

Dietary beta-carotene inhibits mammary carcinogenesis in rats depending on dietary alpha-linolenic acid content.

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20 **Abstract**

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23 To investigate whether dietary α -linolenic acid (ALA) content alters the effect of β -carotene (β C) on
24 mammary carcinogenesis, we conducted a chemically-induced mammary tumorigenesis experiment in
25 rats randomly assigned to four nutritional groups (15 rats/group) varying in β C supplementation and
26 ALA content. Two oil formulae-enriched diets (15%) were used: one with 4% ALA in the essential
27 fatty acids (EFA) ratio linoleic acid to ALA = 5 (EFA 5 diet) and the other with 16% ALA in an EFA
28 ratio = 1 (EFA 1 diet), both designed with a similar linoleic acid content. β C was added (10 mg/kg
29 diet/day) or not to these diets. β C led to decreased tumour incidence and tumour growth when
30 supplemented to the EFA 5 diet, whereas it had no effect when supplemented to the EFA 1 diet. The
31 decrease of tumour growth did not result from an involvement of lipoperoxidation (tumour
32 malondialdehyde content similar between groups) or from an inhibition of tumour cell proliferation
33 (unchanged S phase fraction in tumours). We concluded that an adequate content of ALA in diet is
34 required for allowing a protective effect of β C in mammary carcinogenesis. Whether such an
35 interaction between ALA and β C influences the risk of breast cancer in women needs to be
36 investigated.

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38 Introduction

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42 Several epidemiological studies have consistently shown that individuals with high intake of
43 vegetables and / or fruits had reduced risk of cancer, including breast cancer (Riboli & Norat, 2003). A
44 potential explanation is that antioxidant nutrients, including carotenoids, prevent carcinogenesis by
45 interfering with oxidative damage to DNA, lipids and proteins. However, results of epidemiological
46 studies are inconclusive on the association between β -carotene (β C) and risk of breast cancer (IARC
47 Working Group on the Evaluation of Cancer Preventive Agents., 1998a). Moreover, two intervention
48 trials conducted in male smokers, the Alpha-Tocopherol, β C cancer prevention study (ATBC) and the
49 β C and Retinol Efficacy Trial (CARET), which both used high-dose β C supplements, found an
50 increased incidence of lung cancer in subjects who received supplements in comparison to non-
51 recipients (The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group, 1994; Omenn *et al.*,
52 1996). By contrast, in a trial conducted in healthy men, the Physicians' Health Study (PHS), a high
53 supplementation of β C on alternate days had no effect on cancer incidence (Hennekens *et al.*, 1996).
54 No clear mechanistic explanation has been provided yet to explain these conflicting findings.
55 Nevertheless, some hypothesis have been advanced, involving the form of β C (synthetic/natural,
56 trans/cis), the amount of β C (physiological/pharmacological), the individuals exposed or not to high
57 risk factors of cancer, genetic factors interfering with nutrition, and the possible interaction between
58 β C and other nutrients.

59 Antioxidant vitamins supplements in mammary tumour-bearing rodents have generated
60 contradictory results (IARC Working Group on the Evaluation of Cancer Preventive Agents., 1998b).
61 It is still unclear whether contrasting results are due to differences in animal models, differences in
62 supplement doses, or interference of antioxidants used with other dietary compounds or the combined
63 effects of these confounding factors. In a model of chemically-induced mammary tumours in rats,
64 adding the antioxidant vitamin E to a diet rich in alpha-linolenic acid (ALA, 18:3n-3, the essential
65 fatty acid of the omega-3 family) led to enhanced mammary tumour growth, whereas it had no effect
66 when added to a diet devoid of ALA (Cognault *et al.*, 2000). These data suggest that an interaction

66 between antioxidant compounds and dietary omega-3 fatty acids is a determinant of mammary tumour
67 growth.

68 To determine whether ALA content of diet alters the effect of β C on mammary
69 carcinogenesis, we examined the effects of two oil formulae-enriched diets differing by their ALA
70 content (4% and 16%), with ratios of linoleic acid (LA, 18:2n-6) to ALA of 4.66 and 1.05
71 respectively, in absence or presence of β C (10 mg/kg diet/day) on the characteristics of mammary
72 carcinogenesis, and found that the omega-3 lipid environment of diet modifies the effect of β C.

