Fish oil diet during pre-mating, gestation, and lactation in adult offspring rats on cancer cachexia prevention

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Abbreviations: ω-3 (omega-3); AIN (American Institute of Nutrition); ALT (alanine aminotransferase); AST(aspartate aminotransferase); DEXA(dual-energy x-ray absorptiometry); DHA (docosahexaenoic acid); EPA(eicosapentaenoic acid)

Abstract

Scope: Nutritional supplementation of the maternal diet can modify the cancer susceptibility in adult offspring. Therefore, we evaluated the effects of a fish-oil diet administered to a long-term, during pre-mating, gestation and lactation, in reducing cancer-cachexia damages in adult Walker-256 tumour-bearing offspring. **Methods and results:** Female rats received control or fish oil diet during pre-mating, gestation, and lactation. After weaning, male offspring were fed the control diet until adulthood and distributed in (C) control adult-offspring; (W) adult tumour-bearing offspring; (OC) adult-offspring of maternal fish oil diet; (WOC) adult tumour-bearing offspring of maternal fish oil diet groups. Fat body mass was preserved, muscle expression of mTOR and 4E-BP1 was modified, being associated with lower 20S proteasome protein expression, and the liver ALT enzyme content maintained in the WOC group. Also, the OC group showed reduced triglyceridemia. **Conclusion:** In this experimental model of cachexia, the long-term maternal supplementation was a positive strategy to improve liver function and lipid metabolism, as well as to modify muscle proteins expression in the mTOR pathway and also reduce the 20S muscle proteasome protein, without altering the tumour development and muscle wasting in adult tumour-bearing offspring.

1. Introduction

Cancer cachexia is a multifactorial syndrome, characterised by systemic inflammation and continuous fat and lean body mass wasting, especially skeletal muscle [1,2]. In this syndrome, the upregulated ubiquitin-proteasome pathway [3,4] associated with the downregulated mTOR pathway [5] leads to muscle wasting, reducing its function, which cannot be entirely reversed by conventional nutrition [6]. Therefore, new strategies to prevent this wasting process in cachexia condition are necessary.

One of the most used nutritional supplements in cancer treatment is the omega-3 (ω-3) polyunsaturated fatty acid [7], by its role in modulating protein catabolism and anti-inflammatory properties [7–9]. Omega-3 attenuates the ubiquitin-proteasome pathway [10] and even reduces different types of cancer incidence *in vitro* and *in vivo* models [11]. Rich in omega-3, the cod liver oil, which is often used as a nutritional supplement, has preventive effects because of higher ω-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content. Epidemiological studies with high fish oil consumption, rich in omega-3, showed lower colon cancer rates and decreased risk of prostate cancer incidence [12,13]. Also, the exposure to ω-3 fatty acids during the perinatal period influences the offspring's metabolic development, reducing the plasma and hepatic concentration of esterified fatty acids, such as plasmatic triglycerides, ameliorating peripheral insulin sensibility [14,15]. Also, maternal ω-3 diet affects growth, fat mass and lipid content in foetuses [16], as the genes related to lipid metabolism are more susceptible to changes during gestation and lactation,

influencing these metabolism pathways on offspring [17]. Thus, maternal nutrition interventions alter the offspring health throughout life and the disease susceptibility [18–20].

Previous studies with the same experimental tumour model and a lifelong supplementation with fish oil, to dams and offspring, showed that their adult Walker-256 tumour-bearing offspring improved survival and macrophages and lymphocytes function[21–23]. In these studies, the authors focused mainly on the preventive and therapeutic administration of fish oil, rich in ω-3, in reducing cachexia damages. Instead, in this study and our previous work, we focused on preventing the effects of cancer-induced cachexia by the maternal influence alone. Our previous study with a maternal fish oil diet only during pregnancy and lactation time focused on liver responses to cachexia development [24], showing improved cachexia indexes, liver function, and antioxidant response in adult tumour-bearing rat offspring. Whereas, the mother's initial stock of body fatty acid is recruited during gestation and lactation for foetal development [15], the dietary fatty acid composition before the conception likely influences the ω-3 amount disposable to the offspring. In this way, studies are needed to relate the offspring responses to interventions also in the pre-mating time. Therefore, we designed a new study also to contemplate this phase and evaluated the responses of muscle loss to a maternal diet intervention in order to increase knowledge in this new investigation area.

In this study, we hypothesised that a long-term maternal fish oil supplementation – during pre-mating, gestation, and lactation – could preserve the offspring damages led by the cachexia

development. For this purpose, we evaluated the body composition, liver function, lipid serum parameters, and some of the proteins related to muscle protein synthesis and degradation pathways along with key proteins involved in tumour growth in adult tumour-bearing offspring.

2. Experimental Section

2.1. Animals

Wistar adult rats were obtained from our animal facilities and kept in our Laboratory of Nutrition and Cancer, in individual ventilated cages, with food and water *ad libitum*, under controlled environmental conditions (temperature, 22 ± 2 ºC; light and dark, 12/12 h; humidity, 50-60%). The animals were monitored daily and weighed three times per week.

