

Detection of 2,6-Cyclolycopene-1,5-diol in Breast Nipple Aspirate Fluids and Plasma: A Potential Marker of Oxidative Stress¹

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Abstract

An high-performance liquid chromatography method with electrochemical detection was developed to quantify both 2,6-cyclolycopene-1,5-diol and lycopene in plasma and breast nipple aspirate fluids (NAF). As an example of the utility of this assay, levels in plasma and NAF of 11 women were examined and compared with levels of 8-isoprostane, a commonly used marker of lipid oxidation. Levels of 2,6-cyclolycopene-1,5-diol and 8-isoprostane were higher in NAF than in plasma, but levels of lycopene were lower in NAF than in plasma. Levels of 2,6-cyclolycopene-1,5-diol in plasma and in NAF were significantly correlated with both lycopene and 8-isoprostane levels. This is consistent with the dependence of 2,6-cyclolycopene-1,5-diol levels on both dietary intakes of lycopene and oxidative stress levels. For the correlations between NAF and plasma, lycopene levels were significantly correlated, whereas 2,6-cyclolycopene-1,5-diol levels were not, indicating that levels of 2,6-cyclolycopene-1,5-diol in NAF are difficult to predict from plasma levels. The high levels of 2,6-cyclolycopene-1,5-diol and 8-isoprostane in NAF are consistent with high levels of oxidative stress in the breast.

Introduction

Numerous epidemiological studies have demonstrated that consuming relatively high quantities of fruits and vegetables reduces the risks for many types of human cancers, including that of the breast (1–4). Carotenoids are abundant in fruits and vegetables, and have been suggested to contribute to the cancer-preventive properties of these foods (5–9). One possible mechanism behind the cancer-preventive properties of carotenoids may be their ability to function as antioxidants (7, 10). The facile reaction of carotenoids with oxidants results in formation of many oxidation products. The major oxidation product of lycopene in human serum is 2,6-cyclolycopene-1,5-diol, which is mainly present in isomer form A (Fig. 1). This oxidation product has also been identified in the diet, in breast milk, and in prostate tissue (11, 12).

Our previous work has shown that when compared with

several other carotenoids, lycopene is oxidized most rapidly (13). Oxidation of lycopene also occurred before lipid peroxidation could be detected. Therefore, oxidized lycopene may be a sensitive marker for oxidative stress, which is formed before formation of lipid oxidation products, such as the commonly studied oxidation product of arachidonic acid, 8-isoprostane (14). In this study, a sensitive HPLC³ method was developed for detection of both 2,6-cyclolycopene-1,5-diol A and lycopene in plasma and breast NAF samples.

NAF is ideally suited for studies on biomarkers of breast cancer risk because it can be obtained noninvasively from healthy women (15–18). This fluid bathes the epithelial cells lining the breast ducts. To additionally define the composition of NAF and expand its potential utility for clinical studies, we examined the relationships between plasma and NAF levels of lycopene, 2,6-cyclolycopene-1,5-diol A and 8-isoprostane in a sample of 11 women. The women were all participants of a dietary intervention trial described previously (19), and before intervention both plasma and sufficient volumes of NAF were available from these women for all of the present analyses.

Materials and Methods

Preparation of 2,6-Cyclolycopene-1,5-diol A. Lycopene was extracted from tomato paste using acetone, followed by partitioning into hexane (20). The extract was dried and redissolved in dichloromethane followed with purification by silica flash chromatography (230–400 mesh, 60 Å) using hexane containing 15% dichloromethane for elution. On the basis of comparison to published spectra, this material was estimated by UV-VIS spectrophotometry (21) and HPLC to be >90% *trans*-lycopene. The extracted lycopene was then dissolved in tetrahydrofuran (THF), and it was oxidized by *m*-chloroperoxybenzoic acid at room temperature for 4 h to form lycopene epoxides. This was then hydrolyzed with 0.1% H₂SO₄ for 24 h using published methods (22). The mixture was purified by silica flash chromatography (230–400 mesh, 60 Å), and the fraction containing 2,6-cyclolycopene-1,5-diol A was additionally purified by reverse-phase HPLC, using a semipreparative C18 μ Bondapak column (7.8 × 250 mm; Waters, Milford MA) with an isocratic mobile phase of 85% acetonitrile, 10% methanol, 2.5% hexane, 2.5% dichloromethane and 0.1% *N,N*-diisopropylethylamine pumped for 2 ml/min, and UV detection was at 250 nm.