73 **Material and Methods**

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76 *Animals and experimental carcinogenesis.*

77 Sixty 40-day-old female Sprague-Dawley rats were purchased from Harlan France (Gannat,
78 France). The care of animals was in accordance with institution guidelines. Rats were housed 3 per
79 cage and maintained at constant temperature (22°C) and humidity with a 12 hour light/dark cycle.
80 Mammary tumours were induced by a single dose of N-Nitroso-N-methylurea (NMU) as previously
81 described (Colas *et al.*, 2004). Rats were randomly separated into four dietary groups (15 rats/group).
82 Three weeks after carcinogenesis initiation and for 15 weeks, animals were palpated once weekly for
83 the detection of mammary tumours. The largest length, width and depth of each tumour were
84 measured with a calliper and the tumour area was calculated as the product of the two largest
85 diameters. The *tumour incidence* (percentage of rats bearing at least one malignant mammary tumour)
86 and the *tumour growth* (mean of tumour areas per tumour bearing rat each week) were determined.
87 After 17 weeks of rat monitoring, animals were sacrificed.

88 *Diets.*

89 Until NMU administration, rats were fed a recommended diet for the breeding and rearing of
90 rodents (Harlan Teklad TRM Rat / Mouse Diet, France). Then, they were fed the experimental diets,
91 composed of a common basal diet (APAE, Jouy-en-Josas, France) as already described (Cognault *et*
92 *al.*, 2000) and 15% of an oils mixture (wt/wt). Diets, designed with a similar LA content, were as
93 follows: *EFA 5 diet* containing a mixture of 60.2% African peanut oil and 39.8% European rapeseed
94 oil (Bailly, Aulnay sous Bois, France) resulting in a 4%-ALA content in the recommended essential
95 fatty acids ratio (EFA ratio: LA/ALA) for rats near 5 (actually 4.66) (Potier de Courcy *et al.*, 1989);
96 *EFA 1 diet* containing a mixture of 69% African peanut oil and 31% Linseed oil (ALA enriched oil,
97 Daudruy, Dunkerque, France) leading to a 16%-ALA content in an EFA ratio of 1.05 (Table 1). βC
98 (type I, Sigma, France) was added (10 mg/kg diet/day; + βC) or not (*w/o* βC , controls) to these diets.
99 Animals received commercial and experimental diets and water ad libitum.

100 The weight of rats was controlled weekly until the end of experiment.

101 **Biochemical analyses.**

102 The fatty acid composition of adipose tissue was determined as previously described (Colas *et*
103 *al.*, 2004): after total lipids extraction, triglycerides were purified by preparative thin layer
104 chromatography and fatty acids were methylated with boron trifluoride and analyzed by gas
105 chromatography (Trace GC, Thermofinnigan, France) with a 60 m polar capillary column (BPX 70,
106 SGE, France).

107 The β C absorption was controlled for each nutritional group by measuring the hepatic β C
108 content. After extraction (Lyan *et al.*, 2001), β C was analyzed by a HPLC system (Spectra System,
109 Thermofinnigan, France) with two Adsorbosphere HS C18 3 μ m cartridges (100 mm X 4.6 mm and
110 150 mm X 4.6 mm, Alltech, France) at 37°C and a photodiode array detector (UV6000LP,
111 Thermofinnigan, France) as already described (Steghens *et al.*, 1997).

