

Lifelong n-3 PUFA consumption reduces HER2+ mammary tumour growth and alters immune markers compared to safflower or corn oil-based sources of n-6 PUFA

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Short Title: n-3 PUFA reduces HER2+ tumour growth compared to n-6

Key words: Breast cancer, n-3 PUFA, n-6 PUFA, fish oil, fatty acid, MMTV-neu, eicosapentaenoic acid, docosahexaenoic acid

Abbreviations: **10% CO**, 10% corn oil; **10% SO**, 10% safflower oil; **3% FO 7% CO**, 7% corn oil plus 3% menhaden oil; **3% FO 7% SO**, 7% safflower oil plus 3% menhaden oil; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; eEF2, eukaryotic translation elongation factor 2; CB1, Cannabinoid receptor 1; CB2, Cannabinoid receptor 2; F4/80, Adhesion G Protein-Coupled Receptor E1; CD206, Mannose receptor C-type 1; ARG1, Arginase 1; CD86, Cluster of Differentiation 86; EGR3, Early growth response 3; ID4, DNA-binding protein inhibitor ID-4; JAM3, Junctional adhesion molecule 3; TBX2, T-box 2; ZBP1, Z-DNA binding protein 1.

Supplementary Material

Table S1. Primer sequences.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>eEF2</i>	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
<i>Cnr1</i>	CTGGCCTATAAGAGGATCGTCA	GAGAGGCAACACAGCAATTACTA
<i>Cnr2</i>	CTACAAAGCTCTAGTCACCCGT	CCATGAGCGGCAGGTAAGAAA
<i>Adgre1</i>	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
<i>Mrc1</i>	ATGGATTGCCCTGAACAGCA	TGTACCGCACCCCTCCATCTA
<i>Arg1</i>	CAGCACTGAGGAAAGCTGGT	ACAGACCGTGGGTCTTTCAC
<i>Cd86</i>	TCAATGGGACTGCATATCTGCC	GCCAAAATACTACCAGCTCACT

eEF2: eukaryotic translation elongation factor 2; *Cnr1*: Cannabinoid receptor 1 (CB1); *Cnr2*: Cannabinoid receptor 2 (CB2); *Adgre1* (F4/80): Adhesion G Protein-Coupled Receptor E1; *Mrc1* (CD206): Mannose receptor C-type 1; *Arg1*: Arginase 1; *Cd86*: Cluster of Differentiation 86

Table S2. Body weight and food intake.

Fatty Acid	10% CO	10% SO	3% FO 7% CO	3% FO 7% SO
Initial body weight (g)	17.3 ± 0.29	16.5 ± 0.34	17.2 ± 0.23	17.2 ± 0.30
Final body weight (g)	29.5 ± 0.61 ^a	26.5 ± 0.56 ^b	30.1 ± 0.74 ^a	28.7 ± 1.07 ^{ab}
Body weight gain (g)	12.3 ± 0.51 ^{ab}	10.0 ± 0.34 ^b	12.9 ± 0.69 ^a	11.5 ± 0.97 ^{ab}
Food intake (g/mouse/day)	2.2 ± 0.02 ^{ab}	2.1 ± 0.03 ^b	2.3 ± 0.04 ^a	2.3 ± 0.04 ^a

Body weight and food intake of female MMTV mice fed 10% CO (n=14), 10% SO (n=14), 3% FO 7% CO (n=12) or 3% FO 7% SO (n=14) for 20 weeks. Values are means ± SEM. Within each row, values with different letters indicate a significant difference ($p \leq 0.05$) between groups according to one-way ANOVA followed by Tukey's Studentized Range test. 10% CO, 10% corn oil; 10% SO, 10% safflower oil; 3% FO 7% CO, 7% corn oil plus 3% menhaden oil; 3% FO 7% SO, 7% safflower oil plus 3% menhaden oil.

