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# Dietary omega-3-polyunsaturated fatty acids prevent the development of metastases of colon carcinoma in rat liver

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**Abstract** *Background* Fish oil consisting of omega-3 polyunsaturated fatty acids (PUFA) seems to reduce the incidence of colon cancer. The effect of PUFAs on metastasis of colon carcinoma is still unclear. *Aim* The study was designed to examine the effects of a diet rich in omega-3-PUFAs on a model of colorectal metastasis. *Methods* Thirthy animals (WAG/Rij) were randomly assigned to receive an omega-3 diet or a control diet to evaluate their effect on tumor growth. The target male rats (WAG/Rij) were fed a diet containing 15% omega-3-fatty acids three days before and 28 days after intervention and the control rats received 15% coconut oil at the same time points. CC 531 cells, a moderately differentiated colon adenocarcinoma, were injected into the spleen of each rat. After 28 days of diet, animals were sacrificed. The tumor growth was evaluated macroscopically and microscopically in liver tissue. The tissue was examined after immunostaining and the use of monoclonal antibodies. *Results* PUFAs decreased the index of tumor load from 1.54 in the controls to 0.79 in the treatment group ($P = 0.036$). While 69.2% of the control animals were tumor positive, only 21.4% of the target animals showed tumor after omega-3-fatty acid ($P < 0.05$). *Conclusion* We could show that omega-3-fatty acids may decrease malignant metastatic tumor growth in the liver.

**Key words** colon carcinoma – liver metastasis – nutrition – omega-3 fatty acids – tumor growth

## Introduction

### Background

Colon cancer is one of the most prevalent cancers in the Western world. A relationship has been shown to exist between the incidence of colon cancer and total fat consumption, as well as the quality of fat [1]. In particular, epidemiological studies have shown a reduced incidence of colon cancer among populations consuming a large quantity of n-3 polyunsaturated fatty acids (PUFAs) of marine origin [2–4]. The underlying mechanisms for anti-tumoral effects of PUFAs such as eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) are hypothesized as reduction of PGE-2 and NO synthase levels as well as an increased rate of lipid peroxidation at the cell membrane [5, 6, 1]. Furthermore, the tumor growth suppressing mechanism of omega-3 fatty acids in vitro is thought to be due to the following factors: the generation of eicosanoids mediators with less biological activity compared to those derived from omega-6 PUFAs [7]; and the modulation of signal transduction and gene expression with sub-

sequent induction of apoptosis [8]. In addition, PUFAs decrease cancer cell adhesion to the subendothelial extracellular matrix in vitro [9]. Moreover, experimental studies demonstrated that these fatty acids inhibit carcinogen-induced colon tumorgenesis in rats [10], reduce growth of transplantable colon carcinoma implanted in mice [11, 12], and induce apoptosis in colorectal tumor cells cultured in vitro [13, 14]. In addition, these fatty acids exert additional beneficial effects in neoplastic pathology, such as inhibition of tumor metastasis [15] and angiogenesis [16].

Different studies underlined the anti-metastatic effect of omega-3 PUFAs compared to omega-6 PUFAs in a lung metastasis model [17, 18]. In a study focused on colon carcinoma metastasis in the liver after a diet rich in omega-3 fatty acids, however a promoting effect on cancer growth was shown [19]. Griffini et al. concluded that due to the complexity of liver metabolism and the special PUFA metabolism within the liver cells (i.e. Kupffer cells, macrophages and other immune cells), the anti-tumoral effect of PUFAs was limited [19].

The findings of Griffini et al. have an important impact on the treatment of cancer patients with liver metastasis who suffer from cachexia. However, their results contradict most other studies, which focused on PUFAs and tumor growth. Therefore, we developed a rat model that focuses on the effects of PUFAs and liver metastasis of colon carcinoma. The metastatic cascade involves various processes such as cell-detachment and cell-attachment [20, 21]. The metastatic potential, invasiveness, and tumor progression in different gastrointestinal carcinomas are associated with tumor-dependent cell adhesion molecules such as ICAM-1, VCAM-1 and CD44 [9]. Changes in their expression, can be an indication for metastatic activity [22–24]. Furthermore, the activity of immune competent cells, such as macrophages, correlates with tumor progression [25]. Therefore, the monoclonal antibody HIS36, a marker for the expression of macrophages has been used here to investigate the influence of PUFAs on colon carcinoma liver metastasis. The expression of CC 52, VCAM, HIS 36 and CD44 in the liver parenchyma of rats fed with a omega-3 PUFA enriched diet have been analysed.

