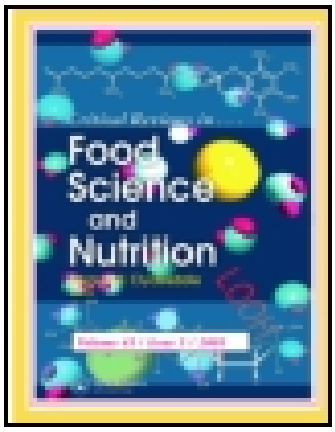

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Long-Chain n-3 PUFA against breast and prostate cancer: which are the appropriate doses for intervention studies in animals and humans?

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Long-Chain n-3 PUFA against breast and prostate cancer: which are the appropriate doses for intervention studies in animals and humans?

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Abstract

The potential antineoplastic effect of the long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) remains a highly controversial issue. Numerous animal studies have supported the anticancer role of these dietary fatty acids, whereas conflicting results have been obtained in population studies, and only a few intervention human trials have been so far performed. In view of the possibility that the anticancer effects may be maximally observed within a defined range of EPA and DHA doses, herein we critically review the results and doses used in both animal studies and human clinical trials focusing on the possible n-3 PUFA protective effects against breast and prostate cancer. Our main aim is to identify the EPA and/or DHA ranges of doses needed to obtain clear anticancer effects. This may be of great help in designing future animal studies, and also in understanding the most appropriate dose for further human intervention studies. Moreover, since the healthy effects of these fatty acids have been strictly related to their increased incorporation in plasma and tissue lipids, we also examine and discuss the incorporation changes following the administration of the effective anticancer EPA and/or DHA doses in animals and humans.

Keywords

Bioavailability, breast cancer, dose, incorporation, n-3 PUFA, prostate cancer.

Introduction

Plenty of preclinical *in vivo* studies have given substantial support to the hypothesis that long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) may exert antineoplastic effects. The earliest studies on animals treated with fish oil (FO) were published in the late eighties/early nineties of the last century (Karmali et al., 1984; Karmali et al., 1987; Rose and Cohen,1988; Rose and Connolly,1993; Hudson et al., 1993). Since then, *in vitro* studies have further and strongly supported the antineoplastic potential of these fatty acids, through the identification of a number of possible biological and molecular mechanisms underlying their anticancer effects (Calviello et al., 2006).

Rodent tumors transplanted in syngeneic animals or human tumor cells injected in immunodeficient mice have been the most used animal models to study the antineoplastic efficiency of n-3 PUFA (see Tables 1 and 3A-3D). But also rodents subject to chemical carcinogenesis (see Tables 4A and 4B) or genetic mouse models of cancers (see Table 2) have been largely used. The animals have been always fed with diets enriched with FO, more or less concentrated in n-3 PUFA, or directly with the two LC n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), either mixed with the diet or administered orally and individually, as ethyl esters or free fatty acids. In order to study the preventive or therapeutic effects of these fatty acids, the treatments have taken place before or after cancer cell transplantation, as well as before or after the beginning of the carcinogenic protocol. Recently, the genetically engineered *Fat-1* mouse model was developed, which constitutionally converts n-6 PUFA into n-3 PUFA by expressing a transgene (ω-3 desaturase) derived from *Caenorhabditis*

elegans, which encodes a n-3 PUFA desaturase not present in mammalians (Kang, 2007). Thus, the *Fat-1* mouse tissues are endogenously enriched in n-3 PUFA obtained from the metabolism of n-6 PUFA added at high levels with the diet. The diet that they receive is comparable to that of most Western populations, with high levels of n-6 PUFA and very low levels of n-3 PUFA. Thus, the use of this model allow to be independent of the dietary treatment with n-3 PUFA, their dose, the length of the dietary treatment, as well as the animal body weight and food intake. On the other hand, the dietary supplementation with n-3 PUFA allows the identification of the doses that could have anticancer effects without exerting toxicity, and consents also to establish which the optimal dietary ratio between n-6 and n-3 PUFA is. Moreover, by using this model it is also possible to calculate the bioavailability of these fatty acids, i.e. the degree at which they become available to the serum or incorporated in erythrocytes or target tissues following an established intake of n-3 PUFA. As we will discuss in a next section, this information may in turn be useful to hypothesize the doses that could have anticancer effects also in humans.

For the sake of clarity, it should be underlined that the animal studies have sometimes drawn criticism (Calviello and Serini, 2010), since the diets often contain levels of fat quite high if compared to other classes of dietary components. However, such high levels were administered with the aim to reproduce human diets at very high levels of fat, such as some Western diets.

In this review we have analysed the range of doses and the forms of supplementation (fish oils more or less concentrated, EPA or DHA given together or individually) of LC n-3 PUFA that were able to inhibit breast and prostate cancer growing in animals. We have also examined the changes in n-3 PUFA incorporation in plasma and tissue lipids obtained with

different doses. We have chosen prostate and breast cancers, since they are among the most frequent human cancers, and plenty of preclinical studies support their susceptibility to the antineoplastic action of n-3 PUFA (Serini et al., 2010). Our aim is to provide a complete view of the doses used so far and the effects that they are able to produce, in order to ease the choice of the doses for those researchers who intend to design preclinical studies *in vivo* or human intervention trials.

Methods for dose calculation and comparison

In our analysis of published literature we have considered only those works that directly compared the effects of diets enriched/supplemented with FO/purified LC n-3 PUFA with control reference diets containing other kinds of fats. Only in a few cases also the control diets contained a low amount of FO. We have included only the articles reporting the amount (in grams or percentage) of oils/FA present in the diets as referred to the weight of the diet (in g/kg or %), and excluded those that instead referred them to the total energy furnished by the diet, unless a detailed description of the diet allowed the conversion of these values. When the effect of combined treatment of LC n-3 PUFA with an antineoplastic therapy were studied, we took into account only the works reporting a control condition in which LC n-3 PUFA were administered alone.

To better compare the highly variable doses of n-3 PUFA administered in the various studies we have converted the amount in grams of n-3 PUFA fed to the animals (both in the form of FO or as purified fatty acid-ethyl esters, FA-EE) into the dose of LC n-3 PUFA expressed in

g/kg of body weight (b. wt.). We have confined our analysis to the most bioactive LC n-3 PUFA contained in FO, i.e. EPA and DHA.

Most of the animal studies have been performed using menhaden oil (MO) as FO. A smaller number used MaxEPA or other FO concentrates. When the actual EPA and DHA contents in the FO used were reported by the authors or by the FO producers, these values were used to calculate the dose in g/kg b. wt. Alternatively, we have used the mean published values for EPA and DHA content in each FO. In particular, we have used the (EPA+DHA) content of 20 g and 30 g for 100 g of MO or MaxEPA respectively, that are those reported in most studies (Braden and Carroll, 1986; Abou-El-Ela 1988; Abou-El-Ela et al., 1989; Karmali et al., 1989; Olivo and Hilakivi-Clarke, 2005).

To calculate the dose in g/kg b. wt. we referred to the "U.S. EPA Recommendations for and Documentation of Biological Values for Use in Risk Assessment (EPA/600/6-87/008)" (http://www.tera.org/Tools/ratmousevalues.pdf), where the ratio between the daily food intake and the animal body weight (Food Factors, *FF*) was calculated for both mice and rats during acute and chronic studies (Fig. 1).

Even though different mean *FF* values for both male and female animals were reported in the "EPA recommendations", in our calculation we used the mean values obtained by summing all the values obtained for both sexes, since there were only slight gender differences.

Practically, to obtain the daily (EPA+DHA) dose in g/kg b. wt., we first calculated the amount (in grams) of (EPA+DHA) present in a kilogram of the ingested diet. Then we applied the following formula:

For instance, if we apply this formula for a mouse diet containing 20.5 % Max-EPA (see Table 1, Karmali et al., 1984) (i.e. 205 g/kg diet), which corresponds to about 67.7 g (EPA+DHA)/kg diet [since the Max-EPA used contained 33% (EPA+DHA)], by applying the formula (a), using the *FF* value (0.20) for mice in acute studies, reported in Fig. 1, we obtain:

67.7 g /kg *x* 0.20 = 13.5 g /kg b. wt. (EPA+DHA)

When the b. wt. gain and amount of food ingested were directly reported by the authors, we were able to calculate the exact *FF* for that case, and used it for our calculations. In any case, it should be underlined that, by using this calculation, we could obtain only an "approximate dose", since the experiments usually lasted several weeks and sometimes months, and during such a long time period it is known that both the b. wt. and the food intake increase, and the increase is also gender-related. However, our approximation can be considered acceptable, since most of the experiments are conducted from weaning onwards, and the variations in both b. wt. gain and dietary intake increase proportionally as the young animals grow to adult age (Lin et al., 1977). Similarly, the dose of PUFA in g/kg b. wt., calculated as described above, can be considered valid even when the experiments are carried out in adult animals (as it was the case in

experiments of chemical carcinogenesis), and both the b. wt. and the dietary intake remain quite stable. Moreover, we are aware that in some models, and following some protocols, the animals become cachectic, anorexic, and lose weight. In any case, the calculated "approximate doses" should be considered as the doses that have been ingested by the animals at the beginning of the experiments, when cachexia was not yet observed. Nevertheless, also the researchers that perform these kinds of experiments are aware that during the late cachectic stage the amount of dietary factors ingested by the animals tends to change and to be more irregular if compared to the amount ingested during all the early non-cachectic phases of the experiments.

