Serum Homocysteine, Cholesterol, Retinol, α-Tocopherol, Glycosylated Hemoglobin and Inflammatory Response during Therapy with Bevacizumab, Oxaliplatin, 5-Fluorouracil and Leucovorin

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Abstract. *Background: Targeted agents present with a new spectrum of side-effects, including toxicities that negatively impact the risk of atherosclerosis. The aim of the present study was to evaluate the effect of the combination of targeted therapy and chemotherapy on serum homocysteine and other laboratory parameters of cardiovascular risk in patients with metastatic colorectal carcinoma. Patients and Methods: Thirty-one patients with metastatic colorectal carcinoma treated with the combination of bevacizumab, oxaliplatin, 5-fluorouracil and leucovorin were studied before and during the therapy. Results: Serum homocysteine decreased significantly throughout the course of treatment. Total cholesterol and low-density lipoprotein cholesterol also decreased significantly during the first month of therapy. In contrast, serum retinol significantly increased during the second and third months of treatment. A significant increase in glycosylated hemoglobin was also observed. After an initial rise, serum C-reactive protein (CRP) and carcinoembryonic antigen (CEA) were significantly lower compared to baseline throughout the course of treatment. Serum ferritin increased throughout most of the course of treatment. A significant correlation was observed between CRP and high-density lipoprotein cholesterol, retinol, ferritin, and CEA. CEA correlated with hemoglobin, retinol, and ferritin. Retinol correlated significantly with hemoglobin. Conclusion: Tumor control, reflected in lower CEA, resulted in suppression of the acute phase response and generally in favorable effects on laboratory parameters indicative of risk factors of atherosclerosis, including lower homocysteine concentrations, and lower total and LDL cholesterol.*

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The prognosis of metastatic colorectal carcinoma has improved substantially during the last decade as a result of the introduction of new effective cytotoxic agents (1), and, more recently, targeted agents, the monoclonal antibodies cetuximab and bevacizumab (2, 3). It has been demonstrated that combination of 5-fluorouracil with either irinotecan or oxaliplatin is, in terms of efficacy, superior to 5-fluorouracil alone (4, 5). The addition of bevacizumab, monoclonal antibody against vascular endothelial growth factor (VEGF), to combination chemotherapy has been shown to increase survival both in the first-line as well as in the second-line setting (2, 6). Although therapy with bevacizumab is, in general, well tolerated, the drug has a peculiar toxicity profile, with the principal side-effects including proteinuria, hypertension and thrombosis. The spectrum of these toxicities is linked to the fact that VEGF is an endothelial growth factor, and VEGF blockade leads to endothelial cell dysfunction (7). These toxicities are usually mild and of little clinical significance in most patients because of their limited life expectancy, but could be of concern in long-term survivors because they are associated with increased risk of atherosclerosis and its complications.

Thus, with the remarkable improvement of long-term prognosis in patients with inoperable metastatic colorectal carcinoma treated with the combination of targeted agents

and chemotherapy that may, when combined with secondary surgery (8), even result in cure in some patients, chronic effects of treatment on the cardiovascular system may become an important consideration. Risk factors of atherosclerosis are complex, and, along with hypertension or hypercholesterolemia, include high serum concentrations of homocysteine, C-reactive protein (CRP) or parameters of oxidative stress (9). The effect of anticancer therapy on laboratory parameters associated with the risk of atherosclerosis, *e.g.* serum lipids, has been well defined for hormonal agents (10, 11), but less is known about the effect of targeted agents or chemotherapy on serum cholesterol or other laboratory parameters of cardiovascular risk.

Homocysteine is an intermediate in the metabolism of methionine. Increased serum concentration of homocysteine is, in most cases, caused by a deficiency of folate or vitamin B12. Hyperhomocysteinemia has been amply documented in patients with vascular disorders, and increased homocysteine levels are a well-defined risk factor of atherosclerosis and thrombosis (12, 13). Hyperhomocysteinemia has also been documented in cancer patients (14-16).

Patients with advanced cancer have laboratory evidence of activation of the acute phase response. CRP is the parameter most widely used to assess the acute phase response. CRP concentrations are also increased in the serum of patients with atherosclerosis, and elevated serum CRP levels predict the risk of future cardiovascular events (17-19).