## Methods

### Animals

Thirty male inbred WAG/Rij rats weighing 150–200 g were kept under standard laboratory conditions. Animals were randomized to the treatment or to the control diet for 28 days starting 3 days before injection of CC531 tumor cells into the spleen. Animals in both groups had access to their diet ad libitum. At the end of the study, all animals were subjected to various analyses as described below. All experimental procedures were approved by the National Research Council and complied with national legislation governing the care and use of laboratory animals. All experiments were carried out in accordance with institutional guidelines for animal care.

### Tumor cell line

CC 531 is a 1,2 dimethylhydrazine-induced weakly immunogenic, moderately differentiated colon adenocarcinoma, transplantable in syngeneic WAG/Rij rats. The cells were cultured in RPMI 1640 medium supplemented with 2% Hepes buffer solution, 1% L-glutamine, 1% streptomycin penicillin and 5% fetal calf serum at 37°C in an atmosphere of 5% carbon dioxide. Before use, cells were washed with phosphate-buffered saline, trypsinized for 5 min, centrifuged for 5 min at 1200 rpm, resuspended and counted. Viability, which always exceeded 95%, was tested with the trypan blue method. To establish tumor growth, 500,000 cells/ml were injected.

### Interventions

#### Feeding model and diets

The experimental diets were prepared by the SSNIFF Spezialdiäten GmbH (Soest, Germany; http://www.ssniff.de/index.php?pcid=9&pdid=15) and were based on a non-purified commercial diet. The composition included crude protein, fat, fiber, mineral water and carbohydrates, which were presented in detail in Table 1. The diets contained either 15% Omega-3-fatty acids (DHA, EPA) or 15% of coconut oil (90.5% saturated fatty acid/SFA), in order to keep them isocaloric and isonitrogenious. The EPA: DHA ratio in the Omega-3 diet was relatively 3:2. The diets were stored in the dark at 3°C. They were not formed in pellets. The animals had free access to the diet and water. The diet was started 3 days before tumor cell injection and was maintained for 4 weeks until the rats were sacrificed.

#### Anesthesia

The rats were anaesthetized by intraperitoneal injection of ketamine 100 mg/kg and xylatinhydrochlond (2%) 10 mg/kg.

#### Intervention and induction of liver metastasis

After a 3 cm conventional median laparotomy and exploration of the abdomen, the spleen was grasped

**Table 1** Calculated contents of the diet supplemented with n3- and n6-PUFA. (http://www.ssniff.de/index.php?pcid=9&pdid=15)

| **Pure nutrients** | **(%)** |
|---|---|
| Dried substance | 87.9 |
| Crude protein | 21.2 |
| Crude fat | 3.8 |
| Crude fiber | 4.4 |
| Starch | 35.0 |
| Sugar | 5.0 |
| **Mineral ingredients** | **(%)** |
| Calcium | 1.00 |
| Phosphorus | 0.70 |
| Natrium | 0.25 |
| Magnesium | 0.21 |
| Kalium | 1.03 |
| **Vitamins** | **(Per kg)** |
| Vitamin A | 15,000 IE |
| Vitamin D3 | 1,000 IE |
| Vitamin E | 110 mg |
| Vitamin K (Menadion) | 5 mg |
| Thiamin (B1) | 18 mg |
| Riboflavin (B2) | 23 mg |
| Pyridoxin (B6) | 21 mg |
| Cobalamin (B12) | 100 µg |
| Nicotinic acid | 115 mg |
| Pantothenic acid | 42 mg |
| Folic acid | 7 mg |
| Biotin | 510 µg |
| Choline-Cl | 3.150 mg |
| Inositol | 100 mg |
| **Fatty acids** | **(%)** |
| C 14:0 | 0.01 |
| C 16:0 | 0.49 |
| C 16:1 | 0.02 |
| C 18:0 | 0.12 |
| C 18:1 | 0.81 |
| C 18:2 | 2.03 |
| C 18:3 | 0.26 |
| C 20:0 | 0.01 |
| C 20:1 | 0.01 |
| C 20:5 = EPA | 1.96 |
| C 22:6 = DHA | 1.27 |

with forceps and punctured with a 0.4-mm cannula under direct vision. The tumor cell suspension (0.1 ml containing 50,000 cells/ml) was injected and the needle was removed. The following 5 min the punctured area was covered by a cotton pad. Afterwards, the abdominal wall was closed using running sutures (Vicryl 4-0). The animals were fed for 25 days according to the randomization. Thereafter, they underwent relaparotomy and the portal vein was cannulated. Two milli liter of PPSB solution was injected into the portal vein to stabilize the liver tissue for immunohistochemistry after portal vein clamping. The animals were sacrificed under general anesthesia. All animals were evaluated as described on the following paragraphs.