Identity and purity of the synthesized 2,6-cyclolycopene-1,5-diol A was confirmed by matching UV-VIS spectra, mass spectra, and ¹H NMR with that of published literature (22). Absorption spectra of the lycopene and 2,6-cyclolycopene-1,5-diol A in hexane were recorded on a HP 8452A diode array spectrophotometer. Mass spectra were obtained with a Micro-

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³ The abbreviations used are: HPLC, high-performance liquid chromatography; NAF, nipple aspirate fluid; BHT, butylated hydroxytoluene.

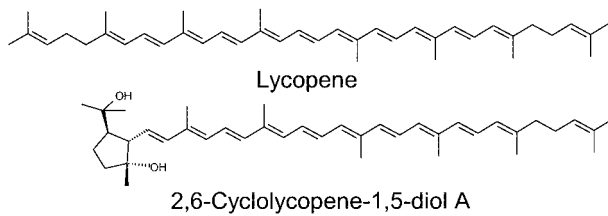


Fig. 1. Chemical structures of lycopene and its metabolite 2,6-cyclolycopene-1,5-diol A.

mass spectrometer, operated in electrospray positive mode, with cone voltage set at 44 V. A Unity-500 spectrometer was used to obtain 500 MHz ^1H NMR spectra after dissolving the samples in CDCl_3 . All of the solvents were HPLC-grade Burdick & Jackson brand (Muskegon, MI), and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Subjects and Samples. The Nutrition and Breast Health study was designed to examine multiple markers of breast cancer risk before and after dietary intervention (19). The women collected fluid every other day for 2 weeks, and 5 of those collections were combined and stored frozen in their home freezers at -20°C in an insulated container during the collection period and at -70°C thereafter. Among the 123 women randomized onto the study, 70 provided NAF samples at baseline. The volume of fluids varied greatly (0–1000 μl), and 11 women had sufficient volume of NAF at baseline for performing both the assays that the study was primarily designed for and the present pilot investigation of oxidized lycopene levels. Fasting plasma samples were obtained at baseline from the same women shortly after the NAF collection period, and these were stored at -70°C until analysis.

Extraction of Lycopene and 2,6-Cyclolycopene-1,5-diol A. Plasma aliquots, 200 μl , were added to 10 μl of Tocol internal standard solution (0.01% in ethanol; a generous gift of Hoffman La Roche, Basel, Switzerland). The proteins were precipitated by adding 200 μl ethanol containing 0.05% BHT. The mixture was extracted twice with 2 ml of hexane (0.05% BHT). The combined hexane extracts were evaporated to dryness under vacuum. The residue was redissolved in 100 μl of ethanol (0.05% BHT) and filtered in 0.45 μm Micro Spin Filter (Alltech, Deerfield, IL) before HPLC analysis.

NAF samples ranging from 6.8 to 54 mg were first diluted to 200 μl with 5% dextrose, 50 mM mannitol, 10 mM Tris (pH 7.4; dextrose solution), then mixed with 10 μl Tocol internal standard. The mixtures were then digested enzymatically in a similar manner as described by Khachik *et al.* (11). Briefly, the solution of NAF in dextrose solution was vortexed with 20 μl of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (24.6%), 20 μl of Na_2CO_3 (12.6%), and 20 μl of bile salts (6%), and the mixture was incubated at 37°C for 15 min. The mixtures were then treated with 4 μl of Pronase E (50 mg/ml, in PBS) and 8 μl of lipase (50 mg/ml, in PBS), and incubated at 37°C for 1 h. After incubation, 300 μl of ethanol with 0.05% BHT was added into each mixture followed by vortexing for 30 s, and two extractions with hexane were used in the same manner as for plasma described above. Plasma extracted after the enzymatic digestion procedure used for NAF had similar levels of 2,6-cyclolycopene-1,5-diol A and lycopene as that without this digestion step (data not shown). The recovery of internal standard was $>85\%$ in all of the cases.