Administration of cytotoxic agents or biological agents is associated with oxidative stress, and disorders of antioxidant balance are thought to be involved in the toxicity associated with radiotherapy and chemotherapy. Oxidative stress also plays an important role in atherosclerosis as the atherogenic potential of serum lipids is dependent on oxidation (20) and is affected by liposoluble antioxidants (21). Moreover, antioxidant administration may retard atherosclerosis (22). Vitamin E represents the major antioxidant in the serum (23). The term vitamin E denotes several naturally occurring tocopherols and tocotrienols, but alpha-tocopherol is responsible for most of the vitamin E activity in animal tissues. Disorders of antioxidant balance involving vitamin E are also thought to be involved in the toxicity associated with radiotherapy (24), and chemotherapy (25). A decrease in serum alpha-tocopherol has been observed during systemic chemotherapy (26-29). Retinol is a major circulating form of vitamin A that has also antioxidant activity and plays an essential role in many physiological functions, including vision, growth, development and differentiation, and immune response (23). Alterations of iron metabolism are also associated with oxidative stress. In some studies, elevated serum ferritin, an indicator of iron stores, was associated with the risk of acute myocardial infarction (30, 31), and progression of atherosclerosis (22). Serum ferritin concentrations are increased in the acute phase response and, along with other indicators of the acute phase response, ferritin is elevated in cancer patients. Recently, we described a marked increase in serum ferritin in breast cancer patients treated with chemotherapy (32). The aim of the present study was to evaluate the effect of combination therapy with bevacizumab, oxaliplatin, 5-fluorouracil and leucovorin on serum homocysteine and some other parameters of cardiovascular risk in patients with metastatic colorectal carcinoma.

Patients and Methods

Patients. Thirty-one patients, 11 women and 20 men, aged 57±10 (range 33-70) years, with histologically verified metastatic colorectal carcinoma were included in the present study. The intravenous regimen consisted in 29 patients of combination of bevacizumab and FOLFOX7 as follows: bevacizumab (5 mg/kg), oxaliplatin (130 mg/m^2 every 2 weeks), leucovorin (400 mg/m^2) and 5-fluorouracil (400 mg/m^2 bolus and 2400 mg/m^2 for 46 hour infusion) every 2 weeks. Two patients were treated with a modified regimen of bevacizumab, oxaliplatin, 5-fluorouracil and leucovorin combining systemic bevacizumab and hepatic arterial infusion of oxaliplatin. Twenty-two patients were treated in the first line of therapy, and 9 patients were treated in the second or higher line of therapy. Two patients had diabetes mellitus and 3 patients had hyperlipidemia controlled with therapy. Standard premedication before each cycle included intravenous granisetrone or ondansetrone and dexamethasone (16 mg). The project was approved by the Institutional Ethical Committee, and the patients signed informed consent.

Measurement of serum homocysteine, lipids and CRP. Blood samples were taken before the start of therapy, after the first cycle, and before each subsequent cycle of combined therapy (maximum 8 cycles). Homocysteine concentration was determined immunochemically (Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, USA). Serum cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and CRP were determined using commercially available kits on a MODULAR analyzer (Hoffmann-La Roche, Basel, Switzerland).

Analysis of alpha-tocopherol and retinol. Serum alpha-tocopherol and retinol were determined before and during the therapy by high-performance liquid chromatography as described elsewhere (33). Blood samples were drawn from a peripheral vein after an overnight fast. The samples were transferred immediately to the laboratory, centrifuged (1600×g for 10 minutes at 16˚C), the serum was separated and then frozen at –20˚C until analysis. In the liquid-liquid extraction procedure, 500 μl of serum were deproteinized by cool ethanol denatured with 5% methanol (500 μl for 5 minutes at 4˚C). Subsequently, 2500 μl of *n*-hexane were added to this mixture and extracted for 5 minutes by a vortex apparatus. After centrifugation (1600×g for 10 minutes at 0˚C), the aliquot (2000 μl) of the clean extract was separated and evaporated under nitrogen (60˚C). The residue was dissolved in 400 μl methanol and analyzed by reversed-phase high performance liquid chromatography using external standard calibration. The analyses were performed using a Perkin Elmer high-performance liquid chromatography set (Norwalk, USA)