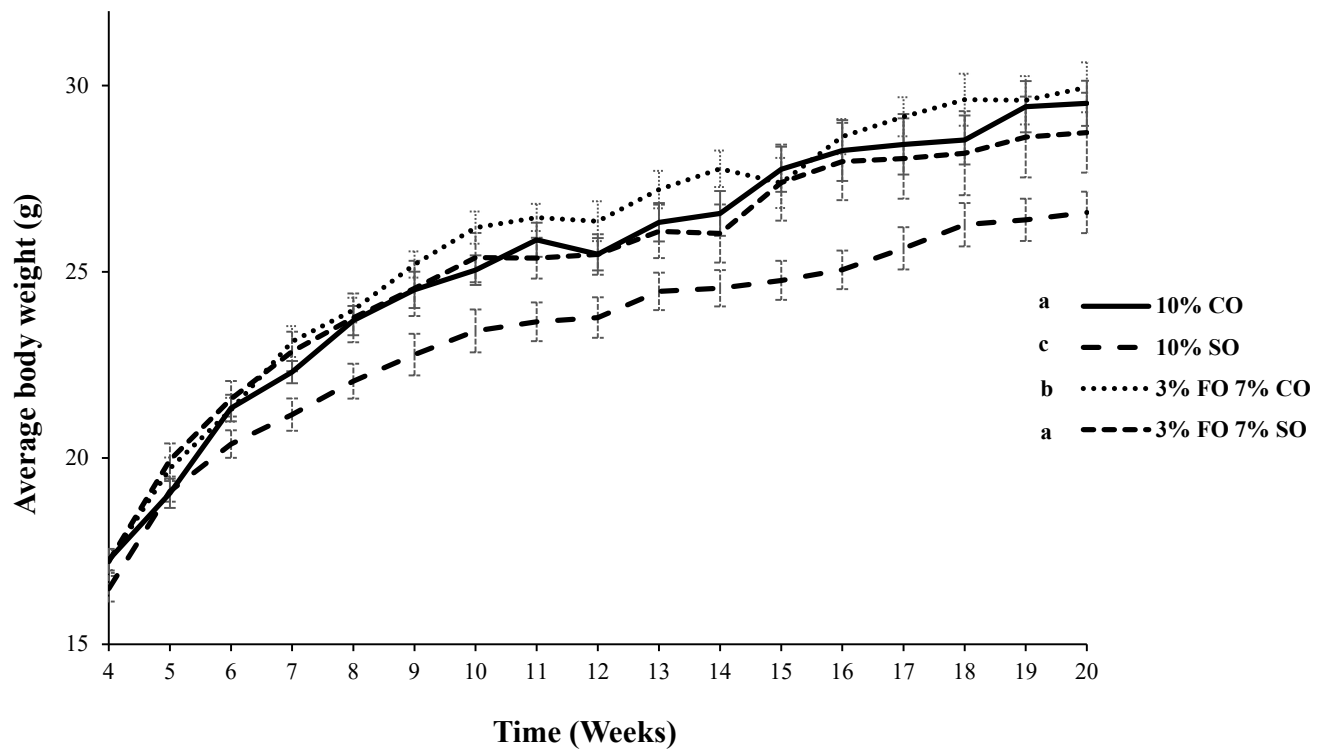


Figure S1. Changes in body weight (g) over 16 weeks (4 to 20 weeks) in female MMTV mice fed 10% corn oil (n = 14), 10% safflower oil (n = 14), 7% corn oil plus 3% menhaden oil (n = 12), and 7% safflower oil plus 3% menhaden oil (n = 14). Groups with a different letter indicate significantly different body weights as determined by a repeated measures analysis ($p \leq 0.05$). Values are mean $\pm$ SEM. 10% CO, 10% corn oil; 10% SO, 10% safflower oil; 3% FO 7% CO, 7% corn oil plus 3% menhaden oil; 3% FO 7% SO, 7% safflower oil plus 3% menhaden oil.

Table S3. Fatty acid composition (%) of mammary tumour phospholipids in SM, PI and PS fractions.