Overall, even though in most cases we cannot deduce all the exact factors to make calculations from the reports (i.e. animals' body weight and food intake, as well as their changes during the different phases of the experiments), the dose values obtained through our calculation procedure and reported in the tables should not differ substantially from the values of the actual dose ingested by the animals, and thus they should not prejudge our conclusions.

LC n-3 PUFA doses used in animal models

Prostate cancer

The data were collected from the works that have used transplant tumor models of prostate cancer and that are analysed in this paragraph and are also summarized in Table 1. Some of these studies (Karmali et al., 1987; Rose and Cohen, 1988; Connolly et al. 1997a) reported that diets enriched in FO (MO or MaxEPA) at high content in EPA and DHA (20% or 30%) efficiently inhibited the growth of the androgen-independent human prostate DU-145 cancer

cells transplanted in nude mice if compared to a diet containing high levels of corn oil (CO, 18-23.5%) or leenseed oil (LO, 18%), which contain high levels of α-linolenic acid (18:3 n-3, LNA). The content of FO in the diets ranged from 17 to 21.5 %, and it was always accompanied by 3-6 % CO to furnish also the essential n-6 PUFA. These diets were administered both before and/or after cell inoculation, for a total period ranging from 7 to 9 weeks. According to our calculation, the mice were supplied with daily (EPA+DHA) doses ranging between about 7 g and 14 g/kg b. wt., that are quite high doses for mice, if compared to most of the doses used more recently. For instance, the antineoplastic efficiency of a lower daily dose of 3.0 g/kg b. wt. of purified EPA was recently observed in castrated testosterone supplemented nude mice implanted with the androgen sensitive CWR22R cell line (McEntee et al., 2008). Marked antineoplastic effects were also observed when similar lower daily doses (2.3-3.0 g/kg b. wt.) of EPA or (EPA+DHA) were administered to immunodeficient mice injected with androgen insensitive human cells (LAPC-4 or CWR22) (Kobayashi et al., 2006; McEntee et al., 2008). Also a treatment for 15 wks with a diet enriched (60% w/w) in the immediate metabolic precursor of EPA, stearidonic acid (SDA 18:4 n-3), (Kelavkar et al., 2006; Kelavkar et al., 2009), significantly retarded the growth of LAPC-4 cells in immunodeficient mice. However, the dose of SDA needed to observe antineoplastic effects in this case was of about 120 g/kg b. wt., that is much higher than the doses generally reported for EPA or (EPA+DHA), and that could be explained by a low metabolic conversion of SDA to EPA. In a more recent report by Wang et al. (2012) nude mice treated with a dose of 4.0 g (EPA+DHA)/kg b. wt. were transplanted with mouse prostate cells carrying the Pten gene deletion, and a marked inhibition of tumor growth was still observed. Actually, the Pten gene deletion model is an immuno-competent mouse

prostate cancer model that has been used mainly by these and other authors for studying the effect of n-3 PUFA on genetic animal models of prostate carcinogenesis (Berquin et al., 2007; Vissapragada et al., 2010; Hu et al., 2010; Wang et al., 2012) (Table 2). The Pten tumor suppressor gene is the most frequently mutated gene in prostate cancer metastases (Suzuki et al., 1998; Vlietstra et al., 1998), and the mice carrying the omozygous deletion of this gene develop prostate cancer with metastases. These mice were treated with a diet enriched with EPA and DHA in triglyceride form [4 g (EPA+DHA)/kg b. wt./day], and having a n-6/n-3 PUFA dietary ratio of 1, were compared to mice fed with diets with a n-6/n-3 PUFA ratio more similar to that observed in Western diets (ranging between 20 and 40), being enriched in different proportion with palm oil as a source of α-linolenic acid (ALA), and CO as a source of linoleic acid (LA) (Berquin et al., 2007; Hu et al., 2010; Wang et al., 2012). The dietary treatment with LC n-3 PUFA reduced significantly prostate tumor growth (clearly observed after 5-21 weeks of dietary treatment), slowed histopathologic progression and increased survival (Berquin et al., 2007). In one of these works (Hu et al., 2010) lower expression of syndecan, a marker of increased malignancy in prostate cancer, was found in the tumors from EPA+DHA-treated mice (Hu et al., 2010). Interestingly, a similar pattern of protection from prostate cancer was found also in Pten-/- mice carrying the *fat-1* gene, that show conspicuous endogenous increase of the n-3/n-6 PUFA ratio both in mouse blood and pancreas.

Vissapragada et al. (2010) used a probasin-driven ErbB-2 transgene mice model with heterozygous loss of *Pten* (PB-ErbB-2 x Pten+/-), that is a sporadic model of prostate cancer showing a 15% incidence rate by 12-14 months of age in basal lab chow (AIN93G) fed mice. They observed that the incidence of prostatic cancer augmented to 100% with diet containing CO

(70%, w/w), and that the presence of n-3 PUFA together with CO in the diet (with a MO/CO ratio of approximately 1:1) did not modify prostate cancer progression. However, the authors lacked to evaluate the effect of a diet with a n-3 to n-6 PUFA ratio of 1, that in Berquin's model had showed high antineoplastic efficacy. In their CO-MO diet the ratio between (EPA+DHA) and LA was of about 3, if we consider only the LC-PUFA deriving from MO and the CO added to the AIN93G diet, but if we consider also the intake of LA deriving from the AIN93G whose 17% fat derived from soy oil contained high levels of LA (54% of total fatty acids) and OA (24%), and a very small amount of ALA (7%) this ratio becomes much higher, and out of the range recommended by nutritionists (Simopoulos et al., 1999). Moreover, the calculated approximate EPA+DHA daily (about 1.4 g/kg b. wt.) was lower than all the others that were used in the other studies so far considered, and the fact that it was also administered in the presence of a conspicuous amount of LA, likely, made the EPA+DHA dietary dose insufficient to prevent cancer progression. Therefore, from all the results obtained in the PTEN mouse model of prostate cancers we observe that both an adequate dose of (EPA+DHA) in the diet (higher than 1.4 g/kg b. wt., and around 4 g/kg b. wt.), as well as a dietary n-6 to n-3 PUFA ratio of about 1 appear crucial to obtain anticancer effects.

The high antineoplastic efficacy of dietary FO administration [corresponding to a (EPA+DHA) dose of about 6.0 g/kg b. wt.] was also recently demonstrated in another transgenic model of prostate cancer, the C3(1)Tag mouse, which develops prostatic intraepithelial neoplasia that progresses to prostate carcinoma due to the expression of the large T antigen in the prostate (Akinsete et al., 2012).

In conclusion, excluding an early work in which a very high dose (13.5 g/kg b. wt.) was administered to mice (Karmali et al., 1987), in the other reports examined comparable dietary (EPA+DHA) doses resulted efficient, and they were all comprised between about 2-3 and 7 g/kg b. wt. (used for 2-10 wks in prostate cancer transplantation models or for 8-33 wks in prostate genetic models), being able to induce clear antineoplastic effects. On the other hand, lower doses (Vissapragada et al., 2010) (about 1.4 g/kg b. wt, accompanied by high levels of LA) did not produce any anticancer effect. Interestingly, we can observe that in the more recent works, there is the tendency to use doses in the lower range as compared to the earliest works performed before the year 2006. This is what generally has happened in works with n-3 PUFA, since, excluding some exceptions, there has been increasing awareness that too high doses of EPA and DHA may not be completely safe, but may produce generalized tissue oxidative stress, and may consequently even induce carcinogenesis (Serini et al., 2011).

Breast cancer

Most of the works performed on animals and reporting an inhibitory effect of a dietary treatment with marine oils on breast cancer made use of either the transplantation or the carcinogenic model. Only two studies were conducted using genetic mouse models of breast cancer [MMTV/v-Ha-ras and FVB/N-TgN(MMTVneu)202Mul transgenic mice] (Table 2), (Fernandes et al., 1995; Yee et al., 2005) and the daily doses that had clear anticancer effects in these cases were comprised in the range of 3.9-8 g/kg b. wt. Interestingly, we can observe that also in this case the more recent work (Yee et al., 2005) tends to use lower doses than the earliest work (Fernandes et al., 1995).

The first two studies on animal models of breast cancer were performed concomitantly by Karmaly et al. (1984) and Jurkowsky and Cave (1985), and are examples of the use of the two different experimental approaches. By using the transplantation model, Karmali et al. (1984, Table 3D) observed that the growth of the transplanted murine R3230AC mammary adenocarcinoma in inbred F344 rats was strongly inhibited by the treatment with diets containing marine oil at increasing doses. The diets provided 0.22-0.88 g (EPA+DHA)/kg rat b. wt. These doses were to a great extent lower than the dose (4 g/kg b. wt., obtained by using a diet containing 20% MO) used by Jurkowski and Cave (1985) (Table 4A) to reduce tumor incidence and prolong the tumor latent period in rats subject to carcinogenesis with 1-methyl-1-nitrosourea (MNU). Therefore, there was a considerable discrepancy between the effective doses between these two first works on rats. It should be noticed, however, that the higher dose used by Jurkowsy and Cave (1985) is in the same range of doses (between 0.7 and 6.0 g n-3 PUFA/kg b. wt.) resulted effective in a number of other later works performed on rats subject to chemical carcinogenesis (Tables 4A and 4B). A dose of about 3.6 g EPA+DHA/kg b. wt. was administered by Gabor and Abraham (1986) in the first transplantation study on mice. The authors observed the inhibition of the growth of a transplanted breast adenocarcinoma (strain not specified), and explained it on the basis of an increased tumor cell loss. This observation is in agreement with much later findings that have demonstrated the powerful activity of n-3 PUFA, and especially of DHA, as inductors of apoptosis in cancer cells (Calviello et al., 2004; Calviello et al., 2005; Calviello et al., 2007; Fasano et al., 2012; Serini et al., 2008; Serini et al., 2009; Serini et al., 2012). Similarly, other authors reporting anticancer effects of n-3 PUFA in mice transplanted with breast cancer cells used approximate daily doses of (EPA+DHA) comprised

between 2.0 to 7.6 g/kg b. wt. (Borgeson et al., 1989; Pritchard et al., 1989; Blank and Cerian, 1989; Gabor et al., 1990; Gonzalez et al., 1991; Gonzalez et al., 1993; Welsch et al., 1995; Shao et al., 1995; Shao et al., 1997) (Tables 3A and 3B).