comprising an LC 200 pump, LC 200 autosampler, LC Column Oven 101 thermostat and LC 235C Diode Array Detector attached to the Perkin Elmer Turbochrom Chromatography Workstation version 4.1. Separation of alpha-tocopherol and retinol was performed using Chromolith Performance RP-18e, 100×4.6 mm monolithic columns (Merck, Darmstadt, Germany). As the mobile phase 100% methanol was used at a flow rate of 2.5 ml min^{-1} and column pressure of 3.3 MPa. The block heater LC Oven 101 (Perkin Elmer) was utilized to keep the analytical column temperature at 25˚C. The injection volume was 50 μl. The detection of alpha-tocopherol and retinol was carried out at 295 nm and at 325 nm, respectively.

Determination of hemoglobin, glycosylated hemoglobin, ferritin and carcinoembryonic antigen. Hemoglobin was measured by a photometric method using sodium lauryl sulfate using a Sysmex XE-2100 blood analyzer (Sysmex, Kobe, Japan). Glycosylated hemoglobin was determined by high-performance liquid chromatography using a Variant II Turbo System (Bio-Rad; Hercules, CA, USA) according the instructions of the manufacturer and expressed according the IFCC reference system (34). Serum ferritin concentrations were determined by an immunoassay using commercial kits (AxSYM; Abbott, Chicago, IL, USA). The assays were performed according the manufacturer's instructions. Serum carcinoembryonic antigen (CEA) was determined by radioimmunoassay using a commercial kit (Immunotech; Marseille, France) as described elsewhere (35).

Statistical analysis. The parameters during the therapy were compared with baseline values using the Wilcoxon paired test. Correlations were examined using Spearman's rank correlation coefficient. The decision on statistical significance was based on $p=0.05$ level. The analyses were performed using NCSS software (Number Cruncher Statistical Systems, Kaysville, UT, USA).

Results

As shown in Table I, serum homocysteine decreased significantly throughout the course of treatment. Serum total cholesterol also decreased significantly during the first month of therapy. This decrease affected LDL cholesterol. After an initial mild decrease, HDL cholesterol significantly increased late during the course of treatment. The decrease of total cholesterol during the first month of therapy was accompanied by a reduction in alpha-tocopherol. In contrast, serum retinol significantly increased during the second and third months of treatment. A significant increase in glycosylated hemoglobin was also observed at several visits. After an initial significant increase, CRP significantly decreased compared to baseline throughout the course of treatment. Hemoglobin concentration remained relatively stable, but serum ferritin increased throughout most of the course of treatment. The course of CEA mirrored concentrations of CRP. After an initial rise, that was, however, not significant, a significant decrease of serum CEA was observed starting from the second month of therapy.

Significant negative correlations were observed between CRP and HDL cholesterol ($r_s=–0.37$, $p<0.05$), retinol ($r_s=–0.70$, $p<0.0001$; Figure 1) and positive correlations

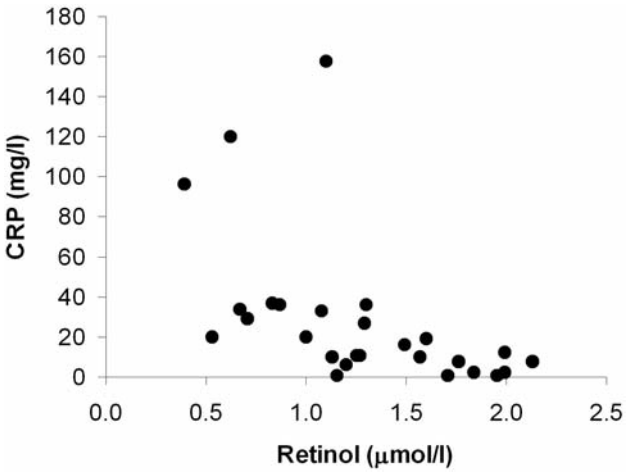

Figure 1. *Correlation between C-reactive protein and retinol ($r_s=–0.70$, $p<0.0001$).*

were noted between CRP and ferritin ($r_s=0.52$, $p<0.01$), and CEA ($r_s=0.60$, $p<0.001$). CEA correlated inversely with hemoglobin ($r_s=–0.36$, $p<0.05$), and with retinol ($r_s=–0.60$, $p<0.001$), and positively with ferritin ($r_s=0.42$, $p<0.05$). Retinol correlated significantly with hemoglobin ($r_s=0.77$, $p<0.00001$). Alpha-tocopherol correlated significantly with total cholesterol ($r_s=0.48$, $p<0.01$) and LDL cholesterol ($r_s=0.42$, $p<0.05$), and total cholesterol correlated significantly with LDL cholesterol ($r_s=0.95$, $p<0.00001$).