## Macroscopic evaluation

For macroscopic evaluation organs were not removed from the abdominal cavity and only superficial lesions were taken into account.

The intraperitoneal tumor growth was scored by diameter and number of tumor nodules, and by a semiquantitative cancer index (Tables 2, 3). The index was assessed by two independent observers. In case of disagreement, the lower score was accepted. Tumor growth was evaluated in the subcutis (abdominal wall metastases), parietal peritoneum, mesentery, liver, kidney, retroperitoneum, and omentum.

## Microscopic evaluation

At the end of the experiment the liver was removed immediately and was split into its four lobes and examined immunhistochemically. For each lobe ten randomly selected areas were evaluated. In each section the immunocompetent cells were quantified in ten standardized microscopic fields using a Zeiss microscope and a Neofluar objective. The number and intensity of the immunohistochemical stained cells were evaluated. Data are expressed in an immuno-reactive score (IRS) (Table 4). The differences were assessed in a semi-quantitative manner.

## Immunostaining and monoclonal antibodies

The liver was immediately frozen in liquid nitrogen and stored at −80°C until analysis. The frozen tissue was cut in 8-µm layers. Monoclonal antibodies of anti-ICAM-1 (clone M-19, Santa Cruz Biotechnology®, Vienna Austria), anti-VCAM-1 (clone, Santa Cruz Biotechnology®, Vienna Austria), anti-CD 44std (clone Pgp-1, Pharmingen®, San Diego, USA), anti-CC52 (ARA01, Dornenburg, The Netherlands) and

**Table 2** Tumor yield and cancer index in control and treatment animals after 28 days

| | No. of rats | Tumor bearing | Mean cancer index | | |
|---|---|---|---|---|---|
| | | | Intra-hepatic | Extra-hepatic | Overall |
| Control group | 13 | 9 | 1.38 ± 0.61 | 1.23 ± 1.39 | 1.54 ± 1.65 |
| Omega-3 group | 14 | 3* | 0.79 ± 1.74** | 0.14 ± 1.29** | 0.79 ± 2.03** |

Tumors were found in 9 out of 13 animals in the control group compared to 3 out of 14 animals in the omega-3 PUFA group (*$P = 0.021$). The average Cancer Index of the control group was significantly higher than that of the omega-3 PUFA group (**$P = 0.036$).

**Table 3** The cancer index applied as a grading system for tumor cells according to the diameter of the metastatic nodules in liver parenchyma

| Tumor grade | Size of tumor nodules (diameter in cm) |
|---|---|
| 0 | No tumor |
| I | <0.5 |
| II | 0.5–1 |
| III | 1.1–2.0 |
| IV | 2.1–3.0 |
| V | >3.0 |

anti-HIS 36 (monoclonal clone, Pharmingen®, San Diego, USA) were respectively used against ICAM-1, VCAM-1, CD 44v6, CC 52 and HIS 36 for immunohistochemistry. For secondary staining unconjugated anti-ICAM-1, anti-VCAM-1, anti-CD44 std and anti-HIS 36 were incubated with rabbit-anti-mouse IgG HRP, (DAKO, Carpinteria, Calif., USA). For tertiary staining swine-anti-rabbit IgG HPR (DAKO, Carpinteria, Calif., USA) was applied for isotype control mouse IgG1; FITC (Bende Med Systems®, Vienna Austria).

### Randomization

Animals were randomly allocated to the dietary groups. A randomization list with the assigned diet for each animal was designed using a commercial statistic randomization program before the trial started. Because of the homogeneity of the groups no stratification was necessary. Animals received the diet in assigned numbered food containers. Each animal received a prepared experimental diet based on a non-purified commercial diet. Both the animal surgeons as well as the two independent observers evaluating the tumor growth were blinded with respect to the diets being fed.

### Statistical analysis

The data is presented as mean standard deviation. To analyze the data for significant differences between groups, the Kruskal–Wallis (global test) and the Dunn test with alpha correction were performed as appropriate.

## Results

In controls 13/15 animal were eligible for analysis while in the study group 14/15 animals completed the whole study (Table 2). The deaths were most likely associated with the surgical cell injection into the spleen. Animals died between postoperative days 4–5.

Weight gain over the 28 days of feeding was not significantly different between the omega-3-fatty acid (11.8 g ± 2.3) and control groups (12.7 g ± 2.6) ($P > 0.05$). Tumors were found in 9 of 13 control animals (69.2 %) and 3 of 14 animals after omega-3-fatty acid diet (21.4%) ($P = 0.021$) (Table 2). Tumor growth was evaluated using the defined cancer index as published by Bouvy et al. [26] (Table 3).