Fatty Acids	SM				PI				PS			
	10% CO	10% SO	3% FO 7% CO	3% FO 7% SO	10% CO	10% SO	3% FO 7% CO	3% FO 7% SO	10% CO	10% SO	3% FO 7% CO	3% FO 7% SO
12:0	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 ± 0.1	n.d.	n.d.	n.d.	0.1 ± 0.1	n.d.	n.d.
14:0	1.9 ± 0.3	1.7 ± 0.4	1.5 ± 0.3	2.2 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
15:0	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	n.d.	n.d.	n.d.	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
16:0	33.1 ± 2.3	32.6 ± 3.0	30.5 ± 3.1	33.6 ± 3.0	6.5 ± 0.5	7.0 ± 0.4	5.8 ± 0.3	6.3 ± 0.3	5.1 ± 0.6	6.3 ± 0.7	5.2 ± 0.5	5.2 ± 0.4
18:0	7.9 ± 1.8	6.5 ± 0.7	6.5 ± 0.9	7.5 ± 0.7	34.4 ± 0.7	34.4 ± 0.7	35.4 ± 1.0	34.7 ± 1.0	35.6 ± 1.1	35.3 ± 0.7	36.1 ± 1.2	35.8 ± 1.2
19:0	0.6 ± 0.2	0.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.7 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
20:0	0.9 ± 0.2	1.5 ± 0.8	1.2 ± 0.4	1.0 ± 0.3	0.3 ± 0.1 ^a	0.2 ± 0.1 ^{ab}	0.3 ± 0.1 ^a	0.1 ± 0.0 ^b	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.1
22:0	2.7 ± 0.8	3.8 ± 1.0	4.2 ± 0.9	3.3 ± 1.0	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.7 ± 0.1
24:0	3.4 ± 1.0	5.1 ± 1.1	5.7 ± 1.4	5.2 ± 1.5	0.3 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.8 ± 0.3	0.3 ± 0.1	0.5 ± 0.3
Total												
SFA	50.9 ± 3.2	52.0 ± 3.2	50.7 ± 3.7	53.8 ± 3.6	42.6 ± 0.9	42.9 ± 0.9	42.7 ± 1.1	42.1 ± 1.1	43.3 ± 1.3	44.3 ± 1.1	43.6 ± 1.3	43.7 ± 1.3
16:1c9	2.5 ± 0.6	1.8 ± 0.4	1.9 ± 0.6	3.0 ± 0.7	0.9 ± 0.1	1.0 ± 0.3	0.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	1.6 ± 0.3
18:1c9	7.6 ± 2.1	4.4 ± 1.4	4.3 ± 1.3	5.8 ± 1.6	8.7 ± 0.5	7.1 ± 0.4	8.4 ± 0.9	8.3 ± 1.0	19.3 ± 1.2	17.6 ± 0.9	19.2 ± 2.0	19.3 ± 1.0
18:1c11	2.9 ± 0.8	1.9 ± 0.6	1.8 ± 0.6	2.6 ± 0.8	3.7 ± 0.2	3.6 ± 0.2	3.7 ± 0.4	3.8 ± 0.5	3.6 ± 0.2	3.4 ± 0.2	3.7 ± 0.6	3.5 ± 0.4
20:1c11	0.8 ± 0.4	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.3 ± 0.1	0.9 ± 0.1 ^{ab}	0.9 ± 0.0 ^{ab}	1.0 ± 0.1 ^a	0.7 ± 0.1 ^b
22:1n9	3.6 ± 0.9	4.7 ± 1.9	3.6 ± 0.6	3.9 ± 1.4	1.7 ± 0.4	1.7 ± 0.4	1.7 ± 0.4	1.8 ± 0.8	3.2 ± 0.8	3.1 ± 0.7	4.0 ± 1.6	3.8 ± 1.6
24:1n9	20.0 ± 7.0	24.5 ± 5.5	28.6 ± 6.7	18.8 ± 6.0	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.2
Total												
MUFA	37.4 ± 7.4	37.9 ± 6.0	40.5 ± 6.9	34.4 ± 6.5	16.2 ± 0.7	14.2 ± 0.7	15.8 ± 1.1	15.4 ± 1.4	28.7 ± 1.5	26.7 ± 1.2	29.8 ± 2.7	29.6 ± 2.0
18:2n6	4.1 ± 1.2	3.3 ± 1.1	3.4 ± 1.0	4.9 ± 1.4	6.9 ± 0.4	7.5 ± 0.5	7.8 ± 0.7	8.3 ± 0.7	8.7 ± 0.3	9.4 ± 0.6	10.6 ± 1.2	10.4 ± 0.6
18:3n6	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.6 ± 0.5	0.1 ± 0.0	n.d.	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.5 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
18:3n3	0.1 ± 0.1	0.1 ± 0.1	n.d.	0.3 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 ± 0.1	0.1 ± 0.1
20:2n6	0.6 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.0 ^{ab}	0.8 ± 0.1 ^a	0.6 ± 0.1 ^{ab}	0.5 ± 0.1 ^b
20:3n6	1.1 ± 0.2	1.1 ± 0.3	0.6 ± 0.2	0.8 ± 0.3	5.3 ± 0.3	5.4 ± 0.4	6.2 ± 0.4	5.4 ± 0.5	5.8 ± 0.3	6.6 ± 0.7	5.5 ± 0.5	5.0 ± 0.6
20:4n6	3.0 ± 0.8	2.4 ± 0.8	1.2 ± 0.3	2.0 ± 0.5	23.0 ± 0.8 ^{ac}	25.1 ± 0.8 ^a	19.0 ± 1.3 ^b	20.6 ± 0.8 ^{bc}	6.3 ± 0.8 ^a	6.6 ± 0.6 ^a	3.2 ± 0.2 ^b	3.8 ± 0.4 ^b
20:3n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:5n3	0.9 ± 0.3	1.0 ± 0.1	1.5 ± 0.3	1.4 ± 0.2	0.4 ± 0.1 ^a	0.4 ± 0.2 ^a	1.7 ± 0.1 ^b	1.5 ± 0.2 ^b	0.9 ± 0.1	0.9 ± 0.1	1.3 ± 0.2	1.3 ± 0.2
22:2n6	0.6 ± 0.1 ^{ab}	1.2 ± 0.2 ^a	0.4 ± 0.1 ^b	0.6 ± 0.1 ^{ab}	0.2 ± 0.1	n.d.	0.1 ± 0.1	n.d.	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
22:4n6	0.2 ± 0.1 ^{ab}	0.4 ± 0.1 ^a	n.d. ^b	n.d. ^b	1.5 ± 0.2 ^a	1.3 ± 0.3 ^{ac}	0.6 ± 0.1 ^{bc}	0.6 ± 0.1 ^b	1.6 ± 0.3 ^a	1.6 ± 0.2 ^a	0.2 ± 0.1 ^b	0.3 ± 0.1 ^b
22:3n3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5n6	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.2	0.8 ± 0.2 ^a	1.0 ± 0.2 ^a	0.1 ± 0.0 ^b	0.1 ± 0.1 ^b	0.9 ± 0.2 ^a	1.0 ± 0.3 ^a	0.2 ± 0.2 ^{ab}	n.d. ^b
22:5n3	0.3 ± 0.2	n.d.	0.7 ± 0.6	0.3 ± 0.2	0.3 ± 0.1 ^a	0.2 ± 0.1 ^a	1.7 ± 0.2 ^b	1.6 ± 0.2 ^b	0.4 ± 0.2 ^a	0.2 ± 0.1 ^a	1.7 ± 0.5 ^b	1.5 ± 0.2 ^b
22:6n3	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	1.9 ± 0.1 ^{ab}	1.3 ± 0.5 ^a	3.1 ± 0.3 ^b	3.4 ± 0.5 ^b	2.1 ± 0.3 ^{ab}	0.9 ± 0.3 ^a	2.7 ± 0.5 ^b	3.2 ± 0.4 ^b
Total n-3	1.5 ± 0.4 ^{ab}	1.1 ± 0.1 ^a	2.4 ± 1.1 ^{ab}	2.4 ± 0.3 ^b	2.7 ± 0.2 ^a	1.9 ± 0.6 ^a	6.5 ± 0.5 ^b	6.6 ± 0.8 ^b	3.3 ± 0.4 ^{ac}	2.0 ± 0.3 ^a	5.8 ± 1.0 ^{bc}	6.2 ± 0.7 ^b
Total n-6	10.1 ± 2.2	9.2 ± 2.3	6.3 ± 1.6	9.5 ± 2.4	38.6 ± 0.9 ^{ac}	41.2 ± 0.6 ^a	35.0 ± 0.9 ^b	36.0 ± 1.1 ^{bc}	24.6 ± 1.2 ^a	26.9 ± 1.0 ^a	20.8 ± 1.3 ^b	20.5 ± 1.1 ^b