HPLC Analysis of Lycopene and 2,6-Cyclolycopene-1,5-diol. The HPLC system for plasma and NAF analyses consisted of a Waters 600E pump (Milford, MA), a Spectra-200

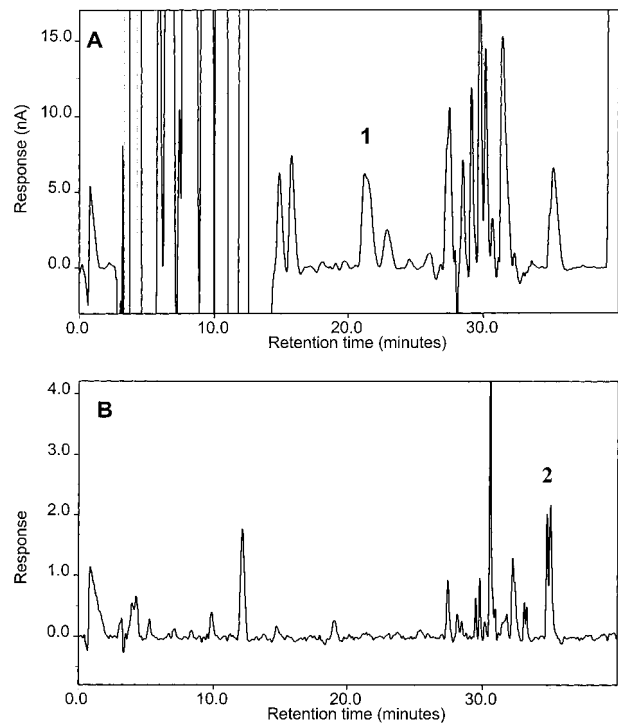


Fig. 2. HPLC chromatograph of a NAF extract. A, electrochemical signal at 300 mV. Peak 1 is 2,6-cyclolycopene-1,5-diol A. B, absorption at 450 nm. Peak 2 was taken as the sum of two peaks: *trans*- and *cis*-lycopene to obtain total lycopene.

UV detector (Spectra-Physics, San Jose, CA) set at 450 nm, and a Coulometric Electrode Array detector (ESA, Chemsford, MA) with channels set at 300, 370, and 440 mV. The column was a YMC 4.6 mm \times 250 mm 3- μm C_{30} carotenoid column with two 4.6 mm \times 15 mm C_{30} guard columns (Wilmington, NC) connected directly to it. Mobile phase mixture A consisted of 80% acetonitrile, 18% methanol, and 2% 1 M ammonium acetate. Mobile phase mixture B consisted of 60% methyl-*tert*-butyl-ether, 20% dichloromethane, 18% methanol, and 2% 1 M ammonium acetate. The flow rate was 1 ml/min. An isocratic mixture of 80% A and 20% B was pumped for 20 min followed by a 7-min linear gradient to 100% B. 100% B was maintained for another 7 min followed by 8 min of re-equilibration. Injections were 50 μl .

The visible signal at 450 nm was used to quantify total lycopene levels. The sum of the areas of electrochemical signals was used to calculate 2,6-cyclolycopene-1,5-diol A and Tocol levels. In all of the samples, signal ratios between electrochemical channels were calculated to confirm identity of peaks. Fig. 2 is a typical HPLC chromatograph of a NAF extract. As shown in Fig. 2, the retention times for 2,6-cyclolycopene-1,5-diol A (peak 1) and lycopene (peak 2) were 21 and 35 min, respectively. Fig. 2A is the 300 mV signal, which is the dominant channel for 2,6-cyclolycopene-1,5-diol A. The signal ratio for 2,6-cyclolycopene-1,5-diol A at the dominant channel and 370 mV was 4:1 and for the dominant channel and 440 mV was 7:1. Standard curves were constructed with synthetic lycopene and 2,6-cyclolycopene-1,5-diol A in ethanol using extinction coefficients of the compounds to determine standard concentrations (lycopene $A_{1\text{cm}}^{1\%} = 3450$ (21); 2,6-cyclolycopene-1,5-diol $A_{1\text{cm}}^{1\%} = 2825$, provided by Dr. Frederick Khachik). The identity of the 2,6-cyclolycopene-1,5-diol A