In the controls, four animals carried metastasis with a diameter of <0.5 cm (Cancer Index Grade I). Metastatic tumor growth was found in hepatic tumor nodules of <2 mm in diameter in only one liver segment. Three control animals had tumor metastasis outside the liver as well. The mean number of nodules found in controls was 3.8 (3–15), while after omega-3 fatty acid enriched diet only 1.6 (0–10) was observed ($P = 0.063$).

The mean cancer index of the omega-3 fatty acid group was less (0.79) than the index of the control group (1.54) ($P = 0.036$) (Table 2), Two out of the three animals that carried tumor metastasis in the omega-3 fatty acid group had a cancer index of five, as did two animals in the controls. Furthermore, two control animals had a cancer index of two and four animals had a cancer index of one. Just one rodent of the omega-3 fatty acid group had metastasis outside the liver (small bowel), compared to six animals of controls. The mean extra- and intrahepatic cancer index in controls was 1.23 and 1.38, respectively. In contrast, after omega-3 fatty acid enriched diet this index was 0.14 and 0.79 respectively (Table 2).

**Table 4** Comparison of the control and treatment groups based on the semi-quantitative immune-reactive scoring (IRS) system, which was measured by the intensity of staining and the number of positive cells

| Immuno-reactive score (IRS) | CC 52 | | VCAM | | CD 44 | | HIS 36 | |
|---|---|---|---|---|---|---|---|---|
| | Control | Omega-3 | Control | Omega-3 | Control | Omega-3 | Control | Omega-3 |
| 0 | 0 | 3 | 0 | 4 | 9 | 11 | 0 | 1 |
| + | 8 | 7 | 6 | 5 | 2 | 2 | 3 | 4 |
| ++ | 1 | 2 | 4 | 3 | – | – | 5 | 6 |
| +++ | 2 | 1 | 1 | 1 | – | – | 3 | 2 |
| ++++ | – | – | – | – | – | – | – | – |

0 No staining
+ Weak staining and few cells
++ Middle strong staining and some cells
+++ Strong staining and many cells
++++ Very strong staining and cell fusion

Immunocompetent cells were mainly found surrounding the bile ducts and periportal endothelium cells. CC 52 and VCAM positive cells were found in all control animals, while three animals of the omega-3 fatty acid group had no positive CC52, and four had no positive VCAM cells (Table 4). ICAM positive cells were found in neither of the two groups. The immune-reactive score (IRS) for tumor cells was semi-quantitatively calculated based on the intensity of staining and the number of positive cells. IRS of HIS36 and CD44 appeared nearly identical in both groups (Table 4).

## Discussion

This study clearly supports the hypothesis that a high-fat diet enriched with omega-3 PUFAs reduces the development of colonic carcinoma metastasis in rat liver. These data corroborate prior reports on omega-3 fatty acids, mainly EPA and DHA, preventing primary tumor growth in vitro [17, 18, 27–29]. For example, Rose et al. showed an inhibitory influence of a diet containing fish oil, compared to a diet enriched with omega-6 PUFAs on primary tumor growth of human breast cancer cells and metastasis in nude mice [30]. In another study, Wu et al. in an in vitro study reported that via activation of a neutral sphingomylinase-mediated pathway, omega-3 PUFAs such as EPA and DHA hindered the tumor growth of breast cancer in rat [31]. Similarly, Calder et al. were able to demonstrate that a high-fat diet enriched with omega-3 fatty acids slowed the growth of human primary colon tumor growth in athymic mice in contrast to a high-fat diet enriched with coconut oil, olive oil and safflower oil [32]. Furthermore it is suggested that omega-3 PUFAs also inhibit primary tumor growth in vivo [33–36]. The study of Calviello et al. investigating the anti-tumor effect of EPAs and DHAs on the growth of Morris hepatocarcinoma 3924A in ACI/T rats found a reduction of tumor growth by both omega-3 fatty acids compared to a control group treated with oleic acid through different mechanisms. The proliferation of tumor cells and apoptosis differed depending on the diet. The authors concluded that EPA has an anti-tumor effect mainly by inhibiting cell proliferation, whereas DHA induces apoptosis [37]. Additionally, Heukamp et al. showed that n-3 PUFA significantly dicreased the number of visible pancreatic tumors and incidence of histological-proven liver metastasis in hamsters with induced ductal pancreatic adenocarcinoma as compared to n-3, n-6 and n-9 PUFAs as well as normal fat diet groups [38].

