method of least squares. Analysis of covariance was used to estimate differences in regression slopes of DHAA on total ascorbic acid. A two-tailed P value < 0.05 was considered statistically significant. Values are given as means ± SDs.

RESULTS

Relations between smoking status, sex, and ascorbic acid are summarized in **Table 1** and **Figure 2**. The concentration of total ascorbic acid in plasma was lower in smokers than in nonsmokers and higher in women than in men (**Figure 3**). Analysis of covariance showed that sex and smoking were significant independent predictors of plasma ascorbic acid concentrations whereas age was not a significant covariate. The concentration of DHAA was higher in smokers (0.78 ± 2.32 μmol/L) than in nonsmokers (0.11 ± 2.36 μmol/L) (P < 0.05).

Further analysis of the factors predicting DHAA concentrations were done by analysis of covariance with sex and smoking as independent variables and age as a covariate. Regardless of whether the DHAA concentration or the fraction of DHAA (%DHAA) of total vitamin C was used as a dependent variable,

TABLE 1
Baseline data for the study population

	Women (n = 100)	Men (n = 106)	Both (n = 206)
Age (y)	47.3 ± 15.5[1]	48.8 ± 16.1	48.0 ± 15.8
Percentage of smokers (%)	40.0	39.6	39.8
Total ascorbic acid (μmol/L)			
All subjects	75.0 ± 28.1[2]	65.4 ± 20.2	70.1 ± 24.8
Smokers	66.4 ± 28.3[2]	59.2 ± 20.8	62.8 ± 24.9[3]
Nonsmokers	80.7 ± 26.6	69.4 ± 19.0	74.9 ± 23.6
%DHAA[4]			
All subjects	0.9 ± 3.5	0.6 ± 3.7	0.8 ± 3.6
Smokers	1.8 ± 3.9	1.8 ± 4.2	1.8 ± 4.0[3]
Nonsmokers	0.3 ± 3.0	−0.1 ± 3.2	0.1 ± 3.1

[1] $\bar{x}$ ± SD.
[2] Significantly different from men, $P < 0.05$.
[3] Significantly different from nonsmokers, $P < 0.05$.
[4] Percentage dehydroascorbic acid of total ascorbic acid.

there was a significant effect of smoking ($P = 0.0009$ and $P = 0.048$, respectively) but not of age or sex ($P = 0.59$ and $P = 0.58$, respectively). In these analyses the negative estimates of DHAA were used; however, if these negative values were substituted with zero the significance of the effect of smoking persisted as a significant difference between the slopes in Figure 2.

Interestingly, the regression lines for %DHAA and DHAA on total ascorbic acid were significantly different in the groups of smokers and nonsmokers, respectively (**Table 2**). There was an inverse correlation between total ascorbic acid and %DHAA in smokers (Figure 2; $r = 0.35$, $P < 0.002$). In nonsmokers, however, the linear-regression slope was not significantly different from zero. A test of parallelism of the slopes of smokers and nonsmokers showed a significant difference ($P < 0.005$), regardless of whether %DHAA or DHAA was used. The
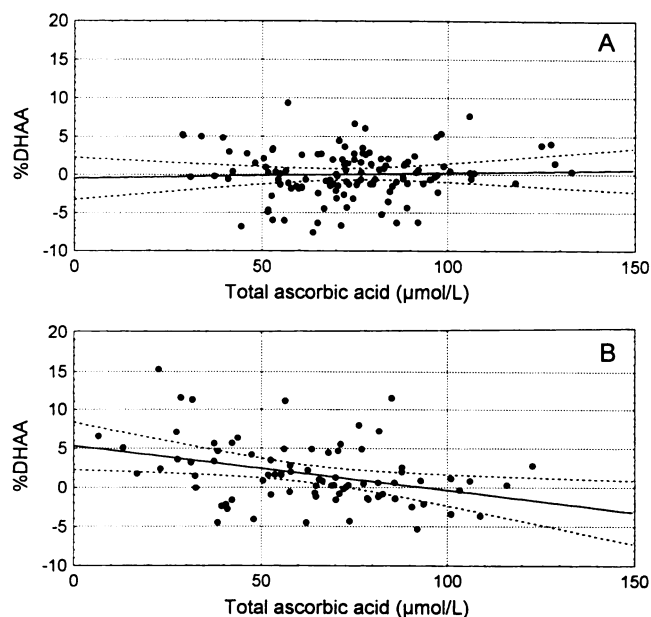