2.2. Diets

The semi-purified diets were prepared following the American Institute of Nutrition (AIN-93) [25], with the same amount of protein, carbohydrate, lipids, minerals, vitamins and fibre. The control diet (C) contained 7% soy oil, and the fish oil diet (O) contained 7% cod liver oil, corresponding to 22.84% of LCPUFAs ω-3, especially EPA and DHA(fish oil: 9.69 ± 0.05 % of EPA and 9.42 ± 0.15 % of DHA), as shown in our previous study [24].

2.3. Experimental procedure

The experimental procedure was in accordance with the United Kingdom Coordinating Committee on Cancer Research [26] approved by the Ethics Committee on Animal Experimentation of

the Institute of Biology at the University of Campinas (protocol number: #4464-1). The long-term nutritional supplementation scheme consisted of feeding the females rats (4 month-old) with C and O diets during 8 weeks pre-mating [27], measuring the food intake. Then, following harem method [28], these females were mated with same-aged males, during one week, distributed in two groups: Control Dams (DC), and Fish oil Dams (DO), feeding the respective diet during gestation and lactation, and placed individually in cages. Dams blood was collected to analyse biochemical parameters at the beginning of the experiment, before mating and after weaning.

After offspring birth, the number of male and female, and pups weight were collected and then the litter were reduced to 8 pups per dam. After weaning, the male pups were fed C diet until adulthood. At 120 days-old, the offspring were redistributed according to maternal diet and tumour implant into the followed groups: control adult offspring (C; n = 8); adult tumour-bearing offspring (W; n = 7); adult offspring of maternal fish oil diet, without tumour (OC; n = 6); adult tumour-bearing offspring of maternal fish oil diet (WOC; n = 10).

Food intake was measured before and during tumour development. Tumour implant consisted of unique subcutaneously injection of $3x10^6$ viable Walker-256 tumour cells in the right flank [29]. After 21 days of tumour growth, all the animals were euthanised by decapitation and collected blood, liver, gastrocnemius muscle, spleen and tumour tissue and the carcasses. The schematic timeline is represented in Figure 1.

2.4. Morphometric and serum parameters

The body composition (bone mass, lean body mass, fat mass) was assessed by dual-energy x-ray absorptiometry (DEXA) Discovery model (Hologic, Marlborough, MA, USA) located in the Nuclear Medicine Service at the State University of Campinas. Total body water content was measured by the difference between the dry and wet carcass weights. The carcass weight represented the whole-body weight without liver, spleen, gastrocnemius muscle and tumour. The body weight variation was calculated as the ratio between carcass weight and initial body weight, as a percentage. The cachexia index was calculated by the following formula: [initial weight – carcass weight + tumour weight + control group weight gain)/(initial weight + control group weight gain)] x 100% [30].

Serum parameters (cholesterol, triglycerides, HDL, LDL, albumin) were quantified spectrophotometrically using commercial kits (Bioclin, Belo Horizonte, Brazil). The liver samples were homogenised in phosphate-buffered saline, and the homogenate was centrifuged at 12.000 x g for 15min at 4 ºC, and supernatants were assessed to measure the hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes contents, using commercial kits (Bioclin, Belo Horizonte, Brazil).

2.5. Western Blot Assay

Gastrocnemius muscles samples were homogenised in buffer (10 mM EDTA, 100 mM Tris, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM phenylmethylsulphonyl fluoride, 0.1 mg/mL aprotinin), centrifuged at 12,000 x g, and total protein

content was assessed using the Lowry method [31]. Skeletal muscle proteins (60 µg) were resolved by SDS-PAGE on 10% or 12% gels, followed by Western blotting assay. Proteins were probed with primary antibodies GAPDH (SC47724) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); 20S (PW8195), 19S (PW9265), and 11S (PW8185) (Enzo Life Sciences, Farmingdale, NY, USA); PI3K (4292), phosphor-PI3K (4228), Akt (4685), phospho-AktThr308 (4056), mTOR (2972), phospho-mTOR (2971), p70S6K (9202), phospho-p70S6KThr421/Ser424 (9204), 4E-BP1 (9452), phospho-4E-BP1Thr70 (9455) (Cell Signalling, Danvers, MA USA); and secondary antibodies goat anti-rabbit (7074) and horse anti-mouse (7076) (Cell Signalling). The band images were captured (Alliance 2.7, UVITEC, Cambridge, UK) and quantified using the UVIband-1D (UVITEC). The protein expression was normalised using the GAPDH as a loading control.

In tumour tissue, the same protocol was followed for protein quantification using the primary antibodies BAX (SC7480), Bcl-2 (SC7382), VEGF (SC507) (Santa Cruz Biotechnology), Cleaved Caspase-3 (9664), P53 (2524), phospho-P53 (9284) (Cell Signalling), and e-Cadherin (07-697) (Upstate, Lake Placid, NY, EUA).

2.6. Statistical Analyses

The results are expressed as the means ± SEM, and statistical comparisons were made with two-way ANOVA followed by the post-hoc Bonferroni test (Graph Pad Prism software, version 7.0, San Diego, CA, USA). For direct comparisons between two groups, the data were analysed using the non-

parametric Mann-Whitney test. When the *p*-value was < 0.05, the results were considered significant [32].