Discussion

The present data demonstrate that bevacizumab administered with combination chemotherapy has a complex short-term effect on laboratory measured risk factors of atherosclerosis that includes changes both favorable and unfavorable for the long-term risk of atherosclerosis. Some of these changes are probably due to the effect of combined therapy on metastatic tumor. Reduced tumor burden results in lower serum CEA and CRP concentrations. Suppression of the systemic inflammatory response also results in high retinol and, possibly, lower homocysteine concentrations.

The serum concentrations of cholesterol and LDL cholesterol decreased markedly during the first cycle of therapy. Non-fasting samples were used for lipid analyses in the present study, similarly to some earlier reports that examined the effect of anticancer agents (36). In cancer patients receiving emetogenic chemotherapy, it is difficult to require an overnight fast before chemotherapy repeatedly. The decrease of serum total and LDL cholesterol was evident mostly during the first month of therapy. Later in the course of treatment, an increase of HDL cholesterol was observed that could also have a favorable impact on the progression of atherosclerosis.

Table I. *Laboratory parameters during therapy.*

Parameter	Baseline	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10
Time from the start of treatment (days)	–2±4	7±1	15±4	35±7	52±14	65±11	81±15	101±14	114±14	135±16
n	31	30	31	31	30	28	27	22	18	11
Homocysteine (μmol/l)	12.9±6.1	9.0±3.1[e]	11.0±4.01	9.9±3.7[b]	9.2±3.4[c]	9.1±2.5[c]	9.8±2.9[b]	8.5±2.0[c]	8.9±2.0[a]	8.0±1.71
Total cholesterol (mmol/l)	5.6±1.7	4.7±1.3[e]	4.8±1.1[b]	5.2±1.4	5.1±1.0	5.5±1.5	5.4±1.0	5.3±1.2	5.2±0.8	4.8±0.8
LDL cholesterol (mmol/l)	3.8±1.4	2.8±1.1[e]	3.1±1.0[b]	3.3±1.3	3.2±0.9	3.4±1.4	3.4±1.1	3.2±0.9	3.3±0.6	2.8±0.6[a]
HDL cholesterol (mmol/l)	1.21±0.37	1.16±0.41[a]	1.21±0.35	1.23±0.34	1.28±0.34	1.38±0.43[a]	1.35±0.38[b]	1.31±0.30	1.31±0.34[a]	1.34±0.33[a]
Retinol (μmol/l)	1.29±0.47	1.31±0.50	1.33±0.48	1.41±0.46[a]	1.44±0.47[a]	1.56±0.54c	1.50±0.60[b]	1.34±0.47	1.37±0.46	1.23±0.53
Alpha-tocopherol (μmol/l)	25.7±5.5	21.5±5.3[d]	22.6±5.93[c]	24.5±5.3[a]	25.2±6.1	26.5±7.0	25.3±6.0	24.2±6.1	25.3±7.0	21.8±5.7
Glycosylated hemoglobin (%)	3.6±0.8	3.7±0.9	3.8±0.8[a]	3.9±0.9[c]	3.8±1.0	3.9±1.0[a]	3.7±1.0	3.8±1.0	3.6±1.1	3.1±0.4
C-reactive protein (mg/l)	35±49	51±54[c]	19±25[b]	19±25[b]	16±19[c]	17±25[b]	15±27[b]	10±13b	10±11[b]	22±26
Hemoglobin (g/l)	125±16	127±14	123±17	123±15	121±14	119±16[a]	121±17	119±21	117±18[a]	119±14
Ferritin (μg/l)	150.3±197.6	332.9±335.1[d]	171.8±169.3[a]	177.1±159.6[a]	152.6±139.5	182.7±151.4[a]	190.0±164.8[a]	393.9±861.9[b]	385.6±773.1	196.1±131.6
Carcinoembryonic antigen (μg/l)	565.2±1071.4	957.3±2122.8	933.8±2194.9	553.8±1264.0[c]	389.1±775.0[c]	401.3±774.9[b]	285.9±724.4[c]	119.2±230.1[b]	175.0±284.1[a]	234.4±375.8[a]

Mean±standard deviation of the respective parameters are shown. [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$; [d]$p<0.0001$ and [e]$p<0.00001$ compared to baseline.