FIGURE 2. Relation between total ascorbic acid concentrations and the percentage of dehydroascorbic acid (%DHAA) of total ascorbic acid in nonsmokers (A) and smokers (B). The dotted lines are the 99% CIs.
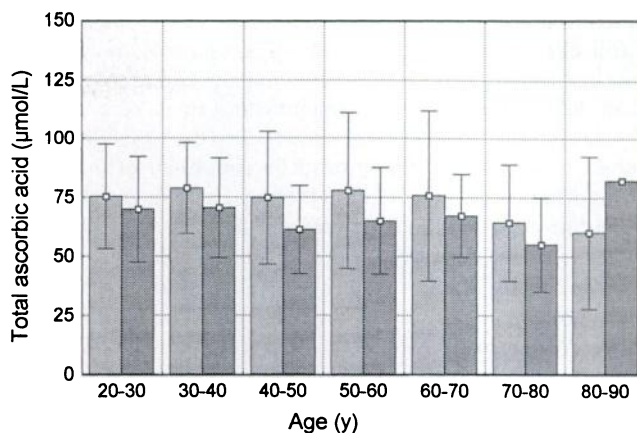

FIGURE 3. Mean (± SD) plasma total ascorbic acid concentrations by 10-y age strata in women (lighter bars) and men (darker bars). There were no significant differences.

regression line on %DHAA for the smokers crossed the x axis at a total ascorbic acid concentration of 94.4 μmol/L; the lower limit of the CI crossed the x axis at a total ascorbic acid concentration of ≈70 μmol/L.

DISCUSSION

In the present study, we found smoking to be the dominant predictor of high plasma concentrations of the oxidized form of ascorbic acid, DHAA. The crude difference between men and women persisted in a multivariate analysis in which smoking and age were taken into account. There were no significant effects of age in any of the age strata, by multivariate analysis.

As stated in the Introduction, true concentrations of DHAA in plasma have been debated for a long time, older reports indicating much higher values than more recent publications. The analytic methodology we developed is highly sensitive and specific (17) and involves well-controlled sample preparation, which has proved crucial to the reliability and reproducibility of the results obtained. Furthermore, our method does not rely on determination of ascorbic acid in the chromatographic void volume but includes retention of ascorbic acid on the column, in contrast with most other published methods (17–26).

It is apparent from Figure 2 that we measured negative DHAA values. In the biological sense, negative values make no sense; however, it should be appreciated that DHAA is mea-

TABLE 2
Results of the regression of the dehydroascorbic fraction of total ascorbic acid (% DHAA) and absolute DHAA concentrations on the total ascorbic acid concentration

	Smokers[1]		Nonsmokers[1]	
	% DHAA	DHAA	% DHAA	DHAA
Intercept	+5.4[2]	+1.48	−0.60	−1.33
Slope	−0.057[2]	−0.011	−0.0091	+0.019
r	−0.35[2]	−0.12	+0.069	+0.19[2]

[1] The regression lines for smokers and nonsmokers are significantly different ($P < 0.05$) for both regression of % DHAA and DHAA concentration on total ascorbic acid.
[2] $P < 0.05$.

sured as a difference between total ascorbate (ie, reduced plus oxidized) and reduced ascorbate. The amounts of total and reduced ascorbate are of the same magnitude and much larger than their difference, which amounts to a small percentage. If the dehydroascorbate value is close to zero the variability of the measurement would clearly result in about half of the values being negative and the other half being positive and the mean would be close to zero. It is obvious that truncating the negative values would introduce a bias and should not be done. Whether such a bias contributes to the discussion about the true concentrations of DHAA is not clear from the published literature. We repeated the calculations (not shown) with such truncation of the data and the difference as depicted in Figure 2 persisted.

Of further interest is the finding depicted in Figure 2 that the regression of the ratio of DHAA to total ascorbic acid on total ascorbate is negative in smokers and clearly different from that of nonsmokers, in whom the slope is zero. The difference is consistent regardless of whether the actual DHAA values or the dehydroascorbate fraction of total ascorbate was used. This is further indication of oxidative stress in smokers and also indicates that the fraction of the dehydroascorbate of total ascorbate might provide useful information about the level of oxidative stress in smokers. Of particular interest is the finding that for plasma concentrations of ascorbic acid > 94 μmol/L, there were no differences in the ratio of DHAA to total ascorbic acid between smokers and nonsmokers as judged from the regression line. On the basis of the 99% CIs of the slope, the value was > 70 μmol/L (Figure 2). Vitamin C is accumulated in cells in part by a sodium-dependent transporter with saturation kinetics at ≈70 μmol/L. The reduction of dehydroascorbate to ascorbate occurs intracellularly and depends on both ascorbic acid and the dominant intracellular reductant glutathione (11). Our findings are therefore consistent with a need for higher vitamin C intakes in most smokers.