3. Results

3.1. Fish oil diet did not change maternal body composition, whereas modified the weight gain, food intake and reduced triglycerides levels

During the 8 weeks of pre-mating, the fish oil diet did not change the dams food intake and body weight evolution, being similar between the DC and DO groups (Figure 2A and 2B). During gestation until the 32nd day (which correspond to 11th lactation day), food intake was similar, but after that, showing an effect of the maternal diet, the DO group had a reduction compared to DC until weaning (Figure 2C). The DO body weight gain was also reduced on the 17th - 20th pregnancy days in relation to DC, followed by a recovery in both groups that lasted until weaning (Figure 2D and 2E).

Also evaluating the dams responses to the maternal diet, the serum cholesterol, triglycerides and albumin levels were analysed, being similar between both groups after pre-mating (Table 1). After weaning, the triglycerides levels reduced in the DO group (p = 0.03), likely indicating a modulation by the maternal diet, with no alterations in the fat and lean body mass contents, water content and relative bone mass (Table 1).

3.2. Long-term maternal fish oil diet modified offspring growth, but not permanently

In relation to the litters, the long-term maternal fish oil diet did not alter the number of pups/litter, the female/male proportion and, especially, the birth weight (Supporting Information Table S1).

In accordance with the birth weight, the offspring had similar body weight in both groups on the second day until the 14th day after birth (Figure 3A). However, the OC pups body weight reduced compared to C group, which was recovered at the 44th day, then, maintaining similar until the tumour inoculation, at 120 days-old (Figure 3B).

3.3. Long-term maternal fish oil diet did not prevent body wasting in adult tumour-bearing offspring but modified hepatic ALT levels and serum triglycerides content

There were no significant differences in the initial body weight neither in the initial food intake among the groups before tumour implant (Table 2). After 21 days of tumour growth, the carcass weight and weight variation in tumour-bearing groups reduced when compared to non-tumour-bearing groups (carcass weight: W < C, p = 0.0012 WOC < OC, p = 0.0063; weight variation: W < C, and WOC < OC, p < 0.0001, Table 2). Also as a consequence of the cachexia [2], the final food intake in W group was 56% lower than in C group, whereas in the WOC group the food intake reduced 43% compared to the OC group (W < C, p < 0.0002; WOC < OC, p = 0.0308, Table 2).

The liver weight was similar among the groups, as well the gastrocnemius muscle weight (Table 2), despite the decrease in the carcass weight. The increased spleen weight indicated an overload of

the immune response in both tumour-bearing groups (W > C, WOC > OC, $p < 0.0001$). The long-term maternal fish oil diet did not alter the tumour weights, and the cachexia indexes in W and WOC, being similar between the groups.

In order to verify whether the maternal diet could influence adult offspring, we accessed the serum parameters and hepatic metabolism, usually deregulated in the cachexia syndrome [1] (Table 3). The serum albumin content reduced in both tumour-bearing groups (W < C; WOC < OC, $p < 0.0001$; being the tumour factor responsible for 74% of the total variation), and the total cholesterol levels had difference only between WOC and C groups (WOC < C, $p = 0.0007$) (Table 3). The LDL-C levels were similar among the groups. Besides, the HDL-C level reduced in both W and WOC (W < C, $p < 0.0001$; WOC < OC, $p = 0.0007$; as the tumour factor accounted for 62.3% of total variation, considered extremely significant). Showing another positive effect led by the maternal diet, the triglycerides levels reduced in OC compared to control group (OC < C, $p = 0.0465$), and W tumour-bearing group (W > OC, $p = 0.0007$), showing a significant effect led by diet factor (26.8%) and by tumour factor (20.4%). Another effect influenced by maternal diet was related to the liver function. Whereas the tumour-bearing group showed an increased hepatic ALT content (where the tumour factor accounted for 25% of total variation, being considered very significant effect), a similar content of this liver enzyme occurred in both fish oil maternal supplemented groups (OC = WOC; Table 3). Additionally, the WOC group had lower ALT content than W group (WOC < W, $p = 0.0087$; where this effect accounted for 14% of total variance), showing another positive effect of this maternal intervention. On the other hand, the AST content was similar among the groups (Table 3).

The lean body mass decreased in both tumour groups (W < C, p = 0.0417; WOC < OC, p = 0.0012). Additionally, the relative fat body mass reduced only in the tumour-bearing group (W < C, p = 0.0115) (Table 4). As a positive effect of maternal nutritional intervention, the fat body mass was maintained in both groups supplemented with fish oil, showing less fat mass spoliation independently of tumour growth. Moreover, the water body content was higher in W and WOC (W > C, p = 0.00168; WOC > OC, p = 0.0022).

3.4. Long-term maternal fish oil diet modified the mTOR pathway and reduced the muscle 20S proteasome protein expression without changing tumour parameters

The mTOR pathway is usually impaired in cachectic animals showing impairment on protein synthesis [33]. The activation of PI3K and Akt, upstream activators of the mTORC1 pathway, had no differences among the groups (Supporting Information Figure S1A and B). Moreover, the mTORC1 activation did not show differences between both tumour-bearing groups (Figure 4A), but the tumour factor accounted for 59,6% of the total variation, which means that the tumour effect was considered extremely significant reducing the mTOR activation compared to the control groups (W < C, p = 0.0605; WOC < OC, p < 0.0016; Figure 4a). The activation of the downstream protein P70S6K1 was similar among the groups (Supporting Information Figure S1C). Conversely, the 4E-BP1 activation reduced in WOC group (WOC < OC, p = 0.0002) and the tumour factor accounted for 52.9% of the total variation which means that the tumour effect is considered extremely significantly

(Figure 4b). In relation to the maternal diet effect, the muscle mTORC1 and 4EBP-1 protein content were higher in the OC group when compared to the W group (OC > W; $p = 0.0005$ and $p = 0.0011$; respectively), but the diet factor accounted for approximately 8,5% of the total variation, where the diet effect was considered not quite significant for this parameter.