With some limitations, serum CEA concentrations may reflect tumor burden (37). With a latency, after an initial rise that was, however, not significant, serum CEA decreased, indicating reduced tumor mass resulting from the effective anticancer therapy. An initial surge of serum CEA concentrations after the start of treatment that is indicative of therapeutic response has been described in patients with metastatic colorectal carcinoma (38, 39).

Serum CEA concentrations correlated significantly with serum CRP, an indicator of the acute phase response. An increase in laboratory parameters of the acute phase response has been well described in patients with metastatic colorectal carcinoma (40). The kinetics of CRP was similar to CEA, including the initial surge. The decrease of CRP concentration was linked to the lowering of the circulating homocysteine concentration, suggesting an association between inflammatory response and homocysteine that has been postulated earlier (14, 41). As CRP is predictive of cardiovascular event risk (9, 17-19, 42), a marked decrease of CRP after combined therapy could have a potentially favorable effect on the risk of cardiovascular events.

In association with the acute phase response, serum ferritin concentrations increased significantly after the start of treatment, but remained, in contrast to serum CRP concentrations, increased compared to baseline. This observation is in agreement with our earlier observation of increased ferritin in breast carcinoma patients treated with neoadjuvant chemotherapy (32). While the early marked increase in serum ferritin may reflect the activity of the acute phase response, sustained elevated ferritin levels may be caused by transient fluctuations of serum iron concentrations that accompany the administration of chemotherapy (32, 43-45).

Homocysteine concentrations decreased significantly throughout the course of therapy. Homocysteine may be produced by tumor cells (15), and the decrease of serum homocysteine after combined therapy may be explained by inhibition of tumor growth resulting from the treatment (16). The published information on homocysteine in patients treated with chemotherapy or targeted therapy is limited. A decrease in serum homocysteine has been documented after the administration of ifosfamide/mesna (46), but an increase was observed after methotrexate administration (47). A decrease of circulating homocysteine has also been described after leucovorin administration (48). The changes of circulating homocysteine concentrations observed in the studies mentioned above are result of the effect of anticancer agents on homocysteine metabolism, rather than the effect on cancer cells. The moderate but significant decrease in serum homocysteine observed in the present study may be caused by administration of high-dose leucovorin during the therapy, but may also reflect inhibitory action of chemotherapy on tumor cell proliferation. The magnitude of changes in homocysteine

concentrations observed in the present study was similar to the decrease observed in an earlier study in patients treated with 5-fluorouracil/leucovorin (48). With the exception of the initial surge, serum homocysteine concentrations followed CEA levels. Homocysteine may have different dynamics from other tumor markers as its concentrations reflect tumor cell proliferation and the number of live cells (15).

Administration of chemotherapy is associated with oxidative stress that results in lower concentrations of circulating antioxidants. Moreover, administration of chemotherapy is associated with small bowel dysfunction, and disturbances of the small bowel function are accompanied by a low serum concentration of retinol (49-51). The present observation of a decrease in alpha-tocopherol is in agreement with earlier reports of decreased alpha-tocopherol concentrations during systemic chemotherapy that were associated with oxidative stress induced by the therapy (26-29). However, in a recent study we observed increased alpha-tocopherol and retinol concentrations during paclitaxel/carboplatin combination chemotherapy (52) and hypothesized that these increases are probably linked to the suppression of the systemic inflammatory response by chemotherapy. Serum concentrations of alpha-tocopherol and retinol are significantly decreased in patients with advanced cancer (53, 54), and this decrease correlates with the systemic inflammatory response (53, 55). In the present cohort of patients, we did not demonstrate an increase in serum alpha-tocopherol, but an increase in serum retinol was evident. Significant negative correlation was observed between CRP and retinol concentrations. The suppression of the systemic inflammatory response associated with tumor control could thus explain the increase of serum retinol observed later during the course of treatment in the present study.