112 Lipoperoxidation was evaluated by measuring total malondialdehyde (MDA) content in
113 tumours. At the time of autopsy, necrotic tissues were carefully removed from tumours before
114 freezing. Fifteen tumours (similarly distributed according to their age and their size) were used for the
115 analysis. A fragment of tumour was cut and thawed at 4°C on Tris-HCl 100 mM, pH 7.4 (KCL 100
116 mM, EDTA 1 mM, butylhydroxytoluene 0.1 mM, Triton X-100 and 0.1%
117 phenylmethanesulfonylfluoride 0.1 mM). The total lysat was centrifuged at 10000 g for 5 min and the
118 supernatant extracted for analysis. The protein content in tumour was measured and standardized at
119 15-20 mg/mL for each sample. As previously described (Steghens *et al.*, 2001), MDA was
120 derivatized with diaminonaphtalene in an acid medium at 37°C to form a MDA diazepinium.
121 Analyses were carried out with a HPLC diode array system and an on line mass-spectrophotometer for
122 confirmation. Results were expressed as nmol/g protein, instead of nmol/g tumours, to avoid MDA
123 content variations due to weight of tumours.

124 **Cell cycle.**

125 The distribution of cells within the cell cycle was assessed by flow cytometry after staining of
126 DNA content with propidium iodide, as previously described (Cognault *et al.*, 2000).

127 **Statistical analyses.**

128 Effects of dietary conditions on carcinogenesis and biochemical parameters were evaluated,
129 using the Statistica 6.0 software (StatSoft, Inc., France), by the following tests (p value < 0.10): 1)
130 Pearson Chi² test for tumour incidence; 2) repeated measures ANOVA with grouping factor (time) for
131 tumour growth; 3) Mann-Whitney test for biochemical analyses and cell cycle. Data are expressed as
132 mean \pm standard error.

133 **Results**

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During the experiment, no significant difference in weight gain among dietary groups was observed (data not shown).

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Fatty acid composition of rat adipose tissue, (an indicator of dietary fatty acid intake), is presented in Table 1. Since β C supplementation did not change this composition whatever the ALA content, the Table 1 presents the results for the groups without β C. We showed that the EFA ratio was 5.6-fold greater in rats fed the EFA 5 diet than in rats fed the EFA 1 diet and that the LA content was similar among groups.

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β C was detected only in livers of rats receiving the dietary β C supplementation. This content was not significantly different between rats fed EFA 5 (0.21 ± 0.04 μ g/g of tissue) and EFA 1 diets (0.27 ± 0.04) ($p = 0.35$).

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No significant difference in tumour incidence at the end of experiment was found between groups without β C: EFA 5 diet, 96% incidence, $n = 14/15$ rats with tumour; EFA 1 diet, 73.3%, $n = 11/15$ ($p = 0.14$). β C supplementation led to a reduced tumour incidence in rats fed the EFA 5 diet: 60.0%, $n = 9/15$ ($p = 0.03$), but not in rats fed the EFA 1 diet: 86.7%, $n = 13/15$ ($p = 0.36$) compared to their respective control (96%; 73.3%). No difference in tumour growth was observed between rats fed the EFA 5 and EFA 1 diets without β C (Figure 1). β C led to a decreased tumour growth in rats fed the EFA 5 diet (around a 50% decrease) but not in rats fed the EFA 1 diet (Figure 1).

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The measure of S phase fraction in tumours was not significantly different between groups: 3.8 ± 0.8 and 4.7 ± 0.9 % w/o and + β C respectively in groups fed the EFA 5 diet; and 4.4 ± 0.7 and 3.7 ± 0.9 % w/o and + β C respectively in groups fed the EFA 1 diet (all $p > 0.2$).

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The average MDA content in tumours was 220.6 pmol/mg proteins and not significantly different between dietary conditions (data not shown).

158 **Discussion**

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The objective of this study was to determine whether dietary ALA content alters the effect of β C on mammary carcinogenesis. We provide evidence that β C had an inhibitory effect on tumour incidence and growth in rats fed the recommended EFA ratio of 5 (4% ALA), but failed to act as a protective agent in rats fed an EFA ratio of 1 (16% ALA), which is pretty far above physiological levels in human diets. These data suggested that such a protective effect of β C on tumour growth may be dependent on ALA content in diets, although other nutritional factors associated with ALA in oils might interfere.