In relation to protein degradation, the muscle proteasome subunits were evaluated, showing some maternal fish oil diet effects. The 19S regulatory subunit and the 20S catalytic subunit (32KDa) showed no differences among the groups (Figure 4c and 4d). On the other hand, for the other 20S catalytic subunit (28KDa), we observed the maternal influence for both maternal supplemented groups. The WOC tumour-bearing group had a reduction in this 20S proteolytic subunit (28KDa) in comparison to the W group (WOC < W, $p = 0.00117$, Figure 4E), and also the OC group showed a reduced expression compared to the W group (OC < W, $p = 0.0138$, Figure 4E, the diet factor accounted for 56% of total variation, being extremely significant).

In order to verify the effects of the long-term fish oil diet on tumour development, proteins related to cellular death (BAX, Caspase-3, Bcl-2), angiogenesis (VEGF), metastasis (e-Cadherin), and tumour suppression (p53) were quantified, and no differences were found between the tumour-bearing groups W and WOC, showing no effects of this nutritional scheme, in accordance with the similar tumour weight found in both groups (Supporting Information Figures S2A-F).

4. Discussion

In this study, as an innovative of maternal nutritional approach against other studies in the literature [21–23], we have shown that a long-term maternal fish oil diet, during the periods of pre-mating, gestation and lactation, influenced a few parameters in adult tumour-bearing offspring. Although fish oil supplementation only during pregnancy and lactation, as demonstrated in our previous work, can decrease the cachexia impairment, reducing cachexia index and ameliorating liver function and reducing its oxidative stress [24], here we showed that with the additional pre-mating diet for 8 weeks, the fish oil diet had no efficient effect in preserving the energy body sources of the tumour-bearing offspring. However, this maternal nutritional scheme influenced the lipid metabolism and modulated the expression of the mTOR and 20S proteasome protein and, similarly, preserved liver function in adult tumour-bearing offspring.

In both dams groups, with the long-term fish oil supplementation, we observed that serum albumin and bone, fat and lean body mass were similar before and after gestation and lactation, probably indicating the safety of this maternal diet supplementation. Besides, as one of the benefits of omega-3-rich diets [34], the reduced triglyceridemia in DO group likely showed a positive effect of this intervention, modifying the lipid metabolism. In addition, considering that the offspring had a similar profile of body weight evolution, except for late weaning and three weeks after weaning, associated to a decrease in serum triglycerides in adulthood, as a similar pattern as observed in their mothers, this fact showed the maternal influence on this modulation. A study with soy oil (rich in

omega-6), and flaxseed oil (rich in omega-3) diets, administered on the last ten days of pregnancy and during breastfeeding, showed that the ω-3 supplemented-offspring had reduced leptin levels [35], which could possibly explain the offspring reduction of body weight on the 14^{th} – 44^{th} day of life followed by recovery and the modulating effect on lipid metabolism in the adult-offspring.

Here we verified that the cachexia state happened in both adult offspring independently of the maternal nutritional scheme. The low food intake and the altered serum parameters, such as serum albumin, triglycerides and HDL-C combined to changed body composition confirmed the cachexia condition in both tumour-bearing animals. The lower lean body and fat body masses, as verified in W group, corroborated these altered serum parameters levels, showing that the tumour growth induced the host energy source waste, especially over lipid and protein content, according to the literature [1,2]. Conversely, the maternal fish oil diet could preserve the fat body mass storage in the tumour-bearing offspring, likely showing an improvement of the lipid metabolism, despite not reflecting in an improvement of the global carcass spoliation. This is an important finding, beyond the muscle wasting, one of the severe effects of cancer cachexia is the spoliation of the host adipose tissue [1]. Thus, this long-term maternal nutrition supplementation could reduce the cachexia impact by preserving this tissue so relevant to the syndrome development. The increased water body content was associated with decreased serum albumin, which likely led to a reduced serum osmotic pressure, inducing oedema, as expected in the cachectic state [33]. Despite having a tumour, the tumour-bearing groups had no differences on the triglyceridemia and cholesterol levels, even with minimal benefit effects led by the maternal nutritional scheme. However, the fish oil diet could

induce changes in the triglyceridemia, with diminished content in the OC group compared with the control group. This could indicate another improvement of lipid metabolism by the maternal diet. Similar results have been observed, as Mennitti and colleagues demonstrated that 21-days old rat offspring from mothers under fish oil supplemented diet reduced serum triglycerides levels [14]. Besides, regarding liver function, the WOC group had hepatic ALT levels different from W group, suggesting that the altered hepatic function found in W group, as a consequence of tumour damage effect, was improved by the influence of maternal supplementation in WOC, as indicated by the preserved content of this liver enzyme. These results likely suggested a modulatory effect of maternal diet over liver responses faced to tumour growth, in accordance with our previous work, showing that the additional diet intervention during pre-mating phase led to the same positive results found in relation to liver function [24].