The vitamin C requirement in smokers has been debated (13, 27–30). Levine et al (30) defined optimal vitamin C requirements based on several measurements, including dose-function relations, availability in the food supply, steady state concentrations in plasma and tissue achieved at each dose, urinary excretion, bioavailability, toxicity, and epidemiologic observations. Schectman et al (12) showed in a large epidemiologic study based on the Second National Health and Nutrition Examination Survey (NHANES II) that smokers have a lower dietary intake of ascorbic acid than do nonsmokers. Because the difference in plasma concentration persisted even when the dietary intake of ascorbic acid was corrected for, they suggested that the depletion of ascorbic acid as a result of cigarette smoking occurs predominantly via mechanisms independent of dietary intake (12), a finding supported by our recent finding in a study on smoking cessation (14).

Levine et al (31) followed up their studies on vitamin C requirements with a carefully designed and thorough study that included determination of plasma total ascorbate concentrations after well-defined vitamin C intakes. They found that in nonsmokers the recommended dietary allowance (RDA; 32) of 60 mg vitamin C/d resulted in plasma concentrations of ≈25 μmol/L, and 200 mg vitamin C/d resulted in plasma concentrations of ≈66 μmol/L; higher doses did not increase plasma concentrations considerably. On the basis of these and other data, Levine et al concluded that the current RDA for vitamin

C should be increased to 200 mg/d. This suggestion is consistent with our finding that plasma DHAA concentrations are close to null when plasma concentrations are > 70 μmol/L. Whether an intake of ≥ 200 mg vitamin C/ d is needed to achieve such concentrations remains to be determined.

No correlations between ascorbic acid or DHAA and age were found in the present study (Figure 3). However, a decrease in plasma total ascorbic acid concentrations with age was observed but it was not significant ($P = 0.11$). The absence of a correlation between age and ascorbic acid agrees with more recent observations (33–35), although a few older studies did find a correlation (36, 37). It has been suggested that a correlation with age would most likely be due to a decreased dietary intake of ascorbic acid with increasing age, rather than to physiologic changes (38). Thus, the correlation with smoking might be of particular interest in elderly persons with a poor diet.

We suggest that the ratio of DHAA to total ascorbate is a relevant measure for further studies on vitamin C needs in smokers, might be a measure of oxidative stress from cigarette smoking, and could be used to measure effects in clinical intervention studies.

REFERENCES

1. Halliwell B, Gutteridge JMC. Free radicals, ageing and disease. In: Free radicals in biology and medicine. Oxford, United Kingdom: Clarendon Press, 1989;2:1–543.
2. Murata A. Smoking and vitamin C. World Rev Nutr Diet 1991;64:31–57.
3. Doll R, Peto R, Wheatley K, Gray R, Sutherland I. Mortality in relation to smoking: 40 years' observations on male British doctors. Br Med J 1994;309:901–11.
4. Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Biochem J 1991;277:133–8.
5. Leanderson P, Tagesson C. Cigarette smoke–induced DNA damage in cultured human lung cells: role of hydroxyl radicals and endonuclease activation. Chem Biol Interact 1992;81:197–208.
6. Pryor WA, Stone K. Oxidants in cigarette smoke—radicals, hydrogen peroxide, peroxinitrate and peroxinitrite. Ann N Y Acad Sci 1993;686:12–27.
7. Kiyosawa H, Suko M, Okudaira H, et al. Cigarette smoking induces formation of 8-hydroxydeoxyguanosine, one of the oxidative DNA damages in human peripheral leucocytes. Free Radic Res Commun 1990;11:23–7.
8. Loft S, Vistisen K, Ewertz M, Tjønneland A, Overvad K, Poulsen HE. Oxidative DNA-damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. Carcinogenesis 1992;13:2241–7.
9. Duthie GG, Arthur JR. Cigarette smoking as an inducer of oxidative stress. In: Sen CK, Packer L, Hänninen O, eds. Exercise and oxygen toxicity. Amsterdam: Elsevier, 1994:297–317.
10. Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci U S A 1989;86:6377–81.
11. Meister A. Commentary: on the antioxidant effects of ascorbic acid and glutathione. Biochem Pharmacol 1992;44:1905–15.
12. Schectman G, Byrd JC, Gruchow HW. The influence of smoking on vitamin C status in adults. Am J Public Health 1989;79:158–62.

13. Schectman G. Estimating ascorbic acid requirements for cigarette smokers. Ann N Y Acad Sci 1993;686:335–45.

14. Lykkesfeldt J, Priemé H, Loft S, Poulsen HE. Effect of smoking cessation on plasma ascorbic acid concentration. Br Med J 1996;313:91.