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Since β C has been shown to display antioxidant (Sies & Stahl, 1995) or pro-oxidant properties (Palozza, 1998), we assessed the involvement of lipoperoxidation in the decrease of tumour growth. We found that the MDA content in tumours was not modified by β C supplementation whatever the dietary EFA ratio. In agreement with our data, Chew *et al.* did not find any significant difference in lipoperoxides products in transplanted mammary tumours of mice fed β C compared to controls without supplementation (Chew *et al.*, 1999). In contrast, β C was found to decrease the MDA content in colon adenocarcinoma cells supplemented with eicosapentaenoic acid (Palozza *et al.*, 2000). These data suggest that β C could act as an antioxidant in presence of long chain omega-3 fatty acids which are more susceptible to peroxidation than ALA.

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The S phase fraction was determined in tumours as an index of cell proliferation. We did not find any significant difference in S phase fractions between groups, suggesting that decreased tumour growth in rats fed the EFA 5 diet with β C was a consequence of cell loss rather than an inhibition of cell proliferation.

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Pathways implicated in the effects of β C in the present study are not known. However, several mechanisms have been proposed, including notably the modulation of apoptotic signalling (Palozza *et al.*, 2004), of the immune response (Chew & Park, 2004), of the gap junction communications or the regulation of the detoxifying enzymes (Stahl *et al.*, 2002).

185 We conclude that dietary ALA content alters the effect of β C on mammary carcinogenesis.
186 Whether ALA content of diet modifies the protective effect of β C on breast cancer prevention in
187 women needs to be determined and implies that more research be carried out in order to understand
188 the effect of dietary β C supplementation along with omega-3 fatty acids in breast cancer.

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Reference

- Chew BP & Park JS (2004) Carotenoid action on the immune response. *J Nutr* **134**, 257S-261S.
- Chew BP, Park JS, Wong MW & Wong TS (1999) A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. *Anticancer Res* **19**, 1849-1853.
- Cognault S, Jourdan ML, Germain E, Pitavy R, Morel E, Durand G, Bougnoux P & Lhuillery C (2000) Effect of an alpha-linolenic acid-rich diet on rat mammary tumor growth depends on the dietary oxidative status. *Nutr Cancer* **36**, 33-41.
- Colas S, Paon L, Denis F, Prat M, Louisot P, Hoinard C, Le Floch O, Ogilvie G & Bougnoux P (2004) Enhanced radiosensitivity of rat autochthonous mammary tumors by dietary docosahexaenoic acid. *Int J Cancer* **109**, 449-454.
- Hennekens CH, Buring JE, Manson JE, *et al.* (1996) Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med* **334**, 1145-1149.
- IARC Working Group on the Evaluation of Cancer Preventive Agents. (1998a) Carotenoids. In *IARC Handbooks of Cancer Prevention*, pp. 82, 87, 112-115 [IARC, editor]. Lyon: The International Agency for Research on Cancer.
- IARC Working Group on the Evaluation of Cancer Preventive Agents. (1998b) Carotenoids. In *IARC Handbooks of Cancer Prevention*, pp. 137-215 [IARC, editor]. Lyon: The International Agency for Research on Cancer.
- Lyan B, Azais-Braesco V, Cardinault N, Tyssandier V, Borel P, Alexandre-Gouabau MC & Grolier P (2001) Simple method for clinical determination of 13 carotenoids in human plasma using an isocratic high-performance liquid chromatographic method. *J Chromatogr B Biomed Sci Appl* **751**, 297-303.
- Omenn GS, Goodman GE, Thornquist MD, *et al.* (1996) Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med* **334**, 1150-1155.
- Palozza P (1998) Prooxidant actions of carotenoids in biologic systems. *Nutr Rev* **56**, 257-265.
- Palozza P, Calviello G, Maggiano N, Lanza P, Ranelletti FO & Bartoli GM (2000) Beta-carotene antagonizes the effects of eicosapentaenoic acid on cell growth and lipid peroxidation in WiDr adenocarcinoma cells. *Free Radic Biol Med* **28**, 228-234.
- Palozza P, Serini S, Di Nicuolo F & Calviello G (2004) Modulation of apoptotic signalling by carotenoids in cancer cells. *Arch Biochem Biophys* **430**, 104-109.
- Potier de Courcy G, Durand G, Abraham J & Gueguen L (1989) Recommandations sur les conditions d'alimentation des animaux de laboratoire (rats et souris). *Sciences des Aliments* **9**, 209-217.