Moreover, when evaluating the muscle protein synthesis, the mTORC1 pathway was less activated in both tumour-bearing groups, likely leading to lower protein synthesis. This fact is corroborated by the reduced lean body mass found in W group, and also in WOC group, suggesting that the long-term maternal supplementation was not efficient in preserving the protein synthesis. However, as a positive effect of maternal fish oil diet, in adult offspring without tumour, this maternal diet scheme could modify the mTOR and 4-EBP1 proteins, increasing their expression. Additionally, by the literature, the damage effects of tumour growth can upregulate some protein degradation pathways, especially the ubiquitin-proteasome pathway [36,37]. Here, interestingly, the 19S, the ATP-dependent regulatory subunit, showed no differences among the groups, while the

expression of 20S (28KDa) catalytic subunit reduced in the maternally supplemented groups (OC and WOC), likely due to epigenetic modifications, downregulating this subunit expression. Indeed, this is in accordance with treated cachectic mice with EPA, which had an attenuated expression of the 20S subunits [38], showing the same effect for the preventive approach. However, maternal supplementation did not prevent lean body mass waste. Thus, other protein degradation pathways could be driving this spoliation, as seen in previous studies with the same tumour model [39,40]. Moreover, it would be interesting to evaluate the proteasomal activity correlating with the proteasomal protein expression levels. This analysis could also be important to clarify the maternal influence over this degradation pathway, which is a limitation of our study.

In addition, the omega-3 supplementation was previously reported as anticarcinogenic and with properties of reducing the tumour growth [11,23,41]. In a maternal preventive administration, there were no differences between the tumour relative weights in W and WOC groups, confirmed by the similar expression of proteins related to cellular death, angiogenesis, metastasis, and tumour suppression. These results suggest that the fish oil maternal supplementation had no effect on altering the epigenome of the offspring regarding the modulation of the Walker 256 tumour microenvironment. Therefore, in further studies, it would be interesting to use an induced tumour model to verify the possible intergenerational influence, observing not only the tumour microenvironment modulation but also the oncogenesis modulation from the host cells.

Summing up, the long-term maternal supplementation with fish oil improved hepatic function and lipid metabolism, preserving the fat body mass, modifying the expression of some proteins of the mTOR pathway and downregulated the expression of 20S muscle protein, related to the ubiquitin-proteasome pathway, in adult offspring. On the other hand, this maternal diet scheme did not affect tumour development and had no effect on preventing lean body mass loss. Overall, the long-term maternal supplementation may influence the epigenetic modifications of the offspring, since it possibly had modulated the maternal metabolism, PUFA sources and therefore the ω-3 amount delivered to foetus and neonates, improving some essential functions in adult offspring, independently of the tumour presence. Thus, more studies are necessary to clarify the involved epigenetic modifications provided by this long-term maternal supplementation.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Figures and tables

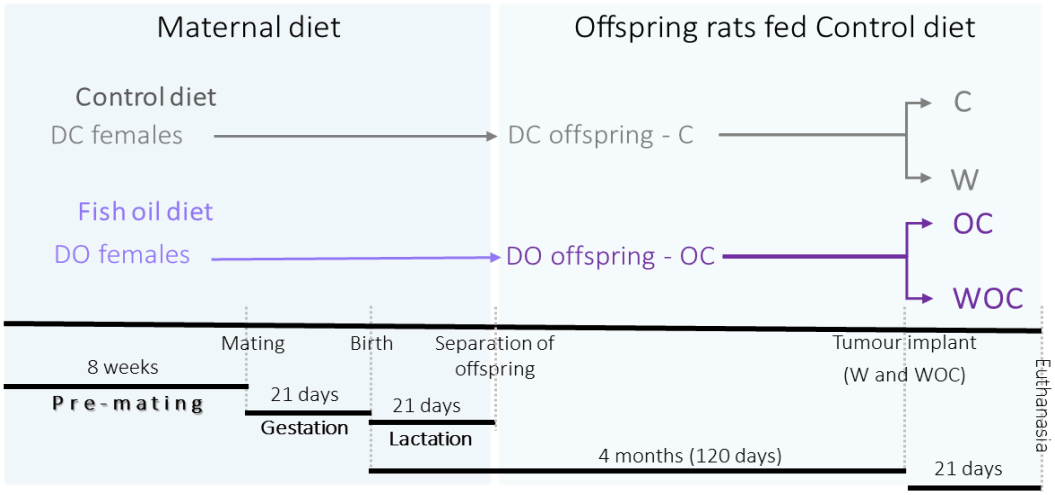

Figure 1: Experimental procedure scheme. DC: Control Dams fed control diet; DO: Omega -3 Dams fed fish oil diet; C: control adult offspring; W: adult tumour-bearing offspring; OC: adult offspring of maternal fish oil diet, without tumour; WOC: adult tumour-bearing offspring of maternal fish oil diet (n ≥ 6, per group).