In earlier studies, low vitamin E intake was associated with toxicity of chemotherapy in children with acute lymphoblastic leukemia (56). The administration of vitamin E has been shown to alleviate some side-effects of radiotherapy (24), and chemotherapy (25). Although serial monitoring of vitamin E levels may be a necessary prerequisite for any therapeutic use of this antioxidant vitamin, serum vitamin E is not being routinely measured in cancer patients, and, in most reports published so far, alpha-tocopherol has been investigated in epidemiological studies in relation to cancer risk. In the present study, low serum alpha-tocopherol was associated with a decreased cholesterol concentration and could be linked to the toxicity of chemotherapy and resulting lower dietary intake. Lower alpha-tocopherol concentration may have unfavorable long-term effects on the progression of atherosclerosis. In contrast to cholesterol or homocysteine, glycosylated hemoglobin increased significantly during the treatment. An increase in circulating VEGF has been described in patients with diabetes mellitus that has been associated with the presence of complications (57-59). The moderate increase of glycosylated hemoglobin may be caused by the administration of dexamethasone as part of premedication or the effect of chemotherapy rather than bevacizumab.

Thus, the administration of bevacizumab and combined chemotherapy has effects on laboratory parameters that both increase and decrease the risk of atherosclerosis. Along with hypertension (7), VEGF inhibition causes proteinuria, another risk factor of cardiovascular mortality (60). Data from the present cohort also indicate that the administration of combined treatment may be associated with impaired glucose tolerance. The concentrations of alpha-tocopherol, the major liposoluble antioxidant, decreased during the treatment, but retinol concentrations increased. Alterations in iron metabolism, reflected in increased ferritin concentrations, and associated oxidative stress may also accelerate the progression of atherosclerosis. On the other hand, therapy led to lower cholesterol and homocysteine concentration and a decrease in systemic inflammation reflected in lower CRP levels. The suppression of the systemic inflammatory response could have also resulted in increased HDL-cholesterol levels. Most of these unfavorable changes were moderate in magnitude and of limited duration, so it cannot be expected that these changes would have a dramatic effect on the progression of atherosclerosis.

In the past, the long-term risk of atherosclerosis was of little concern in patients with metastatic cancer. In the last decade, data have emerged indicating a significant rise of the incidence of complications of atherosclerosis in survivors of metastatic cancer curable with chemotherapy, *e.g.* germ cell tumors (61-63). Although the cure rate of metastatic colorectal carcinoma is much lower compared, for example, to metastatic testicular carcinoma, the survival of patients with metastatic colorectal carcinoma has improved dramatically over the past two decades (1), with median survival currently being close to 2 years (64). In addition, a significant proportion of patients with initially unresectable tumors become candidates for secondary resection. In a recently published report, the 5- and 10-year survival of patients undergoing secondary resection was 33% and 27%, respectively (8). Thus, a significant proportion of metastatic colorectal carcinoma patients survive, under therapy, more than 5 years, and the question of how chronic therapy affects the long-term risk of cardiovascular disorders is of legitimate concern. Thus, the observation that the combination of VEGF-targeted therapy and chemotherapy has, besides unfavorable effects on hypertension and proteinuria, also favorable effects on laboratory risk factors of atherosclerosis is reassuring from the point of view of clinical practice.

In conclusion, combination therapy with bevacizumab, oxaliplatin, 5-fluorouracil and leucovorin is an effective therapy that results in marked decrease of serum CEA. Tumor control also resulted in suppression of the acute phase response, reflected in CRP concentrations. CRP correlated negatively with retinol and HDL-cholesterol, and positively with CEA and

ferritin. Tumor control after administration of combined therapy resulted in generally favorable effects on laboratory parameters indicative of risk factors of atherosclerosis, including decreased homocysteine concentrations, lower total and LDL cholesterol, but some changes associated with the treatment, including increased ferritin and increased glycosylated hemoglobin might have a negative impact on the risk of atherosclerosis.

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