15. Rose RC. Transport of ascorbic acid and other water-soluble vitamins. Biochim Biophys Acta 1988;947:335–66.

16. Vanderslice JT, Higgs DJ, Beecher GR, Higgs HE, Bouma J. On the presence of dehydroascorbic acid in human plasma. Int J Vitam Nutr Res 1992;62:101–2.

17. Lykkesfeldt J, Loft S, Poulsen HE. Determination of ascorbic acid and dehydroascorbic acid in plasma by high-performance liquid chromatography with coulometric detection—are they reliable biomarkers of oxidative stress? Anal Biochem 1995;229:329–35.

18. Okamura M. An improved method for determination of L-ascorbic acid and L-dehydroascorbic acid in blood plasma. Clin Chim Acta 1980;103:259–68.

19. Lunec J, Blake DR. The determination of dehydroascorbic acid and ascorbic acid in serum and synovial fluid of patients with rheumatoid arthritis (RA). Free Radic Res Commun 1985;1:31–9.

20. Schreiber J, Lohmann W, Unversagt D, Otten A. Determination of ascorbic acid, dehydroascorbic acid and isoascorbic acid in blood. Fresenius Z Anal Chem 1986;325:473–5.

21. Lee W, Davis KA, Rettmer RL, Labbe RF. Ascorbic acid status: biochemical and clinical considerations. Am J Clin Nutr 1988;48:286–90.

22. Nyyssönen K, Pikkarainen S, Parviainen MT, Heinonen K, Mononen I. Quantitative estimation of dehydroascorbic acid and ascorbic acid by high-performance liquid chromatography—application to human milk, plasma, and leukocytes. J Liq Chromatogr 1988;11:1717–28.

23. Nagy E, Degrell I. Determination of ascorbic acid and dehydroascorbic acid in plasma and cerebrospinal fluid by liquid chromatography with electrochemical detection. J Chromatogr 1989;497:276–81.

24. Margolis SA, Ziegler RG, Helzlsouer KJ. Ascorbic and dehydroascorbic acid measurement in human serum and plasma. Am J Clin Nutr 1991;54(suppl):1315S–8S.

25. Dhariwal KR, Hartzell WO, Levine M. Ascorbic acid and dehy- droascorbic acid measurements in human plasma and serum. Am J Clin Nutr 1991;54:712–6.

26. Capellmann M, Becka M, Bolt HM. A note on distribution of human plasma levels of ascorbic acid and dehydroascorbic acid. J Physiol Pharmacol 1994;45:183–7.

27. Kallner AB, Hartmann D, Hornig DH. On the requirements of ascorbic acid in man: steady-state turnover and body pool in smokers. Am J Clin Nutr 1981;34:1347–55.

28. Levine M, Dhariwal KR, Washko PW, et al. Ascorbic acid and in situ kinetics: a new approach to vitamin requirements. Am J Clin Nutr 1991;54(suppl):1157S–62S.

29. Schectman G, Byrd JC, Hoffmann R. Ascorbic acid requirements for smokers: analysis of a population survey. Am J Clin Nutr 1991;53:1466–70.

30. Levine M, Dhariwal KR, Welch RW, Wang Y, Park JB. Determination of optimal vitamin C requirements in humans. Am J Clin Nutr 1995;62(suppl):1347S–56S.

31. Levine M, Cantilena CC, Wang Y, et al. Pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc Natl Acad Sci U S A 1996;93:3704–9.

32. National Research Council. Recommended dietary allowances. 10th ed. Washington, DC: National Academy Press, 1989.

33. Jacob RA, Otradovec CL, Russell RM, et al. Vitamin C status and nutrient interactions in a healthy elderly population. Am J Clin Nutr 1988;48:1436–42.

34. Blanchard J, Conrad KA, Watson RR, Garry PJ, Crawley JD. Comparison of plasma, mononuclear and polymorphonuclear leucocyte vitamin C levels in young and elderly women during depletion and supplementation. Eur J Clin Nutr 1989;43:97–106.

35. Blanchard J, Conrad KA, Mead RA, Garry PJ. Vitamin C disposition in young and elderly men. Am J Clin Nutr 1990;51:837–45.

36. Morgan AF, Gillum HL, Williams RI. Nutritional status of ageing. J Nutr 1955;55:431–48.

37. Burr ML, Sweetnam PM, Hurley RJ, Powell GH. Effects of age and intake on plasma ascorbic acid levels. Lancet 1974;1:163–4.

38. O'Sullivan DJ, Callaghan N, Ferriss NB, Finucane JF, Hegarty M. Ascorbic acid deficiency in the elderly. Irish J Med Sci 1968;7:151–6.