Table 1 Levels of lycopene, 2,6-cyclolycopene-1,5-diol A, and 8-isoprostane in plasma and breast NAF from 11 women (mean \pm SD, range)

	Plasma	NAF	<i>P</i> ^a
Lycopene (ng/ml)	452 \pm 193	173 \pm 188 ^b	0.120
Range	130–760	0–572	
2,6-cyclolycopene-1,5-diol A (ng/ml)	22 \pm 9	34 \pm 24 ^c	<0.001
Range	6–36	0–83	
2,6-cyclolycopene-1,5-diol A/lycopene	0.049 \pm 0.0095	0.15 \pm 0.16 ^d	0.014
Range	0.038–0.073	0–0.49	
Total 8-isoprostane (pg/ml)	131 \pm 58	32,110 \pm 35,870	0.035
Range	71–261	2,259–117,500	

^a *P*s are from paired *t* tests for the difference between plasma and NAF.

^b Four women had levels below the limit of detection (53 ng/ml), and 0 ng/ml was assigned to them.

^c One subject had levels below the limit of detection (5 ng/ml), and 0 ng/ml was assigned to her.

^d Total 7 values.

peak in chromatographs of plasma and NAF was verified by HPLC retention times, coelution with synthetic 2,6-cyclolycopene-1,5-diol A, signal ratios between electrochemical channels (300 mV, 370 mV and 440 mV) and by UV-VIS spectra of the collected HPLC peak.

Total 8-Isoprostane Levels. Total 8-isoprostane levels in plasma and NAF were measured using an enzyme immunoassay (EIA) kit from Cayman Chemical Co. (Ann Arbor, MI) using a modified Sep-Pak procedure. Briefly, 200 μ l plasma or NAF diluted with 5% dextrose solution were mixed with tritium-labeled prostaglandin F_{2 α} , followed by addition of 200 μ l 15% KOH and incubation at 40°C for 1 h. This was followed by addition of 400 μ l of ethanol containing 0.05% BHT and vortexing for 30 s. Samples were diluted with 2.2 ml of water, and acetic acid (60 μ l) was added to bring the pH below 4.0. These solutions were then purified with Waters Oasis HLB 3cc Extraction Cartridges (Waters Corporation, Milford, MA) for which we used a modified elution procedure: washing with 3 ml of 40% methanol and eluting with 3 ml of 65% methanol. The eluates were dried under vacuum and dissolved in 400 μ l of ELISA buffer supplied in the kit. Levels were determined according to the manufacturer's recommendations.

Statistics. Statistics were done with Prism software from Graph-Pad (San Diego, CA) and *P*s \leq 0.05 were considered significant. Data series were first confirmed to be normally distributed, and Pearson correlation coefficients and *P*s were then calculated using two-tailed testing. Two-tailed testing was also used for the paired *t* tests.

Results

Both plasma and NAF collected from 11 women who had enrolled in a dietary intervention trial were analyzed for levels of lycopene, 2,6-cyclolycopene-1,5-diol A, and total 8-isoprostanes, and these data are summarized in Table 1. 2,6-Cyclolycopene-1,5-diol A was detected in all 11 of the plasma samples and in 10 of 11 NAF samples. Lycopene was detected in 11 plasma samples and 7 of 11 NAF samples. Of the 4 NAF samples with no lycopene detected, 3 did have 2,6-cyclolycopene-1,5-diol A present and 1 of them had neither compound in detectable levels. With this latter sample, volume of NAF was not limiting but the subject did have low plasma levels of lycopene (178 ng/ml).