Notably, a series of similar works were conducted by the group of Rose and Connolly (Rose and Connolly, 1993; Rose et al., 1995; Rose et al., 1996; Connolly et al., 1997b; Connolly et al., 1999). They found that the minimal doses of (EPA or DHA) that resulted effective in suppressing breast cancer growth and metastasis were comprised between 4 and 8 g/kg mouse b. wt. The model used by these authors was the injection of the estrogen-independent MDA-MB-435 human breast cancer cell line, showing a propensity for lung metastasis, into the thoracic mammary fat pad of nude mice. In the different works mice were shifted from basal or CO-enriched diets to diet enriched with n-3 PUFA (obtained by adding MO, DHA-EE or EPA-EE) at different phases during the experimental protocol: a) before cell injection; b) immediately after the cell injection, c) as the tumors became palpable, and d) after tumor excision. The treatment length ranged from 6 to 12 wks and the growth of the tumors in these animals were compared to that of tumors growing in mice fed diet at high n-6 PUFA content. Suppression of primary tumor growth and reduced occurrence and severity of lung metastases were observed, as well as induction of apoptosis, inhibition of cell proliferation and reduced neovascularisation of primary tumors.

More recently, several other works were performed (Hardman et al., 2001; Hardman et al., 2005; Ghosh-Choudhury et al., 2009, Wu et al., 2005, Mandal et al., 2010) by transplanting the same MDA-MB-435 human breast cancer cell line in nude mice fed diets enriched with n-3 PUFA. These more recent reports based on the older findings already obtained, were especially

aimed to investigate the molecular mechanisms underlying the antitumor action of dietary n-3 PUFA or the effects of combined treatments with these fatty acids and conventional antineoplastic therapy. Wu et al. (2005) besides confirming the inhibitory effect of dietary MO on the growth of MDA-MB-435 cells injected in nude mice, observed that the treatment induced in the cells a sphingomyelinase/ceramide-driven apoptosis. In this case the calculated approximate dose of (EPA+DHA) amounted to 3.6 g/kg b. wt. In their two studies Hardman et al. (2001, 2005) reported the antitumor efficiency of two different fish oils (fish oil concentrate, FOC or AAFA) at high content of EPA+DHA (58 or 55%). In the first work (Hardman et al., 2001), the diet enriched in FOC was supplemented in the post-implantation phase, and accompanied or not by a treatment with doxorubicin (DOX). The mice received an approximate daily EPA+DHA dose of 3.5 g/kg b. wt. The authors found that the effectiveness of DOX was enhanced by the FO treatment, without increasing the toxicity to the host mice. In the second work from this group (Hardman et al., 2005) the diet enriched with AAFA was supplemented to mice in the post-implantation phase. In a group of animals the treatment was performed in combination with a radiation treatment (both before and in concomitance with the radiation treatment). The mice received an approximate daily EPA+DHA dose of 5.5 g/kg b. wt. The authors observed that the FO treatment alone resulted to be equally or more efficient, but safer, than the FO treatment in combination with ionizing radiations in retarding tumor growth and vascularisation.

Recently Ghosh-Choudhury et al (2009) and Mandal et al. (2010) used the same experimental model of MDA-MB-435 human breast cancer cell implantation in nude mice fed a diet containing 10% MO [corresponding approximately to 4g (EPA+DHA)/kg b. wt./day]. The

mice were dietary treated both in the pre- and post-implantation phases (for a total of 4-6 wks). The authors (Ghosh-Choudhury et al., 2009) demonstrated that the PI3K/PTEN/Akt pathway was involved in the growth-inhibitory and pro-apoptotic effect observed. Moreover Mandal et al. (2010) associated the inhibited levels of CD44 adhesion protein observed in the tumor cell surface of MO-treated mice to the suppressed formation of metastases to bone. Another strain of human breast cancer cells (KPL-1) was injected by Senzaki et al. (1998) in nude mice fed a diet containing quite high levels of EPA (9.5% w/w, corresponding to a daily dose of 19 g EPA/kg b. wt.), together with low levels of LA (0.5% w/w). These cells have a specific propensity for axillary lymph node metastasis and the authors found that the mice showed a dramatic reduction in tumor cell growth and metastasis in comparison to mice fed a diet containing 10% LA (LA diet).

The rest of the transplantation studies were performed by implanting cells of murine origin in syngeneic immune-competent mice or rats. A mammary tumor cell line (4526 breast cancer cells) originally spontaneously arisen in mice was transplanted in Balb/c mice fed a diet containing 20% MO (Hubbard et al., 1998; Mukutmoni-Norris, 2000), and the authors observed that tumor latency, growth, vascularisation and metastasis were beneficially affected by this diet. The calculated EPA+DHA dose corresponded in this case to about 8 g/kg b. wt. Other studies were performed transplanting breast cancer cells in syngeneic rats, as in the first transplantation study that we mentioned above (Karmali et al., 1984). The same rat model of the first study was also used in a more recent study by Robinson et al. (2001) that, by using a dose of (EPA+DHA) of about 0.8 g (EPA+DHA)/kg b. wt., i.e. in the range of those used earlier, observed the inhibition of tumor growth. Later, in three studies performed by the same group, the rats were

trasplanted (Togni et al., 2003; Pizato et al., 2005; Mund et al., 2007) instead with a strain of murine breast carcinoma originally arisen spontaneously in Wistar rats (Walker 256 rat mammary cancer cells) . The peculiarity of the first of these studies (Togni et al., 2003) was that the FO (1g/kg, consisting in a mixed marine triacylglycerol preparation containing 30% EPA+DHA) was supplemented by gavage to the rats (Table 3D) throughout two generations. In the first part of the study the female rats were supplemented, both before and during pregnancy and lactation, and then the FO supplementation was given to the male offsprings from weaning until 90 days of age, when the tumor was implanted, and afterwards for further 30 days. The daily approximate dose of (EPA+DHA) corresponded in this case to about 0.3 g/kg b. wt. A marked decrease (60%) in tumor growth was observed, and the rat survival was markedly increased (50%) as well as cachexia prevented. The same dose was used in a following study of the same group (Mund et al., 2007) on Wistar rats at weaning, and besides the inhibition of tumor growth, induction of apoptosis and increased peroxidation were observed in tumor tissue. In the third work Pizato et al. (2005) fed the weaning rats for 10 wks (2 wks before and 8 week after implantation) with a diet enriched in FO (20% w/w), corresponding to a much higher daily dose of EPA+DHA (in the range of 6.5 g/kg b. wt.). Again, they observed that the diet inhibited strongly (by 60 %) the growth of the Walker 256 tumors as compared to a control diet at low level of fat (4%), and containing even 2.5 fold lower levels of LA and just some trace of EPA and DHA. These results suggested that the presence of high levels of n-3 PUFA in the diet is a more important factor for the antineoplastic effect than the low levels of dietary fat in general and of LA in particular.

All the studies of chemical breast carcinogenesis were also performed on rats. In the earliest work of Jurkowsky and Cave (1985), already mentioned above, MNU was the carcinogen used. This carcinogen was also used in several other later works (Colas et al., 2006; Jourdan et al., 2007; Manni et al., 2010; Manni et al., 2011) performed on rats treated with purified EPA and/or DHA. Inhibition of tumor incidence, multiplicity, tumor regression and decreased tumor vascular density (Yuri et al., 2003; Colas et al., 2006; Jourdan et al., 2007; Manni et al., 2011) were observed by the different authors by using this model. Interestingly, when EPA and DHA were fed separately, especially DHA was efficient (Yuri et al., 2003). In these studies EPA and/or DHA were administered for 6-20 weeks post-initiation by adding them to the diet to obtain 0.7-4.5 g DHA or (EPA+DHA)/kg b. wt./rat. Only in one case (Colas et al., 2006) the rats were treated for 3 weeks in the pre-initiation phase. Moreover, in these studies (Colas et al., 2006; Manni et al., 2010) it was also observed that n-3 PUFA at these doses caused an enhancement of the efficacy of the chemotherapic agents epirubucin or tamoxifen in this breast carcinogenesis cancer model. Hamid et al. (1999) observed that when about 3.6 g EPA+DHA/kg b. wt. was added to the diet as MO (18% in a diet containing only 5% CO) reduced the expression of COX-2 as compared to animals treated with high levels of CO (23%) in MNU-induced mammary tumors. Actually, even though this result was interesting, since increased expression of COX-2 has been considered a marker of increased malignancy in many kinds of cancer, in this case the decreasing effect could be ascribable not only to the increased intake of n-3 PUFA with MO, but also to very low levels of CO (5%) received by these animals together with MO.