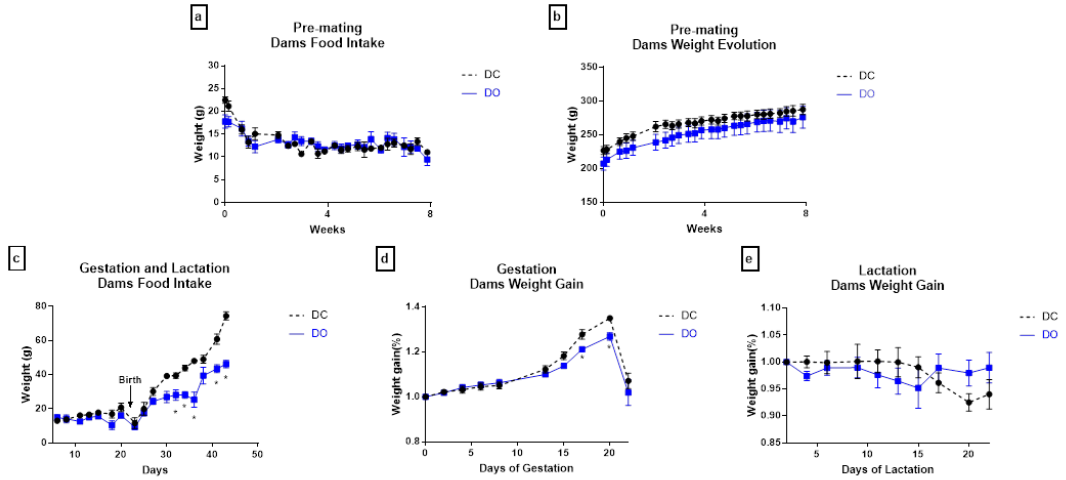

Figure 2. Food intake and body weight gain in both dam groups. (a) Food intake during the pre-mating period (g). (b) Body weight evolution during the pre-mating period (g). (c) Food intake during pregnancy and lactation periods (g). (d) Body weight gain during pregnancy (%). (e) Body weight gain during lactation (%). DC: Control

Dams (n = 4); DO: Fish Oil Dams (n = 6). The values are means ± SEM, and for some points, the error bars would be shorter than the symbol size. * Compared to DC group, $p < 0.05$, detected by Mann-Whitney test.

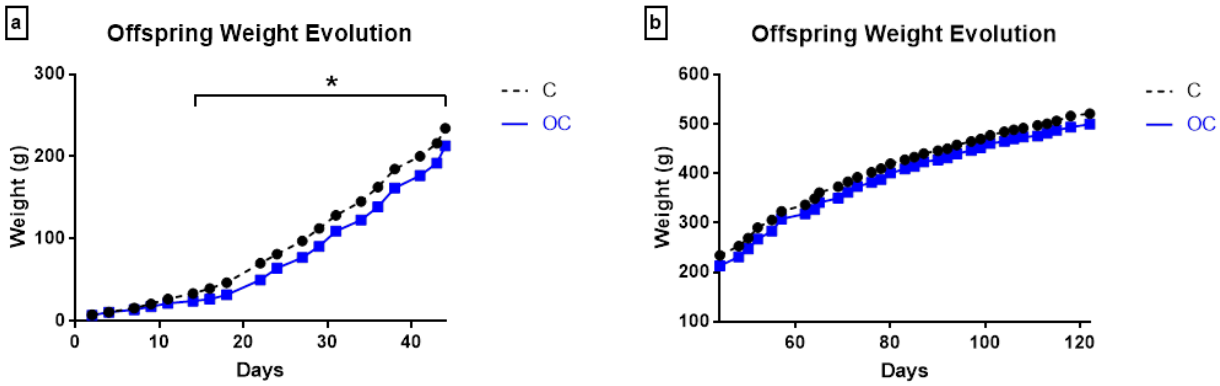

Figure 3. Offspring growth evolution from birth until adulthood from both experimental diet schemes. (a) Body weight evolution from the 2nd to the 44th day old (g). Each litter was weighed and distributed by the number (n = 8) of pups/litter. (b) Body weight evolution from the 44th to the 141st day-old (g). C: control offspring (n = 15); OC: adult offspring of maternal fish oil diet (n = 16). The values are means ± SEM, and for some points, the error bars would be shorter than the symbol size. * Compared to C, $p < 0.05$, detected by Mann-Whitney test.