The mean level of 2,6-cyclolycopene-1,5-diol A in NAF

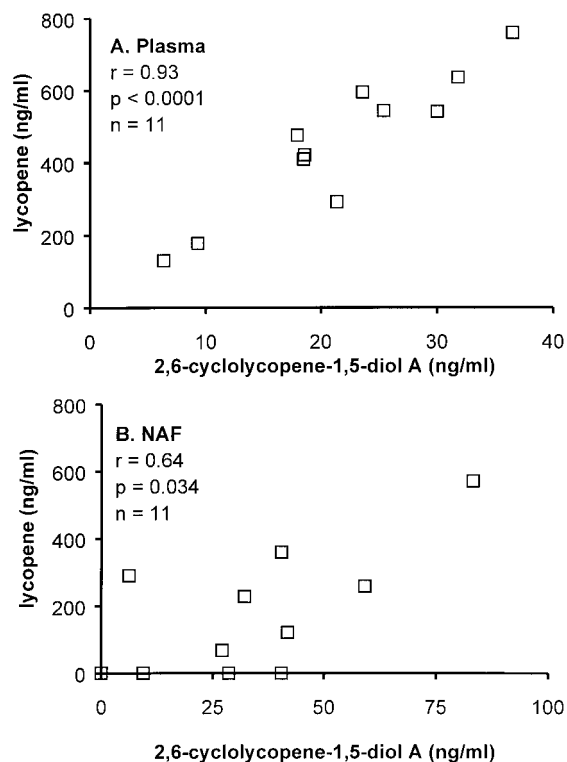


Fig. 3. Correlation of lycopene and 2,6-cyclolycopene-1,5-diol A in plasma and NAF. The Pearson correlation coefficients and *P*s are shown.

(34 ng/ml) was >50% higher than that in plasma (22 ng/ml), whereas the mean level of lycopene in NAF (173 ng/ml) was less than half that in plasma (452 ng/ml). The ratios of 2,6-cyclolycopene-1,5-diol A to lycopene were calculated when lycopene levels were detectable. The average ratio of 2,6-cyclolycopene-1,5-diol A to lycopene in NAF (0.15) was two times greater than that in plasma (0.049; Table 1). The mean 8-isoprostane level in NAF (32,110 pg/ml) also was much higher than that in plasma (131 pg/ml).

The correlation coefficient between lycopene and 2,6-cyclolycopene-1,5-diol A levels was high in both plasma ($r = 0.93$; Fig. 3A) and NAF ($r = 0.64$; Fig. 3B). These associations were similar and remained significant when carotenoids were expressed per amount of cholesterol in either plasma or NAF (data not shown). Lycopene levels in plasma and NAF also were significantly associated ($r = 0.61$; Fig. 4A), but a correlation between plasma and NAF levels of 2,6-cyclolycopene-1,5-diol A was not evident (Fig. 4B). There was a significant positive association between levels of 2,6-cyclolycopene-1,5-diol A and 8-isoprostane in both plasma and in NAF (Fig. 5).

Discussion

Although 2,6-cyclolycopene-1,5-diol had been identified previously in human serum, breast milk, and prostate tissue (11, 12), this article for the first time reports the presence of 2,6-cyclolycopene-1,5-diol in NAF. The significant association of plasma and NAF levels of lycopene (Fig. 4A) agrees with a previous report that plasma and NAF levels of total carotenoids were associated in recently postweaned women (23). Lycopene levels in plasma may well determine NAF levels. However, there was no significant correlation of 2,6-cyclolycopene-1,5-