Several other studies were instead performed using the carcinogen 7,12-dimethylbenz(a)-anthracene (DMBA) and Sprague-Dawley rats (Tables 4A and 4B). The animals were generally fed diets containing MO or MaxEPA (from 10 to 20% w/w), accompanied or not by 3-5% CO (w/w) (Braden and Carrol, 1986; Abou-El-Ela et al., 1988; Abou-El-Ela et al., 1989; Karmali et al., 1989; Olivo and Hilakivi-Clarke, 2005) and compared to animals fed diets containing 6.5-20% CO (w/w). The doses of (EPA+DHA) administered ranged from about 0.8 to 6 g/kg b. wt., and tumor incidence always decreased. In some studies the administration was also performed by gastric intubation and similar protective effects were obtained. In these cases the rats were supplemented with MaxEPA [corresponding to a (EPA+DHA) dose of about 1.0 g/kg b. wt.] (Manna et al., 2007; Manna et al., 2008; Chatterjee et al., 2010), or with 0.7 g/kg b. wt. EPA- or DHA-ethyl esters (EPA-EE or DHA-EE) (Minami and Noguchi, 1996; Noguchi et al., 1997). Notably, in the last paper a reduction of the total number of tumors and cancer cell proliferation was observed when EPA-EE and DHA-EE were supplemented to rats fed a high fat diet (20% CO). No effect was instead observed when the supplementation was combined to a diet at much lower content of fat (0.5% CO), thus suggesting that a low level of dietary fat is protective *per se* against breast carcinogenesis, irrespective of the kind of the dietary PUFA present. Therefore, these results emphasize the importance of an adequate level of dietary n-3 PUFA to contrast the carcinogenic action of n-6 PUFA, particularly when high levels of n-6 PUFA are present in the diet, as it often happens in some Western diets.

Among these studies carried out with the breast carcinogenic DMBA model is worth noting that by Olivo and Hilakivi-Clarke (2005). In fact, in this case the diet at low (3.5% MO+3.5% CO) or high n-3 PUFA content (7% MO+12% CO) were fed to the nursing dams of

rats in prepubertal age. The amount of (EPA+DHA) ingested by the nursing dams was either of about 0.9 g and 1.5 g/kg rat b. wt., accompanied by about 1.9 g or 6.5 g n-6 PUFA/kg rat b. wt., respectively. However, the actual dose of n-3 PUFA received by the litters from the milk was not evaluated. At weaning these rats were fed with identical diets of the nursing dams, and then, at 50 day-age exposed to the DMBA carcinogenesis induction. A lower tumor incidence was observed in the rats receiving the low n-3 PUFA diet vs. those receiving the reference low n-6 PUFA diet (0.5% MO and 6.5% CO). On the contrary, the high n-3 PUFA diet (containing, however, a double amount of CO in the reference diet) increased the mammy tumor incidence, whereas a high n-6 PUFA diet (1.5% MO and 17.5% CO) had no effect. These results suggest that high doses of n-3 PUFA taken by the nursing mothers or present in milk formulas may have promoting effects later in the life. However, a further important confirmation would be the treatment of the nursing dams with purified EPA and DHA, since the concomitant presence of very high levels of CO in the diet could have led to some misinterpretations. Moreover, the knowledge of the actual levels of n-3 PUFA (and n-6 PUFA) in the milk would be essential to better support the authors' hypothesis.

Interesting results have been also obtained in a recent work (Zhu et al., 2011) having a quite complicate experimental design. The authors demonstrated that among several diets furnishing various combined levels of n-3 or n-6 PUFA and having increasing n-3/n-6 PUFA ratios, the only diet that induced a significant antineoplastic effect in rats subject to the MNU carcinogenic treatment was that furnishing 11.6 g (EPA+DHA)/kg b. wt. and having the highest n-3/n-6 PUFA ratio (25:1). This diet was compared to another furnishing about one tenth of EPA and DHA [1.2 g (EPA+DHA)/kg b. wt.] accompanied by 22 g/kg b. wt. CO (n-3/n-6 ratio, 1:1).

It should be underlined that the comparison was made between two diets containing both n-3 PUFA at high levels. Instead (and unexpectedly on the basis of all the other works analysed), diets furnishing 3.6 g (EPA+DHA)/kg b. wt. plus 0.7 g/kg b. wt. CO (n-3/n-6 ratio, 5:1), and 0.4 g (EPA+DHA)/kg b. wt. plus 48 g CO/kg b. wt. (n-3/n-6 PUFA ratio, 1:5) were not different in terms of antineoplastic activity. The short period of exposure to the diets (only 8 wks after cancer induction) can be invoked to explain why in this case the only diet that inhibited tumor growth was that furnishing the highest dose of n-3 PUFA. In any case, it is worth noticing that only the dose showing antineoplastic efficiency was able to significantly decrease the mammary gland density when administered to normal weaning rats for 2 wks. Thus, the author suggested that gland density could represent a factor strictly related to carcinogenesis and subject to the protective action of n-3 PUFA.

Overall, most of the doses used in the studies performed with mouse models of transplanted and genetic breast cancer and shown to be effective ranged between 2-4 and 8 g/kg b. wt., with some exceptions in which higher doses were used. Sometimes, however, the higher doses confirmed the effects observed in the same work with lower doses (Rose et al., 1995; Rose et al., 1996). In one work (Senzaki et al., 1998), however, it was tested only a very high dose (19 g/kg b. wt.), but the human cell line (KPL-1 human carcinoma cells) was different from the human cell lines used by all the others (mostly MDA-MB-435 cells, someone MX-1cells). Also in these studies there was a tendency to use doses comprised in the lower range in more recent times. In the rat models doses of 0.2-0.9 g/kg b. wt. were used, with only one exception in which a markedly higher dose was administered (6.5 g/kg b. wt.) (Pizato et al., 2005). A wider range of doses were tested in the carcinogenic rat models. DMBA-induced carcinogenesis was inhibited in most cases by doses

comprised between 0.7 and 4.0 g/kg b. wt. [with the exception of one work in which 6.1 g/kg b. wt. was needed (Karmali et al., 1989)]. Doses in the same range showed antineoplastic efficacy also as carcinogenesis was induced by MNU, with the sole exception of the EPA+DHA dose of 11.6 g/kg b. wt. that was used in just one work (Zhu et al., 2011) where, however, the comparison was made with a diet containing already 1.0 g (EPA+DHA)/kg b. wt.

It can be concluded from our examination of the literature that the antineoplastic potential of LC n-3 PUFA in breast and prostate cancers has been robustly demonstrated by a number of preclinical *in vivo* studies. In all these studies, the groups of animals ingesting diets enriched with n-3 PUFA have been generally compared with groups ingesting either the basal chow diet, in which n-3 PUFA are almost absent, or, more often, isoenergetic diets containing high levels of n-6 PUFA, especially LA. It is interesting to consider that the FA composition of these control diets containing high levels of LA and scarce amounts of n-3 PUFA are comparable to that of diets currently used by some human Western populations. Instead, the diets given to animals that were enriched in EPA and DHA appear more similar to the diets used by the same Western populations in the early 20th century and before. This dietary change occurred along the 20th century in some Western populations has been related to the increased consumption of soybean oil (by about 1000-fold in the USA) (Blasbalg et al., 2011), containing very high levels of LA. It has led to a large decline in the amount of EPA and DHA incorporated in tissues (EPA and DHA tissue status).Therefore, a new hypothesis has been put forward, also on the basis of the results of the preclinical studies, according to which these new dietary conditions could have the potential to favor the development and progression of cancer also in humans, unless the proportion of dietary fatty acids is not reverted to that existing prior to the 20th century. Therefore, it is

believed that a dietary change allowing the maximal incorporation of EPA and DHA in human tissues could inhibit the development and progression of cancer, and also that the supplementation of diet with these FA could represent an adjuvant therapeutic strategy to improve the beneficial effects of the currently used antineoplastic agents. However, as we will see in the next section, this hypothesis has been verified for prostate and breast cancer only in a few human intervention studies.