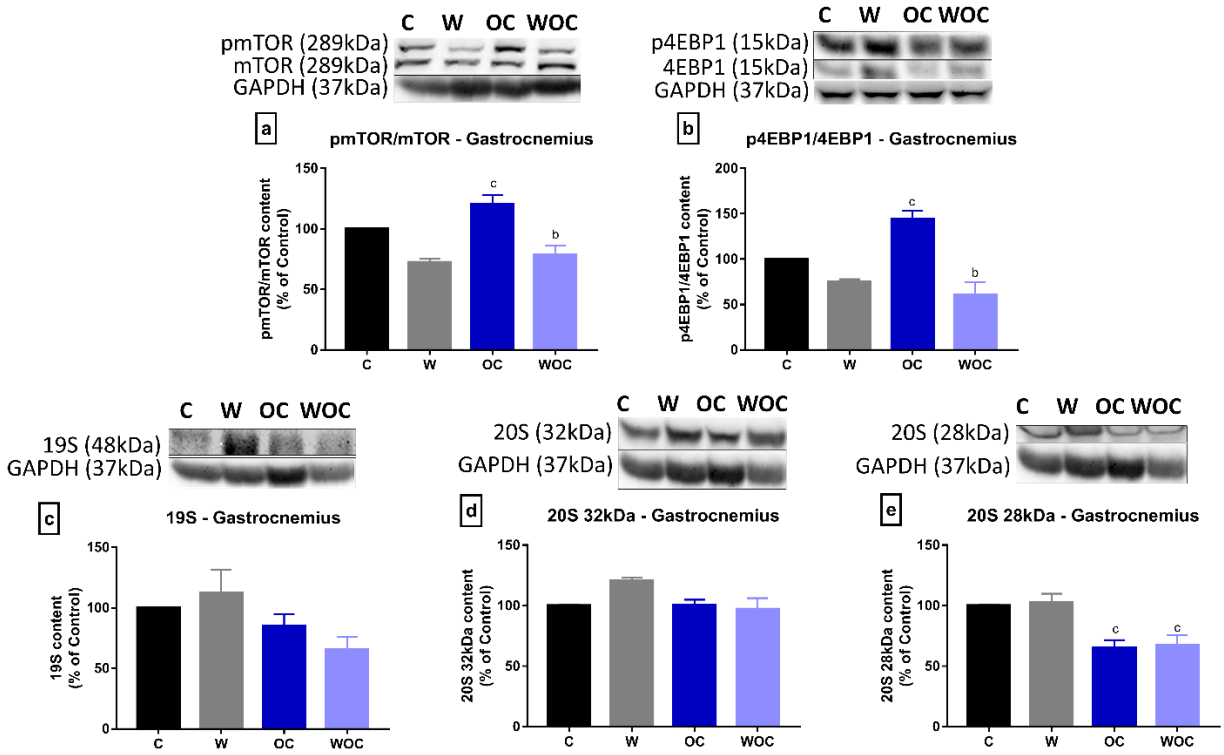

Figure 4. mTOR pathway and proteasome subunits proteins expression in gastrocnemius muscle. (a) pmTOR/mTOR content; (b) p4E-BP1/4E-BP1 content; (c) 19S content; (c) 20S (32 KDa) content; (e) 20S (28KDa) content. The results represent the quantified and normalised bands by GAPDH expression. Western blot images were representative of all different groups. Values are expressed as the relative percentage of the C group, considered as 100%, and are presented as means ± SEM. C: control adult offspring (n = 6); W: adult tumour-bearing offspring (n = 6); OC: adult offspring of maternal fish oil diet (n = 6); WOC: adult tumour-bearing offspring of maternal fish oil diet (n = 7). Significant differences detected by two-way ANOVA showed that: for mTOR analysis, tumour factor accounted for $p < 0.0001$, diet factor for $p = 0.0558$ and interaction effect for p=0.2761; for 4EBP1 analysis, tumour factor accounted for $p = 0.0001$, diet factor for $p = 0.1128$ and interaction effect for $p = 0.0476$; 19S analysis, tumour factor accounted for $p = 0.792$, diet factor for $p = 0,0275$

and interaction effect for p = 0.2297; 20S (32kDa), tumour factor accounted for p = 0.2063, diet factor for p = 0.1034 and interaction factor for p = 0.0994; 20S (28kDa), tumour factor accounted for p = 0.5261, diet factor for p = 0.0002 and interaction effect for p = 0.2297. Differences: [a] Compared to C, $p < 0.05$; [b] compared to OC, $p < 0.05$, [c] compared to W, $p < 0.05$, detected by the post-hoc Bonferroni test.

Table 1. Dams parameters after pre-mating and weaning.

Dams serum parameters		
After pre-mating	DC (n = 4)	DO (n = 6)
Cholesterol (mg/dL)	133.78 ± 1.89	129.79 ± 3.94
Triglycerides (mg/dL)	109.27 ± 3.83	106.13 ± 3.81
Albumin (g/dL)	3.20 ± 0.21	3.18 ± 0.20
After weaning	DC (n = 4)	DO (n = 6)
Cholesterol (mg/dL)	131.16 ± 5.93	125.20 ± 2.56
Triglycerides (mg/dL)	124.10 ± 15.06	92.03 ± 2.64*
Albumin (g/dL)	3.59 ± 0.02	3.71 ± 0.07

Dams Body Composition		
After weaning	DC (n = 4)	DO (n = 6)
Fat Mass (%)	17.50 ± 2.59	15.93 ± 0.64
Lean Body Mass (g)	39.78 ± 3.87	36.81 ± 1.88
Water Content (%)	62.10 ± 1.23	63.22 ±0.61
Bone Mass (%)	4.10 ± 0.14	3.89 ± 0.22

Legend: DC: dams fed control diet; DO: dams fed fish oil diet. The values are means ± SEM. * Compared to DC, $p < 0.05$, detected by Mann-Whitney test.

Table 2. Adult offspring morphometric parameters.