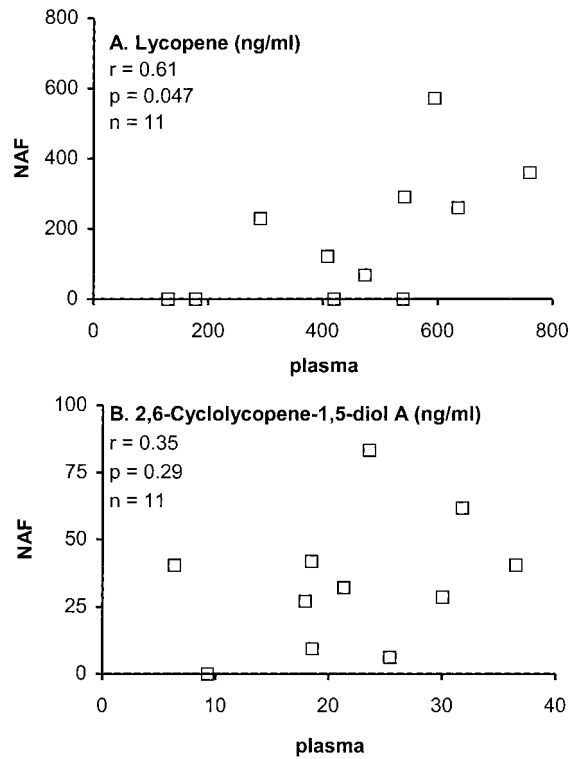


Fig. 4. NAF and plasma levels of lycopene and 2,6-cyclolycopene-1,5-diol A. The Pearson correlation coefficient and P is shown.

diol A levels in NAF and plasma (Fig. 4B). This indicates that the presence of 2,6-cyclolycopene-1,5-diol in the breast does not solely reflect plasma levels. Wrensch *et al.* (24) similarly found that levels of cholesterol and cholesterol β -epoxide were not correlated in serum and NAF.

2,6-Cyclolycopene-1,5-diol in plasma and NAF could originate from the *in vivo* oxidation of lycopene or from dietary intakes (25). The correlation between levels of 2,6-cyclolycopene-1,5-diol A and lycopene (Fig. 3) is consistent with either source of cyclolycopene-1,5-diol. In NAF, turnover rates of lycopene are not known, but they are likely to be much lower than in plasma. Furthermore, because the average ratio of 2,6-cyclolycopene-1,5-diol A to lycopene in NAF was 3-fold that in plasma (Table 1), this data suggest that much of the 2,6-cyclolycopene-1,5-diol A in NAF is likely to be an *in vivo* oxidation product of lycopene and a marker of oxidative stress in NAF. This hypothesis is also supported by the correlation between levels of 2,6-cyclolycopene-1,5-diol A and 8-isoprostane in both plasma and NAF (Fig. 5). 8-Isoprostane is a commonly used marker for oxidative stress (14), and it was significantly correlated with levels of 2,6-cyclolycopene-1,5-diol A in this study despite the small sample size.

Our results showing high 2,6-cyclolycopene-1,5-diol A levels in NAF are indicative of high oxidative stress levels in the breast. The levels of 8-isoprostane were especially high. This is analogous to the high levels of cholesterol epoxides in NAF described by Petrakis *et al.* (26). Interestingly, 8-isoprostane, lycopene, and 2,6-cyclolycopene-1,5-diol A all exhibited large interindividual variation, which may well be related to breast cancer risk, as has been suggested for cholesterol epoxides (26).

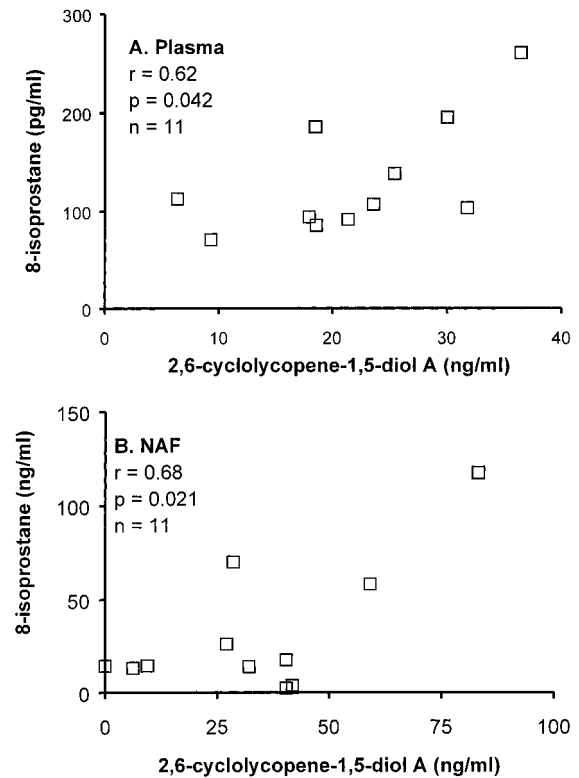


Fig. 5. Plots of 8-isoprostane levels versus 2,6-cyclolycopene-1,5-diol A levels in plasma (A) and in NAF (B). The Pearson correlation coefficient and P is shown.