LC n-3 PUFA doses used in human intervention studies

Prostate cancer patients

Even though most of the preclinical studies which support an anticancer effect for n-3 PUFA have been performed on animal model of prostate cancer, and the first of these studies were conducted over twenty years ago, just one clinical intervention trial on prostate cancer patients treated with n-3 PUFA has been so far published (Aronson et al., 2011). The study was a phase II randomized trial conducted on patients undergoing radical prostatectomy and eating a diet with reduced fat levels and supplemented with 5 g/day of FO for 4-6 weeks. The n-6/n-3 dietary ratio of these patients was seven folds lower than that of control patients following a Western diet (2:1 vs. 15:1). The patients subject to the low fat/FO dietary regimen received a daily dose of 2.6 g of EPA+DHA (EPA, 0.9 g; DHA, 1.7 g), that is considered an absolutely safe dose (EFSA Journal, 2012). Among the secondary outcomes of this study the authors found lower tissue n-6/n-3 ratios in benign and malignant prostate tissues of FO/low fat patients than in the control patients, as well as reduced prostate cancer cell proliferation evaluated both *in vivo*

(as Ki-67 index), and *ex vivo* (by a bioassay using patient sera applied to prostate cancer cells *in vitro*). These results are very worth noting, and the authors conclude that their validation in further studies having proliferation as the primary outcome will support the performance of long-term dietary intervention trials with clinical progression endpoints. Certainly, since in this study the appropriate controls were lacking (patients consuming a high-fat diet supplemented with FO or a low-fat diet without FO), it is not possible to distinguish whether the antiproliferative effect could be ascribable just to the increased intake of n-3 PUFA or also to concomitant decreased levels of fat. Another intervention study recently published by Chan et al. (2011) is not of great help in our discussion. A group of men with favorable-risk prostate cancer were treated for 3 months with 3 g/day FO (containing 1,098 mg EPA and 549 mg DHA). The authors investigated the expression of molecular factors (COX-2 and IGF-1) reported to be involved in prostate carcinogenesis, but did not find any change in the normal prostate microenvironment in men with low-burden prostate cancer. However, in this case the effect on prostate cell proliferation was not investigated. Moreover, as the authors themselves affirm, it is possible that these nutrients may have greater effects on the expression of genes that they did not assess in normal tissues around the tumors, i.e. LC n-3 PUFA may act as efficient antineoplastic agents by altering other molecular factors involved in prostate carcinogenesis that were not evaluated. Furthermore, since the period of dietary LC n-3 PUFA treatment showing antineoplastic efficacy in mice ranges from 5 to 15 weeks in most of the studies analysed, it is probable that the treatment of about 4-12 weeks performed in the studies by Aronson and Chan (Aronson et al., 2011; Chan et al., 2011) could not be sufficient to detect a clear antineoplastic effect in humans. For instance Bougnoux et al. (2009) treated for 6 months the breast cancer patients to obtain a

clear anticancer effect. When we compare time parameters between mice and humans (i.e. life expectancy, time to reach sexual maturity or gestation time), they are several times longer in humans than in rats (approximately, from 10 to 80 times longer). Thus, presumably, the length of the dietary n-3 PUFA treatment in humans should be several times longer than that generally used with animals in order to prevent the growth of tumors that develop in times much longer than in mice. This is further suggested also by the observations that, whereas only 7-10 days are required by rodents (Calviello et al., 1997) to reach a new steady-state in the level of PUFA incorporated in tissues, (that, as discussed below, has been strictly related to the health benefits produced by n-3 PUFA), a month is instead needed to humans (Arterburn et al., 2006).

At the moment other trials are ongoing in this field, and, one pilot study conducted on advanced prostate cancer at Wake forest University (NCT00996749, termination at the end of 2013) could add valuable information about the optimal dose to be used in prostate cancer patients. The main aim of this study is to determine the dosage of n-3 PUFA to achieve an n-3 to n-6 ratio of 1:1, by evaluating the levels of PUFA and the tolerability of supplementations in the patients. The clinical impact of the n-3 PUFA supplementation is also being investigated. Another interesting study (NCT00253643), started several years ago (2005) and still ongoing, could add information on the dose. It examines the effect of a supplementation with FO, combined or not with green tea, but one arm of the study, so far still not published, is investigating patients receiving only n-3 PUFA. The study is examining some prostate cancer markers of change in lipid metabolism (Fatty Acid Synthase, FAS; Sterol Regulatory Element Binding Protein, SREBP), cell proliferation and survival, and membrane phospholipid enrichment in n-3 PUFA.

Breast cancer patients

Recently, Bougnoux et al. (2009) published the results of a human phase II trial performed supplementing DHA (1.8 g/day) for about 6 months to breast cancer patients with visceral metastases treated with FEC 75 chemotherapy (cyclophosphamide + fluorouracil + epirubicin). They found that that addition of DHA to chemotherapy was devoid of adverse side effects and improved the outcome of the metastatic breast cancer patients. They observed that the DHA potential to increase the activity of chemotherapy was proportional to its degree of incorporation in plasma phospholipids (PL). When patients were stratified according to this factor, time to progression (TTP) was significantly higher in patients with high-DHA incorporation (8.7 months vs 3.5 months in patients with low-DHA incorporation). Moreover, they found that the overall survival (OS) was almost doubled in patients with high-DHA incorporation (34 months vs 18 months). The study added new and noteworthy information on the possible application of n-3 PUFA in the therapy of human cancer, even though it was performed on a quite small number of patients. The authors themselves concluded that the results warranted confirmation through a larger placebo-controlled randomised phase III clinical trial stratified on patients' DHA incorporation. In any case this study indicated that 1.8 g/d DHA had the potential of being an efficient and safe dose in humans (Bougnoux et al., 2009). With a similar dose of DHA (2 g/d) Arterburn et al. (2006) had previously obtained maximal plasma response in healthy human adults and had observed that the concentrations of DHA equilibrated

in the plasma in approximately one month, remaining stable later on, even continuing the supplementation.

EPA/DHA plasma and tissue incorporations in humans and animals

Overall, our analysis of the literature shows that only two intervention studies (Bougnoux et al., 2009; Aronson et al., 2011) have so far been published in prostate and breast patients that give direct information about the dose of EPA and/or DHA able to produce anticancer activity in humans. In both cases a clear anticancer activity was shown following a supplementation of EPA+DHA of about 2 g/day. As already discussed above, additional studies would be, however, needed to confirm the anticancer potential of these FA in these patients, as well as to definitively identify a range of safe and efficient doses. One reasonable therapeutic approach would be the combination of these fatty acids with conventional or innovative anticancer agents, as showed by Bougnoux et al. (2009). We have already discussed elsewhere that a relationship may exist between the scarcity of intervention studies and the contrasting results obtained by the epidemiologic non-intervention human studies published in this field (Calviello and Serini, 2010). Even though we have already and comprehensively discussed elsewhere this issue (Calviello and Serini, 2010;Calviello et al., 2006), we would like to emphasize here that, in spite of the abundance of preclinical studies with promising results, the conflicting outcomes of the epidemiologic non-intervention human studies may have not helped with additional scientific basis the projecting and funding of new intervention studies. We know that, whereas in the

preclinical studies the animals are supplemented with controlled concentrations of EPA and DHA, on the contrary the n-3 PUFA consumed by the subjects are established on the basis of questionnaires inquiring about the fish servings consumed by the subject per week or month. This may have led to misinterpretations, since the various methods used for calculating the n-3 PUFA content in the fish were not homogeneous, and, usually, it was not even taken in consideration if the species of fish eaten were lean or at high fat content, as well as if the subjects under studies belonged to populations basically composed by high fish consumers or not (Calviello and Serini, 2010). However, in some of the most recent non-intervention studies the level of EPA and/or DHA incorporation in plasma or red blood cells (RBC) lipids (Calviello and Serini, 2010) was evaluated. This represents an important improvement, since it has now been widely recognized that the health benefits obtained with these FAs are strictly related to their bioavailability, i.e. their incorporation in plasma and tissue lipids, considered reliable biomarkers of n-3 PUFA dietary exposure (Harris et al., 2004). A paper recently published by Brasky et al. (2013) used this new approach in a large prospective study and found an increased cancer risk in men with high LC n-3 PUFA percentage (of total fatty acids) in plasma phospholipids. This is in keeping with what hypothesized by us in a previous review, where we discussed the possibility that extremely high doses of n-3 PUFA may have also carcinogenic effects (Serini et al., 2011). In the same article Brasky et al. (2013) performed also a meta-analysis of the results of many similar studies, and showed how conflicting were the results obtained so far. It should be underlined that the erythrocyte incorporation, better if expressed in quantitative terms, seems to be a more stable biomarker of n-3 PUFA intake than plasma lipid n-3 PUFA content, which appears to be subject to large variations in relation to the more recent intake of n-3 PUFA (Tynan

et al., 1995; Katan et al., 1997). As a matter of fact, in the cardiovascular field, the "omega-3 index", i.e. EPA and DHA as the percent of total RBC fatty acids, has been considered an acceptable and easily measurable biomarker reflecting the EPA and DHA tissue status (Harris et al., 2004), and its low value has been even comprised among the main risk factors for some cardiovascular disorders. Interestingly, in his work Bougnoux et al. (2009) found a strict relationship between the anticancer efficacy of DHA and its incorporation in plasma PL, which was quite variable among the breast patients. Whereas a two-fold average increase in DHA incorporation was registered following the 1.8 g/day DHA supplementation, there were also the "best responders", i.e. those patients that showed the maximal anticancer effect, that resulted to be "high incorporators", being their plasma PL DHA level increased up to 5 folds. Arterburn et al. (2006) in a recent meta-analysis observed that the maximal incorporation of DHA (2-3 fold average increase) was achieved with a 2.0 g/day DHA supplementation, confirming the 2.5 folds increase in incorporation of EPA+DHA previously observed also by Blonk et al. in plasma PL (1990) after a supplementation of 3.0 g FO/day (containing 1.8 g EPA plus 1.2 g DHA). Also Yee et al. (2010) demonstrated a near maximal incorporation of DHA in plasma total lipids of women at high risk of breast cancer treated with 2.5 g/day EPA+DHA. In fact, they demonstrated that also a higher dose (5 g/day of EPA+DHA: 2.8g EPA+2.2g DHA) produced a similar incorporation, and that all the doses were well tolerated by the patients. In agreement with these results in breast cancer patients, recently also West et al. (2010) observed that in familiar polyposis patients an anticancer effect was observed with the administration of 2 g/day EPA alone (for 6 months), that was accompanied by an increase of EPA+DHA of about 1.5 folds in lipids of colonic mucosa. In keeping, a work performed in our laboratory about twenty years

ago (Anti et al., 1994) showed that a daily dose of 2.5 g/day (EPA+DHA) supplemented for 6 months to patients at high risk for colon cancer for sporadic polyposis was able to revert to normality the atypical proliferation of their mucosal crypt cells and to increase mucosal EPA and DHA incorporation.