Our data support the hypothesis that tumor growth is reduced by PUFAs. However, our focus was primarily on liver metastases of colon carcinoma, since the liver is the main organ which metabolizes omega-3 fatty acids. It is likely that the antiproliferative, antiangiogenic, and antimetastatic effects of PUFAs are at least in part due to a reduction in tumor-derived inducible NO (iNOS) [39–41]. This phenomenon has a great influence on the complex mechanism of development of liver metastasis.

Metastatic cells reach a distant secondary focus via lymphogenous or hematogenous spread [20], where they attach to the local endothelium, extravasate and proliferate. EPA is known to decrease cancer cell adhesion in vitro [9]. Due to the fact that almost all animals in our study had immunocompetent cells within the periportal zone, especially around the endothelium cells, we conclude that the metastatic cascade was mainly seen at the stage of cell attachment four weeks after cancer cell administration. The macroscopic findings underline this hypothesis, since only one rodent of the omega-3 fatty acid group developed metastasis outside the liver, compared to five animals in the control group.

High expression of VCAM is believed to promote the adhesion of circulating tumor cells to the endothelium [21, 23, 25]. Moreover, it has been reported that DHA attenuates the expression of VCAM-1 [42, 43]. This hypothesis was confirmed in our trial, as the higher rate of liver metastasis in the control group correlated with VCAM positive cells in all animals as compared to VCAM positive cells in nine animals of the omega-3 fatty acid group (69.2%). The total expression of CD 44 and ICAM in both groups was only marginal. However, macroscopic tumor growth differed significantly between the two groups concerning tumor load ($P = 0.021$) and the cancer index ($P = 0.036$). In addition, the average number of tumor nodules differed (3.8 in the control group and 1.5 in the omega-3 fatty acid group); however this difference was not significant ($P = 0.063$).

A diet with a moderate concentration of omega-3 fatty acids can possibly reduce tumor growth of colon carcinoma cells by the reduction of tumor spread at an early stage. These results stand in contrast to the findings of Griffini et al. [19] In their study the proliferation of colon cell tumors in the liver was not reduced by omega-3 fatty acids. This difference is even more interesting as their study design included a colon metastasis model in rat liver using the same cancer cell line (CC 531) we were using. The authors concluded that omega-3 fatty acids inhibit the growth of primary tumors (concerning the results of the studies described above) and promote the growth of colon cancer metastasis in the liver. Griffini et al. explained these results through the type of proliferation and aggressiveness of colon cell tumors and the increased uptake of adenosine and guanosine [19], caused by omega-3 fatty acids [44]. According to the authors, the increase in purine uptake within the

cancer cells may be partly responsible for the rapid growth of colon cancer cells in the liver on a fish oil diet. At a first glance, our results seem to contradict Griffini et al. The control groups (low fat and safflower oil to coconut oil in our study) and the concentration of omega-3 fatty acids in the diet (70% to 15% in our study) differed fundamentally. The extreme diet rich in fat selected in the trial of Griffini et al. represents a highly artificial situation with the consequence that the results may not reflect the physiological situation. In addition, the time of feeding before the administration of colon cancer cells was longer (3 weeks) in their study than in our study (3 days). If the effect of omega-3 fatty acids, as Griffini et al. suggest, is liver specific, this effect might depend on the time period of feeding and the concentration of the diet. This hypothesis corroborates the findings of Beck et al. [45] who could describe an early reduction of tumor growth followed by an enhanced growth of the tumor.

In conclusion, omega-3 fatty acids reduced the development of metastasis and prevented tumor growth in vivo, but the mechanism of prevention is unclear. One possible mechanism is an affect on apoptosis. This was demonstrated in a study on the preventive effects of omega-3 PUFAs on colorectal carcinogenesis in patients polypectomized for colorectal adenomas/tumors, which demonstrated a promotion of apoptosis of normal colon mucosa by increased intake of omega-3 PUFAs [46]. Other possibly beneficial effects or induction of apoptosis by pre-treatment with PUFAs, arachidonic (AA, 20:4, omega-6) or docosahexaenoic (DHA, 22:6, omega-3) acids on HT-29 cells (a human colon adenocarcinoma cell line) might be caused by an alteration of cell membrane lipid composition and potentiation of oxidative processes, which are accompanied by changes in mitochondria and followed by stimulation of cell apoptotic cascade [47].

Further investigations should examine this protectiove mechanism in order to clarify to what extent dietary interventions have an influence on metastasis and whether its effect is liver-specific. This is of great importance since omega-3 PUFAs seem to play a key role in diets that prevent tumor associated cachexia.

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