In addition to its possible role as a marker of oxidative stress, oxidized lycopene metabolites also may have intrinsic biological properties of relevance to cancer risk (27, 28). The HPLC method we developed in this study, which uses electrochemical detection for sensitive analysis of 2,6-cyclolycopene-1,5-diol A in small amounts of plasma and NAF, may be convenient and useful for future studies on the biological significance of this compound. In particular, levels of 2,6-cyclolycopene-1,5-diol A in NAF may be more closely related to breast cancer risk than levels in blood.

Acknowledgments

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References

- Kant, A. K., Schatzkin, A., Graubard, B. I., and Schairer, C. A prospective study of diet quality and mortality in women. *JAMA*, 283: 2109–2115, 2000.
- Terry, P., Terry, J. B., and Wolk, A. Fruit and vegetable consumption in the prevention of cancer: an update. *J. Intern. Med.*, 250: 280–290, 2001.
- World Cancer Research Fund in Association with American Institute for Cancer Res. *Food, Nutrition and Prevention of Cancer: A Global Perspective*. Washington, 1997.
- Ames, B. N., Gold, L. S., and Willett, W. C. The causes and prevention of cancer. *Proc. Natl. Acad. Sci. USA*, 92: 5258–5265, 1995.
- Giovannucci, E., Rimm, E. B., Liu, Y., Stampfer, M. J., and Willett, W. C. A prospective study of tomato products, lycopene, and prostate cancer risk. *J. Natl. Cancer Inst.*, 94: 391–398, 2002.