The antineoplastic effects induced in animals by n-3 PUFA have been also strictly related to the EPA and DHA incorporation in plasma or tissue lipids (Kim et al., 2010), and, in fact, several of the works examined here reported these evaluations. In most of these reports their incorporation in tumor lipids (total lipids or PL) was measured, whereas just a few of them reported the incorporation in plasma PL or in the lipids extracted from normal tissues, and a strict correspondence between the incorporation in plasma or tumor lipids was observed (Noguchi et al., 1997).

Since the incorporation values published in the animals studies were not uniformly expressed (i.e, percentage of plasma, tumor or tumor microsome total lipids or PL, µM/ml, etc.), for each study we calculated the changes (in folds) that were observed in the incorporation of these FA in order to compare them. From this calculation we have obtained that in most cases the antitumoral effects were accompanied by an increase of (EPA+DHA) incorporation ranging from 3 to 5 folds in tumors. This is comparable with the incorporation observed also by us in Morris hepatocarcinoma 3924A transplanted in Aci/t rats supplemented with 0.2 g/day EPA or DHA, and is not much far from the increase of incorporation observed in humans (Bougnoux et al., 2009) and described above. By the examination of the values published in the animal studies that measured the incorporations, we obtained a calculated average (EPA+DHA) incorporation increase of 4.8 ± 0.6 folds in tumors of mice bearing breast cancer (calculated from references in

Tables 3A, 3B and 3C), and of 4.0 ± 0.9 in mice bearing transplanted prostate cancer (calculated from references in Table 1). A value of 3.1 ± 0.1 fold increase was also obtained in the incorporation of EPA+ DHA in the breast tumors of rats subject to chemical carcinogenesis (calculated from references in Tables 4A and 4B).

Conclusions

From the analysis of the existing literature we have observed that the antineoplastic efficacy of the administrations of EPA and/or DHA to animals (either furnished inside the diets or supplemented by gastric intubation) was tested mostly in comparison to the effects of diets that mimicked Western diets for their high n-6 PUFA content. We have observed that most of the (EPA+DHA) doses that have shown antineoplastic efficacy in different kinds of mouse models of prostate cancer (genetic and transplanted) were comprised between the values of 2.0 and 7.0 g/kg b. wt., Instead a dose lower than 2.0 g/kg b. wt. resulted ineffective. Likewise, in the studies using the equivalent genetic or transplantation models of breast cancer in mice most of the effective anticancer doses reported ranged between 2.0 and 8.0 g/kg b. wt., with just a few exceptions in which higher doses were used. We observed that there was the tendency to use the lowest doses (i.e. about 2.0-4.0 g/kg. b. wt.) included in these ranges of values in the most recent of these studies on both prostate and breast cancer. Instead, we have noticed that the ranges of n-3 PUFA doses that have shown anticancer effects in rats depended on the experimental models used. Doses of 0.2-0.9 g/kg b. wt. were those most used and effective in the transplantation model, whereas a wider range of n-3 PUFA doses were used in the chemical carcinogenesis

models (mostly, doses between 0.7 and 4.0 g/kg b. wt., both in the DMBA- and MNU-induced carcinogenesis models).

In most cases the antitumoral effects observed were accompanied by an increase of (EPA+DHA) incorporation in plasma or tumor lipids ranging between 3 and 5 folds. This is comparable with the 2-5 fold increase in the incorporation of (EPA+DHA) observed in human plasma lipids following a supplementation of cancer patients with doses of n-3 PUFA around 2.0 g/day (Bougnoux et al., 2009; West et al., 2010; Anti et al., 1994; Yee et al., 2010). This dose was the minimal one able to produce the maximal incorporation in tissues and was found able to produce protective effects against several kinds of cancers in humans (Bougnoux et al., 2009; West et al., 2010, Anti et al., 1994). Besides these direct evidences on the human doses with antineoplastic activity, our analysis of published data may also be of help in the choice of the appropriate EPA and DHA dose to be used in human studies. Indeed, we have observed that a supplementation to humans with safe and feasible doses of EPA+DHA of about 2 g/day produces a tissue enrichment in these fatty acids comparable to that observed in plenty of the animal studies, where clear antineoplastic effects of these FA were reported. Thus, also this observation leads to the same conclusion that a n-3 PUFA dose of about 2 g/day could be the more appropriate for clinical trials investigating possible anticancer effects of n-3 PUFA in humans, similar to those already reported by most of the animal studies.

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Table 1. Animal Models of Prostate Cancer: Trasplanted Tumors in mice

Implanted Prostate Cancer Cell	**Animals**	**n-3 PUFA diet: FO or n-3 PUFA added to diet (% w/w)**	**References Diets: Oils or PUFA added to diets (% w/w)**	**Dietary Intervention Lenght (wks)**	**Approximate LC n-3 PUFA daily dose (g/kg b wt)**	**Anticancer effect(s)**	**Ref.**
Human DU-145 (androgen independent)	Swiss (nu/nu) nude mice	20.5 (MaxEPA)§ + 3.0 (CO)	23.5 (CO)	8 (at implantation)	13.5 (EPA+DHA)	↓ tumor growth	[Karmali et al., 1987]
Human DU-145 (androgen independent)	nude mice	17 (MO) + 6 (CO)	23.5 (CO)	3 + 6 *	6.8 (EPA+DHA)	↓ tumor growth #	[Rose and Cohen 1988]
Human DU-145 (androgen independent)	nude mice	18 (MO) + 5 (CO)	18 (CO) + 5 (LO); or 5 (CO) +18 (LO)	1 + 6 *	7.2 (EPA+DHA)	↓ tumor growth	[Connolly et al., 1997a]
Human LAPC-4 (androgen sensitive)	SCID mice	5.7 (MO) + 2.6 (CO)	8.3 (CO)	2 + 8 *	2.3 (EPA+DHA)	↓ tumor growth; ↓ serum [PSA]	[Kobayashi et al., 2006]
CWR22 (androgen sensitive) or CWR22R (androgen insensitive)	castrated testosterone-supplemented nude mice	1.5 (EPA)	1.5 (OA or AA)	2 (starting after detection of tumors)	3.0 (EPA)	↓ responses to ablation therapy; ↓ number of days to tumor relapse; ↓ progression to the androgen-insensitive phenotype	[McEntee et al., 2008]

Mouse cells carrying *Pten* gene deletion	castrated nude mice	2 (EPA + DHA) + 8 (PO) + 3 (OO)	6.5 (PO) + 6.5 (SO)	1 (precastration: age-wk 9 to 10) + 6 (post-implantation: from age-wk 10 to16)	4.0 (EPA+DHA)	↓ tumor growth	[Wang et al., 2012]

CO:Corn Oil; SO: Safflower oil; MO: Menhaden oil; § MaxEPA: (33% EPA+ DHA)
PO:Palm oil; OO: Olive oil; LO: Leenseed oil
*: wks before + wks after implantation; §: cells implanted at 1 or 5 x10(6); #:effect observed only in mice injected with 1x10(6) cells.

Table 2. Genetic Animal Models of Prostate and Breast Cancer

	n-3 PUFA diet: n-3 PUFA, FO or other oils added to diet (% w/w)	**References Diets: Oils added to diets (%w/w)**	**Dietary Intervention Lenght (wks)****	**Approximate LC n-3 PUFA daily dose (g/kg b wt)**	**Anticancer effect(s)**	**Ref.**
Prostate Cancer						
Mice carryng *Pten* gene deletion	2 (EPA + DHA) + 8 (PO) + 3 (OO)	6.5 (PO) + 6.5 (SO)	19 (from 5 wk age)	3.8 (EPA+DHA)	↓ tumor formation and ↑ progression; survival	[Berquin et al., 2007]
Mice carryng *Pten* gene deletion	3.5 (MO) + 3.5 (CO)	7 (CO)	5 or 21 (from 8 wk age)	1.4 or 1.3# (EPA + DHA)	No effect	[Vissaprag ada et al., 2010]
Mice carryng *Pten* gene deletion	2 (EPA + DHA) + 8 (PO) + 3 (OO)	6.5 (PO) + 6.5 (SO)	8	4.0 (EPA+DHA)	↓ prostate syndecan expression (a marker of increasing malignancy)	[Hu et al., 2010]
Mice carryng *Pten* gene deletion	2 (EPA + DHA) + 8 (PO) + 3 (OO)	6.5 (PO) + 6.5 (SO)	1st exp: 4 mo post-castration 2nd exp: from wk 3 to mo 2 postcastration; from 2 mo to 6 mo postcastration	4.0 or 3.8# (EPA+DHA)	1st exp: ↓ number of castration-resistant tumors; 2nd exp: no difference	[Wang et al., 2012]

C3(1)Tag mice#	5 (FO concentrate*) + 5 (Canola Oil)	10 (CO)	17 or 33 (from 7 wk age)	5.7 (EPA + DHA)	↓incidence of intraepitelial dysplastic prostatic lesions	[Akinsete et al., 2012]
Breast Cancer						
MMTV/v-Ha-ras transgenic mice	20 (MO) + 1(CO)	20 (CO)	from weaning until death (max 32 mo)	7.6 (EPA + DHA)	no effect on survival and tumor incidence	[Fernandes et al., 1995]
FVB/N-TgN(MMTVneu)202Mul transgenic mice	9.7 (MO) + 0.97 (CO)§	10.7 (CO)§	15 mo (from 7-8 wk-age)	3.7 (EPA+DHA)	↓ tumor incidence and multiplicity; ↓ mammary gland dysplasia	[Yee et al., 2005]

CO:Corn Oil; SO: Safflower oil; SDA: Stereadonic acid; MO: Menhaden oil; FO: Fish oil; PO: palm oil; OO: Olive oil; *: FO contining 60% EPA + DHA

**: months only if specified; # Mice that bear a transgene for the SV40 large T antigen with a C3(1) rat prostatic steroid-binding protein promoter.