Morphometric parameters							
Parameter	Group				*p-value		
	C (n = 8)	W (n = 7)	OC (n = 6)	WOC (n = 10)	Tumour factor	Diet factor	Interaction effect
Initial weight (g)	532.34 ± 15.53	526.71 ± 16.02	510.26 ± 10.87	509.71 ± 4.41	0.8048	0.1255	0.8389
Carcass weight	541.79 ±	448.48 ±	521.45 ±	438.70 ±	<0.0001*	0.3467	0.7396

(g)	17.56	21.43[a]	13.84 [c]	7.42[a,b]			
Weight variation (%)	1.71 ± 0.62	-15.08 ± 2.16[a]	2.56 ± 0.76 [c]	-13.90 ± 1.47[a,b]	<0.0001*	0.5991	0.8046
Initial food intake (g)	19.39 ± 0.80	16.98 ± 0.50	18.23 ± 0.61	20.71 ± 2.01	0.9789	0.3675	0.0928
Final food intake (g)	19.20 ± 0.60	7.40 ± 1.68[a]	19.15 ± 0.68[c]	11.76 ± 2.19[b]	<0.0001*	0.2144	0.2044
Liver (g)	15.77 ± 0.85	15.72 ± 0.64	13.64 ± 0.43	14.87 ± 0.55	0.3812	0.0333*	0.3466
Gastrocnemius (g)	3.27 ± 0.16	3.10 ± 0.21	3.30 ± 0.15	2.85 ± 0.11	0.0635	0.5112	0.3811
Spleen (g)	0.84 ± 0. 04	2.09 ± 0.15[a]	0.85 ± 0.06 [c]	2.22 ± 0.20[a,b]	<0.0001*	0.6752	0.6864
Tumour (g)	-	63.93 ± 5.24	-	59.14 ± 6.59	-	-	-
Cachexia index (%)	-	32.83 ± 2.62	-	30.21 ± 2.02	-	-	-

Legend: C: control adult offspring; W: adult tumour-bearing offspring; OC: adult offspring of maternal fish oil diet; WOC: adult tumour-bearing offspring of maternal fish oil diet. The values are means ± SEM. *Significant difference accounted for tumour factor, diet factor, or interaction effect, analysed by two-way analysis of variance (ANOVA). [a] Compared to C, $p < 0.05$; [b] compared to OC, $p < 0.05$, [c] compared to W, $p < 0.05$, detected by two-way ANOVA, followed by the post-hoc Bonferroni test.

Table 3. Adult offspring serum and hepatic parameters.

Serum and hepatic parameters							
Parameter	Group				*p*-value		
	C (n = 8)	W (n = 7)	OC (n = 6)	WOC (n = 10)	Tumour factor	Diet factor	Interaction effect
Albumin (mg/dL)	3.55 ± 0.20	2.90 ± 0.13[a]	3.42 ± 0.21[c]	2.92 ± 0.14[a,b]	<0.0001*	0.3574	0.2315
Cholesterol (mg/dL)	132.84 ± 9.66	124.08 ± 5.54	126.08 ± 4.29	120.37 ± 3.80[a]	0.0033*	0.0296	0.5675
HDL-C (mg/dL)	28.93 ± 3.08	12.62 ± 1.64[a]	26.29 ± 1.78[c]	12.88 ± 1.35[a,b]	<0.0001*	0.5730	0.4923
LDL-C (mg/dL)	2.89 ± 0.49	3.89 ± 0.57	2.34 ± 0.54	3.53 ± 0.70	0.1070	0.4971	0.8862
Triglycerides (mg/dL)	135.40 ± 8.16	154.98 ± 9.90	102.78 ± 3.88[a,c]	132.70 ± 7.56	0.0048*	0.0016*	0.4857
Hepatic AST (%)	100.00 ± 5.21	88.67 ± 3.15	92.77 ± 4.46	100.60 ± 2.70	0.6658	0.5660	0.0256
Hepatic ALT (%)	100.00 ± 5.59	240.80 ± 44.11[a]	88.84 ± 2.44[c]	120.50 ± 14.68[c]	0.0023*	0.0155*	0.0404*

Legend: C: control adult offspring; W: adult tumour-bearing offspring; OC: adult offspring of maternal fish oil diet; WOC: adult tumour-bearing offspring of maternal fish oil diet. The values are means ± SEM. *Significant difference accounted for tumour factor, diet factor, or interaction effect, analysed by two-way analysis of

variance (ANOVA). [a] Compared to C, $p < 0.05$; [b] compared to OC, $p < 0.05$, [c] compared to W, $p < 0.05$, detected by two-way ANOVA, followed by the post-hoc Bonferroni test.

Table 4. Adult offspring body composition.

Body composition							
Parameters	Groups				*p-value		
	C (n = 8)	W (n = 7)	OC (n = 6)	WOC (n = 10)	Tumour factor	Diet factor	Interaction
Lean body mass (g)	60.52 ± 5.39	43.42 ± 3.88[a]	58.07 ± 3.54	37.79 ± 2.81[a,b]	0.0002*	0.3270	0.6967
Fat body mass (%)	31.68 ± 2.19	24.88 ± 1.26[a]	31.28 ± 0.67[c]	26.23 ± 0.96	0.0002*	0.7191	0.5116
Water body content (%)	55.03 ± 1.76	60.22 ± 0.87[a]	54.02 ± 0.68[c]	60.53 ± 0.59[a,b]	<0.0001*	0.7500	0.5471

Legend: C: control adult offspring; W: adult tumour-bearing offspring; OC: adult offspring of maternal fish oil diet; WOC: adult tumour-bearing offspring of maternal fish oil diet. The values are means ± SEM. *Significant difference accounted for tumour factor, diet factor, or interaction effect, analysed by two-way analysis of variance (ANOVA). [a] Compared to C, $p < 0.05$; [b] compared to OC, $p < 0.05$, detected by two-way ANOVA, followed by the post-hoc Bonferroni test.