6. Sies, H., Stahl, W., and Sundquist, A. R. Antioxidant functions of vitamins. Vitamins E and C, β -carotene, and other carotenoids. *Ann. N. Y. Acad. Sci.*, **669**: 7–20, 1992.
7. Palozza, P., and Krinsky, N. I. Antioxidant effects of carotenoids *in vivo* and *in vitro*: an overview. *Methods Enzymol.*, **213**: 403–420, 1992.
8. Rock, C. L., Kusluski, R. A., Galvez, M. M., and Ethier, S. P. Carotenoids induce morphological changes in human mammary epithelial cell cultures. *Nutr. Cancer*, **23**: 319–333, 1995.
9. Zhang, S., Hunter, D. J., Forman, M. R., Rosner, B. A., Speizer, F. E., Colditz, G. A., Manson, J. E., Hankinson, S. E., and Willett, W. C. Dietary carotenoids and vitamins A, C, and E and risk of breast cancer. *J. Natl. Cancer Inst.*, **91**: 547–556, 1999.
10. Burton, G. W., and Ingold, K. U. β -Carotene: an unusual type of lipid antioxidant. *Science (Wash. DC)*, **224**: 569–573, 1984.
11. Khachik, F., Spangler, C. J., Smith, J. C., Canfield, L. M., Steck, A., and Pfander, H. Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. *Anal. Chem.*, **69**: 1873–1881, 1997.
12. Kucuk, O., Sarkar, F. H., Sakr, W., Djuric, Z., Pollak, M. N., Khachik, F., Li, Y. W., Banerjee, M., Grignon, D., Bertram, J. S., Crissman, J. D., Pontes, E. J., and Wood, D. P., Jr. Phase II randomized clinical trial of lycopene supplementation before radical prostatectomy. *Cancer Epidemiol. Biomark. Prev.*, **10**: 861–868, 2001.
13. Chen, G., and Djuric, Z. Carotenoids are degraded by free radicals but do not affect lipid peroxidation in unilamellar liposomes under different oxygen tensions. *FEBS Lett*, **505**: 151–154, 2001.
14. Roberts, L. J., and Morrow, J. D. Measurement of F(2)-isoprostanes as an index of oxidative stress *in vivo*. *Free Radic. Biol. Med.*, **28**: 505–513, 2000.
15. Wrensch, M. R., Petrakis, N. L., Miike, R., King, E. B., Chew, K., Neuhaus, J., Lee, M. M., and Rhys, M. Breast cancer risk in women with abnormal cytology in nipple aspirates of breast fluid. *J. Natl. Cancer Inst.*, **93**: 1791–1798, 2001.
16. Gann, P., Chatterton, R., Vogelsong, K., Dupuis, J., and Ellman, A. Mitogenic growth factors in breast fluid obtained from healthy women: evaluation of biological and extraneous sources of variability. *Cancer Epidemiol. Biomark. Prev.*, **6**: 421–428, 1997.
17. Sauter, E. R., Ross, E., Daly, M., Klein-Szanto, A., Engstrom, P. F., Sorling, A., Malick, J., and Ehya, H. Nipple aspirate fluid: a promising non-invasive method to identify cellular markers of breast cancer risk. *Br. J. Cancer*, **76**: 494–501, 1997.
18. Petrakis, N. L. ASO Distinguished Achievement Award Lecture. Studies on the epidemiology and natural history of benign breast disease and breast cancer using nipple aspirate fluid. *Cancer Epidemiol. Biomark. Prev.*, **2**: 3–10, 1993.
19. Djuric, Z., Uhley, V. E., Depper, J. B., Brooks, K. M., Lababidi, S., and Heilbrun, L. K. A clinical trial to selectively change dietary fat and/or energy intake in women: the Women's Diet Study. *Nutr. Cancer*, **34**: 27–35, 1999.
20. Britton, G., Liaaen-Jensen, S., and Pfander, H. (eds.). *Carotenoids. Isolation and Analysis*. Basel: Birkauer Verlag, 1995.
21. Britton, G., Liaaen-Jensen, S., and Pfander, H. (eds.). *Carotenoids. Spectroscopy*. Basel: Birkauer Verlag, 1995.
22. Khachik, F., Steck, A., Niggli, U. A., and Pfander, H. Partial synthesis and structural elucidation of the oxidative metabolites of lycopene identified in tomato paste, tomato juice, and human serum. *J. Agric. Food Chem.*, **46**: 4874–4884, 1998.
23. Nantais-Smith, L. M., Covington, C. Y., Nordstrom-Klee, B. A., Grubbs, C. J., Eto, I., Lawson, D. M., Pieper, B. A., and Northouse, L. L. Differences in plasma and nipple aspirate carotenoid by lactation status. *Nurs. Res.*, **50**: 172–177, 2001.
24. Wrensch, M. R., Petrakis, N. L., Gruenke, L. D., Miike, R., Ernster, V. L., King, E. B., Hauck, W. W., Craig, J. C., and Goodson, W. H., 3rd. Breast fluid cholesterol and cholesterol β -epoxide concentrations in women with benign breast disease. *Cancer Res.*, **49**: 2168–2174, 1989.
25. Khachik, F., Pfander, H., and Traber, B. Proposed mechanism for the formation of synthetic and naturally occurring metabolites of lycopene in tomato products and human serum. *J. Agric. Food Chem.*, **46**: 4885–4890, 1998.
26. Petrakis, N. L., Gruenke, L. D., and Craig, J. C. Cholesterol and cholesterol epoxides in nipple aspirates of human breast fluid. *Cancer Res.*, **41**: 2563–2565, 1981.
27. King, T. J., Khachik, F., Borkiewicz, H., Fukuxhima, L. H., Morioka, S., and Bertram, J. Metabolites of dietary carotenoids as potential cancer preventive agents. *Pure Appl. Chem.*, **69**: 2135–2140, 1997.
28. Yeh, S. L., and Hu, M. L. Induction of oxidative DNA damage in human foreskin fibroblast Hs68 cells by oxidized β -carotene and lycopene. *Free Radic. Res.*, **35**: 203–213, 2001.