§ data originally expressed in % of energy in AIN-93G diet; #: values calculated for both the acute or chronic treatments.

Table 3A: Models of breast cancers: transplanted tumors in mice

Implanted Breast Cancer Cells or Tumors	Implanted Animals	n-3 PUFA diet: FO or PUFA added to the diets (% w/w)	References Diets: Oils or FA added to diets (% w/w)	Dietary Intervention Lenght (wks)	Approximate LC n-3 PUFA daily dose (g/kg b wt)	Anticancer effect(s)	Ref.
Balb/c mouse adenocarcinoma	Balb/c athymic mice	10 (MO)	10 (CO)	7.5 (after implantation)	4.0 (EPA + DHA)	↓tumor growth ↑tumor cell loss	[Gabor and Abraham, 1986]
Human MX-1 adenocarcinoma	Balb/c nu/+ mice	10 (MaxEPA)**	10 (CO)	10 (pre-implantation)	5.6 (EPA + DHA)	↓tumor growth	[Borgeson et al., 1989]
Human MCF-7 cells	MF1 nu/nu/ola mice	10 (FO) §	none or 10 (OO)	1 + 5 *	6.6 (EPA + DHA)	↓tumor growth	[Pritchard et al., 1989]
MCF-7 and MDA-MB231 cells	Athymic nude mice	19 (MO) + 1 (CO) 15 (MO) + 5 (CO) 10 (MO) + 10 (CO) 5 (MO) + 15 (CO)	20 (CO)	5-8 (starting 7-10 d after implantation)	2.0 -7.6 (EPA + DHA)	↓tumor growth ↑tumor cell loss°	[Gonzalez et al., 1991 and 1993; Welsch et al., 1995]
Human MX-1 adenocarcinoma	Balb/c nude mice	11 (MaxEPA) $	11 (CO or Lard)	5 + 4 *	4.8 (EPA + DHA)	↓tumor growth	[Blank and Cerian, 1989; Gabor et al., 1990]

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Human MDA-MB-435 cells	NCr-nu/nu mice	18 (MO) + 5 (CO)	18 (CO) + 5 (MO)	12 (starting 1 wk after implantation)	7.2 (EPA+DHA)	↓tumor growth	[Rose and Connolly, 1993]

CO:Corn Oil; SO: Safflower oil; MO: Menhaden oil; FO: Fish oil.
*:wks before + wks after cell implantation.
°: effect observed only with the 3 highest doses of MO; §:FO containing 33% EPA + DHA; **: MaxEPA: 28.2% EPA + DHA; $: MaxEPA: 22% EPA + DHA.

Table 3B: Models of breast cancers: transplanted tumors in mice

Implanted Breast Cancer Cells	Implanted Animals	n-3 PUFA diet: FO or PUFA added to the diets (% w/w)	References Diets: Oils or FA added to diets (% w/w)	Dietary Intervention Lenght	Approximate LC n-3 PUFA daily dose (g/kg b wt)	Anticancer effect(s)	Ref.
Human MDA-MB-435 cells	NCr-nu/nu mice	20 [(EPA-EE) + SO/CC)]° or 20 [(DHA-EE) + SO/CC)] °°	20 (SO + CC)§	7 wks + 12 wks*	8.0- 16.0 EPA or DHA	↓ tumor growth and metastasis	[Rose and Connolly, 1995]
Human MX-1 adenocarcinoma fragments	Balb/c nu/+ mice	20 (MO) + 5 (CO)	5 (CO)	20 d + (30 or 60) d*	8.0 (EPA + DHA)	↓ tumor growth	[Shao and Pardini, 1995]
Human MDA-MB-435 cells	NCr-nu/nu mice	2-8 (EPA-EE) 2-8 (DHA-EE)	8 (LA)	1st exp: 1 wk before tumor excision; 2nd exp: 8 additional wks after excision	4.0 - 16.0 (EPA+DHA)	↓metastasis	[Rose et al., 1996]
Human MDA-MB-435 cells	NCr-nu/nu mice	18 (MO) + 5 (CO)	23 (CO)	12 wks	7.2 (EPA+DHA)	↓ tumor growth and metastasis	[Connolly et al., 1997b]

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KPL-1 human carcinoma cells	NCr-nu/nu mice	9.5 (EPA) + 0.5 (LA)	10 (LA)	19 d + 43 d*	19 (EPA)	↓ tumor growth and metastasis	[Senzaki et al., 1998]
Mouse 4526 cells	BALB/cAnN mice	20 (MO)	20 (SO)	1 st exp: [4 wks + the time needed to reach 1 cm(3) tumor volume]* + 3 wks after tumor excision; 2nd exp: 4 + 3 wks *	8.0 (EPA + DHA)	↓ Tumor latency and growth ↓ metastasis and angiogenesis	[Hubbard et al.,1998; Mukutmoni-Norris et al., 2000]

CO:Corn Oil; CC: Coconut oil; DHA-EE: DHA-ethyl ester; EPA-EE: EPA-Ethyl-ester; LA: linoleic acic; MO: Menhaden oil; SO: Safflower oil.

°: providing 4 % or 8 % (EPA-EE) + 8 % or 4 % (LA), respectively; °°: providing 4 % or 8% (DHA-EE) + 8 or 4 % (LA), respectively; §: providing 8 % LA.

*:weeks or days before + weeks or days after cell implantation.

Table 3C. Animal Models of Breast Cancer: Transplanted Tumors in Mice

Implanted Breast Cancer Cells or Tumors	Implanted Animals	n-3 PUFA diet: FOs or PUFA added to the diets (% w/w)	References Diets: Oils or FA added to diets (% w/w)	Dietary Intervention Lenght (wks)	Approximate LC n-3 PUFA daily dose (g/kg b wt)	Anticancer effect(s)	Ref.
Human MDA-MB-435 Cells	NCr-nu/nu mice	20 [RBD-DHASCO + (SO/CC)]°	20 (SO + CC)°°	1 + 12 *	4.0 - 8.0 (DHA)	↓ tumor growth	[Connolly et al., 1999]
Human MDA-MB-435 Cells	athymic nu/nu mice	3 (FOC) # + 2 (CO)	5 (CO)	6 (starting 3 wks after cell implantation)	3.5 (EPA + DHA)	↓ tumor growth ↑ oxidative stress in tumor	[Hardman et al., 2001]
Human MDA-MB-435 Cells	athymic nu/nu mice	5 AAFA oil§ + 5 Canola oil	10 (CO)	3 or 5 (starting 6 wks after implantation)	5.5 (EPA+DHA)	↓ tumor growth and vascularization	[Hardman et al., 2005]
Human MDA-MB-435 Cells	nu/nu mice	9 MO + 1 CO	10 (CO)	3	3.6 (EPA+DHA)	↓ tumor growth	[Wu et al., 2005]
Human MDA-MB-435 Cells	nu/nu mice	10 MO	No addition to a normal chow diet	1 + 3 or 2 + 4*	4 (EPA+DHA)	↓ tumor growth bone metastasis	[Ghosh-Choudhury et al., 2009; Mandal et

							al., 2010]

CC: Coconut oil; CO:Corn Oil; SO: Safflower oil; SDA: Stereadonic acid; MO: Menhaden oil; FO: Fish oil; LO;
*:weeks before + weeks after cell implantation; #:FO concentrate containing 58% (EPA + DHA); §: AAFA: containing 55% (EPA + DHA).
°: providing 4% (DHA)+ 8% or 4% (LA), respectively; °°: providing 4% or 8% (LA).