- Riboli E & Norat T (2003) Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr* **78**, 559S-569S.
- Sies H & Stahl W (1995) Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. *Am J Clin Nutr* **62**, 1315S-1321S.
- Stahl W, Ale-Agha N & Polidori MC (2002) Non-antioxidant properties of carotenoids. *Biol Chem* **383**, 553-558.
- Steghens JP, van Kappel AL, Denis I & Collombel C (2001) Diaminonaphtalene, a new highly specific reagent for HPLC-UV measurement of total and free malondialdehyde in human plasma or serum. *Free Radic Biol Med* **31**, 242-249.
- Steghens JP, van Kappel AL, Riboli E & Collombel C (1997) Simultaneous measurement of seven carotenoids, retinol and alpha-tocopherol in serum by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* **694**, 71-81.
- The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* **330**, 1029-1035.

TABLE 1. Fatty acid composition of commercial and experimental diets and triacylglycerides in rats' adipose tissue.

<u>Fatty acids</u> (mole % of total fatty acids)	<i>TRM diet</i> ¹		<i>EFA 5 diet</i>		<i>EFA 1 diet</i>		
	Diet ²	Diet ³	Adipose tissue ⁴		Diet ³	Adipose tissue ⁴	
			Mean	SD		Mean	SD
Saturates							
16:0	16.3	8.5	15.4	0.4	9.3	17.5	0.5
18:0	2.7	2.4	2.6	0.07	3.4	2.9	0.09
Total ⁵	20.5	13.7	19.5	0.4	15.8	21.9	0.6
Monounsaturates							
18:1n-9 _{cis}	20.6	58.6	56.7	0.4	47.1	48.2	1.5
Total ⁶	24.7	63.0	62.4	0.3	49.8	54.6	1.4
n-6 PUFA							
18:2n-6 _{cis}	48.4	18.3	13.8	0.2	16.7	13.2	1.1
Total ⁷	48.4	18.4	14.3	0.2	17.3	13.5	1.1
n-3 PUFA							
18:3n-3	4.7	3.9	1.3	0.05	16.3	7.0	0.4
Total ⁸	4.8	3.9	1.4	0.05	16.4	7.4	0.4
Ratio							
18:2n-6 _{cis} / 18:3n-3	10.3	4.66	10.63	0.4	1.05	1.9	0.1

¹ Harlan Teklad TRM Rat / Mouse Diet, Harlan, France. ² Fatty acid composition given by the supplier. ³ Fatty acid composition of one sample of each diet. ⁴ Ten rats were randomly selected in each dietary group to provide tissues for fatty acid analyses. ⁵ Including: 14:0, 15:0, 17:0, 20:0, 21:0, 22:0, 23:0 and 24:0. ⁶ Including: 14:1, 16:1, 17:1, 18:1n-7_{cis}, 20:1, 22:1 and 24:1. ⁷ Including: 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:2n-6 and 22:4n-6. ⁸ Including: 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

LEGEND TO FIGURES

FIGURE 1. Effects of β -carotene (β C) on mammary tumour growth according to EFA 5 and EFA 1 diets.

After chemical induction of mammary tumours, rats were randomly assigned to 4 nutritional groups (15 rats/group). Rats were fed either the EFA 5 diet (with an essential fatty acid (EFA) ratio LA to ALA = 5) or the EFA 1 diet (with an EFA ratio of 1), supplemented with β C (10 mg/kg diet/day, +) or not (w/o).

*: In rats fed the EFA 5 diet, tumour growths (*means \pm SEM*) are significantly different between animals with β C and those w/o β C ($p = 0.09$, repeated measures ANOVA with time as grouping factor).

Tumor growths are not significantly different between rats fed the EFA 5 and EFA 1 diets w/o β C or between rats fed the EFA 1 diet with β C and w/o β C ($p = 0.99$, repeated measures ANOVA with time as grouping factor).

FIGURE 1.