Table 3D . Animal Models of Breast Cancer: Transplanted Tumors in Rats

Implanted Breast Cancer Cells or Tumors	Implanted Animals	n-3 PUFA diet: FO or PUFA added to the diets (% w/w)	References Diets: Oils or FA added to diets (% w/w)	Dietary Intervention Lenght (wks or days)	Calculated Approximate Daily LC n-3 PUFA dose (g/kg b wt)	Anticancer effect(s)	Ref.
Rat R3230AC breast tumor fragments	Fischer 344 rats	None [100-400 µl (Max-EPA)* by gavage]	None (rat chow diet: 5% fat)	1 + 4 **	0.2 - 0.9 (EPA+DHA)	↓ tumor growth	[Karmali et al.,1984]
Rat R3230AC breast tumor fragments	Fischer 344 rats	5% FO° + 15 % mix of safflower oil, hard beef tallow, linseed oil	20% mix of safflower oil, hard beef tallow, linseed oil	3 + 2.5**	0.8 (EPA+DHA)	↓ tumor growth	[Robinson et al., 2001]
Rat Walker 256 (ascitic form)	Wistar rats	None [1 g/kg b. wt. (FO)°° by gavage]	None [equal volume of (CC) by gavage]	To mother rats: 10 wks before conception, and during gestation and lactation; to offsprings: 90 + 30 days*** (starting from weaning)	0.3 (EPA + DHA) by gavage	↓ tumor growth and cachexia survival	[Togni et al., 2003]
Rat Walker 256 (ascitic form)	Wistar rats	19.8 g (FO)°°° + 9.9 g (CCO)	None or 19.8 (SF)	8 wks + 2 wks**	6.5 (EPA + DHA)	↓ tumor growth	[Pizato et al., 2005]
Rat Walker 256 (ascitic form)	Wistar rats	None [1 g/kg b. wt. (FO)°° by gavage]	None [equal volume of (CCO) by gavage]	7 wks + 3 wks**	0.3 (EPA + DHA) by gavage	↓ tumor growth, COX-2 expression,	[Mund et al., 2007]

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						↑apoptosis and tumor peroxidation	

CC: coconut oil; CO:Corn Oil; SF: Sunflower oil, SO: Safflower oil; MO: Menhaden oil; FO: Fish oil;

*MaxEPA: containing 330 mg (EPA + DHA)/ml (as reported by the authors)

°FO: (Nisshin Flour Milling Ltd, Tokio); **:weeks before + weeks after cell implantation; ***days before+ days after implantation

°° FO :mixed marine triacylglycerol preparation containing 18% EPA and 12% DHA. °°°FO: mixed marine triacylglicerol preparation containing 13% EPA and 20% DHA

Table 4A. Animal Models of Breast Cancer: Chemical Carcinogenesis

Carcinogen used	**Animals**	**n-3 PUFA diet: FO or LC-PUFA added to the basal diet (% w/w)**	**References Diets: Oils or PUFA added to the basal diets (% w/w)**	**Dietary LC n-3 PUFA intervention lenght (wks)**	**Approximate daily LC n-3 PUFA dose (g/kg b wt)**	**Anticancer effect(s)**	**Ref.**
MNU	BUF rats	20 (MO)	20 (CO)	1 wk preinduction + the time to obtain 1-2 cm tumor diameter	4.0 (EPA + DHA)	↓ tumor incidence, burden and latent period	[Jurkowsky and Cave, 1985]
DMBA	Sprague-Dawley rats	10 -20 (MO)	10 -20 (CO)	Non specified (from 1 wk after induction until sacrifice)	2.0 - 4.0 (EPA + DHA)	↓ tumor incidence and multiplicity	[Braden and Carroll, 1986]
DMBA	Sprague-Dawley rats	20 (MO) or 15 (MO) + 5 (CO)	20 (CO)	13 (starting 3 wks after induction)	2.4 - 3.2 (EPA + DHA)	↓ tumor incidence and multiplicity	[Abou-El-Ela et al., 1989]
DMBA	Sprague-Dawley rats	20.5 (MaxEPA) # + 3 (CO)	23.5 (CO)	2 + 4 °	6.1 (EPA + DHA)	↓ tumor incidence and multiplicity	[Karmali et al.,1989]
DMBA	Sprague-Dawley rats	None [0.5 ml (EPA-EE) or 0.5 ml (DHA-EE) + 0.5 ml (CO) (by gavage)]§	20 (CO)	19 wks (by gavage, 2 times/week)	0.7 (EPA-EE or DHA- EE)	↓ tumor incidence and multiplicity	[Minami and Noguchi, 1996; Noguchi et al., 1997]

CO:Corn Oil; SO: Safflower oil; MO: Menhaden oil; FO: Fish oil; LO; #: Max-EPA: containing about 300 mg (EPA + DHA)/ml
#: (given by gavage to rats fed with an high CO diet; §: basal diet containing 20% CO; °: wks preinduction + wks postinduction.

Table 4B. Animal Models of Breast Cancer: Chemical Carcinogenesis

Carcinogen	Animals	n-3 PUFA diet: FO or LC n-3 PUFA added to diet (g % w/w)	References Diets: Oils or PUFA added to diets (g % w/w)	Dietary LC n-3 PUFA Intervention Lenght	Approximate LC n-3 PUFA daily dose (g/kg b wt)	Anticancer effect(s)	Ref.
DMBA	Sprague-Dawley rats (nursing dams or prepubertal rats)	3.5 (MO)§ + 3.5 (CO) (low fat n-3 PUFA)	0.5 (MO)§ + 6.5 (CO) (low fat n-6 PUFA)	from 5 to 15 days of age via milk, from 15-25 days of age via diet	0.8 (EPA+DHA)	↓ tumor incidence ↓ cell proliferation ↑ apoptosis	[Olivo and Hilakivi-Clarke, 2005]
MNU	Sprague-Dawley rats	1.5 (DHASCO)*	8 (PO)	20 wks (starting 2 days after induction)	0.6 (DHA)	↓ tumor growth and angiogenesis	[Colas et al., 2006]
MNU	Sprague-Dawley rats	8 (DHASCO)**	8 (PO)	17 wks (starting 1 day after induction)	2.6 (DHA)	↓ tumor incidence ↓ BRCA1 levels	[Jourdan et al., 2007]
DMBA	Sprague-Dawley rats	none [0.5 ml (Max-EPA°) by gavage]	no addition or 0.5 ml (CO) by gavage	2 wks + 35 wks^	0.8 (EPA + DHA)	↓ tumor incidence and growth ↑ latency period	[Manna et al., 2007 and 2008]
MNU	Sprague-Dawley rats	17 g (FO)*** + 3 g (CO)	20 (CO)	7-8 wks (post-induction)	4.5 (EPA+DHA)	↓ tumor multiplicity and growth §§ ↓ cell proliferation in preneoplastic lesions	[Manni et al., 2010 and 2011]

MNU	Sprague-Dawley rats	14.5 (Omega3/90EE)**** + 0.05 SO #	0.9 (CC) +2.2 (CO) + 6.0 (MO) + 4.3 (PO) + 1.2 (SU) ##	8 wks (starting 1 wk after induction)	11.6 (EPA + DHA)	↓ cancer incidence and burden	[Zhu et al., 2011]

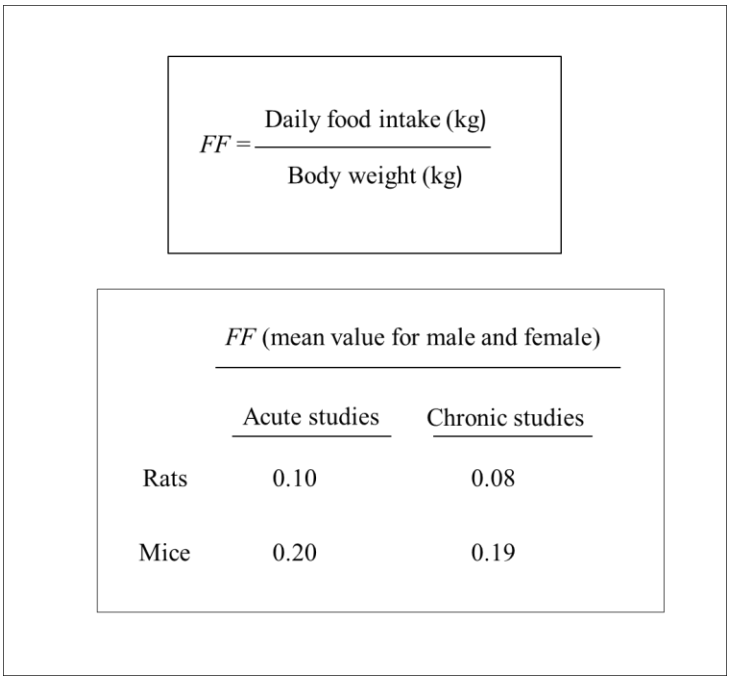
CC: Coconut oil; CO:Corn Oil; SO: Safflower oil; ; MO: Menhaden oil; FO: Fish oil; PO: Palm oil; SU: Sunflower oil; ° Max-EPA: 150 mg (EPA + DHA)/ml

§MO: 22% EPA + DHA; *DHASCO: 45 % DHA; **: DHASCO: 40 % DHA. ° Effects observed in prepubertal rats. ^:wks preinduction + wks postinduction.

***FO: Virginia Prime Gold: 26.30% (EPA + DHA); §:dose given to nursing dams (from days 5-15 of puppies' age) and to puppies (from 15 to 25 days of age);

****: Omega3/90EE: about 80% (EPA+ DHA). #: 25:1 n:3/n:6 PUFA ratio; ##: 1:1 n:3/n:6 PUFA ratio: 1.2 g (EPA + DHA)/kg b.wt.

§§: Transient effects with FO alone; Stable and marked effects in combination with suboptimal doses of tamoxifen.



$$FF = \frac{\text{Daily food intake (kg)}}{\text{Body weight (kg)}}$$

	FF (mean value for male and female)	
	Acute studies	Chronic studies
Rats	0.10	0.08
Mice	0.20	0.19

Figure 1. Food factors for rats and mice. Modified from "U.S. EPA Recommendations for and Documentation of Biological Values for Use in Risk Assessment (EPA/600/6-87/008)" (http://www.tera.org/Tools/ratmousevalues.pdf). *FF*: Food factor.