n-6/n-3	5.3 ± 1.9	8.8 ± 2.6	3.9 ± 1.0	3.9 ± 0.7	14.8 ± 1.2 ^a	22.7 ± 8.8 ^a	5.5 ± 0.4 ^b	5.9 ± 0.7 ^b	7.8 ± 0.8 ^a	15.5 ± 2.8 ^b	4.1 ± 0.6 ^c	3.5 ± 0.4 ^c
Total												
PUFA	11.5 ± 1.5	10.9 ± 1.5	8.6 ± 1.3	11.8 ± 1.7	41.4 ± 1.0	43.0 ± 1.3	41.4 ± 1.6	42.5 ± 1.3	28.1 ± 1.1	28.8 ± 1.3	26.5 ± 1.6	26.5 ± 1.1

Fatty acid composition (%) of mammary tumors from MMTV mice (n = 6/diet group) fed 10% CO, 10% SO, 3% FO 7% CO or 3% FO 7% SO at 20 weeks of age. Within each phospholipid class, values with different letters indicate significant difference ($p \leq 0.05$) between groups according to one-way ANOVA followed by Tukey's Studentized Range test. Values (expressed as % composition of total mammary tumour) are means ± SEM. 10% CO, 10% corn oil; 10% SO, 10% safflower oil; 3% FO 7% CO, 7% corn oil plus 3% menhaden oil; 3% FO 7% SO, 7% safflower oil plus 3% menhaden oil; SM, Sphingomyelin; PI, Phosphatidylinositol; PS, Phosphatidylserine; n.d., Not detected.

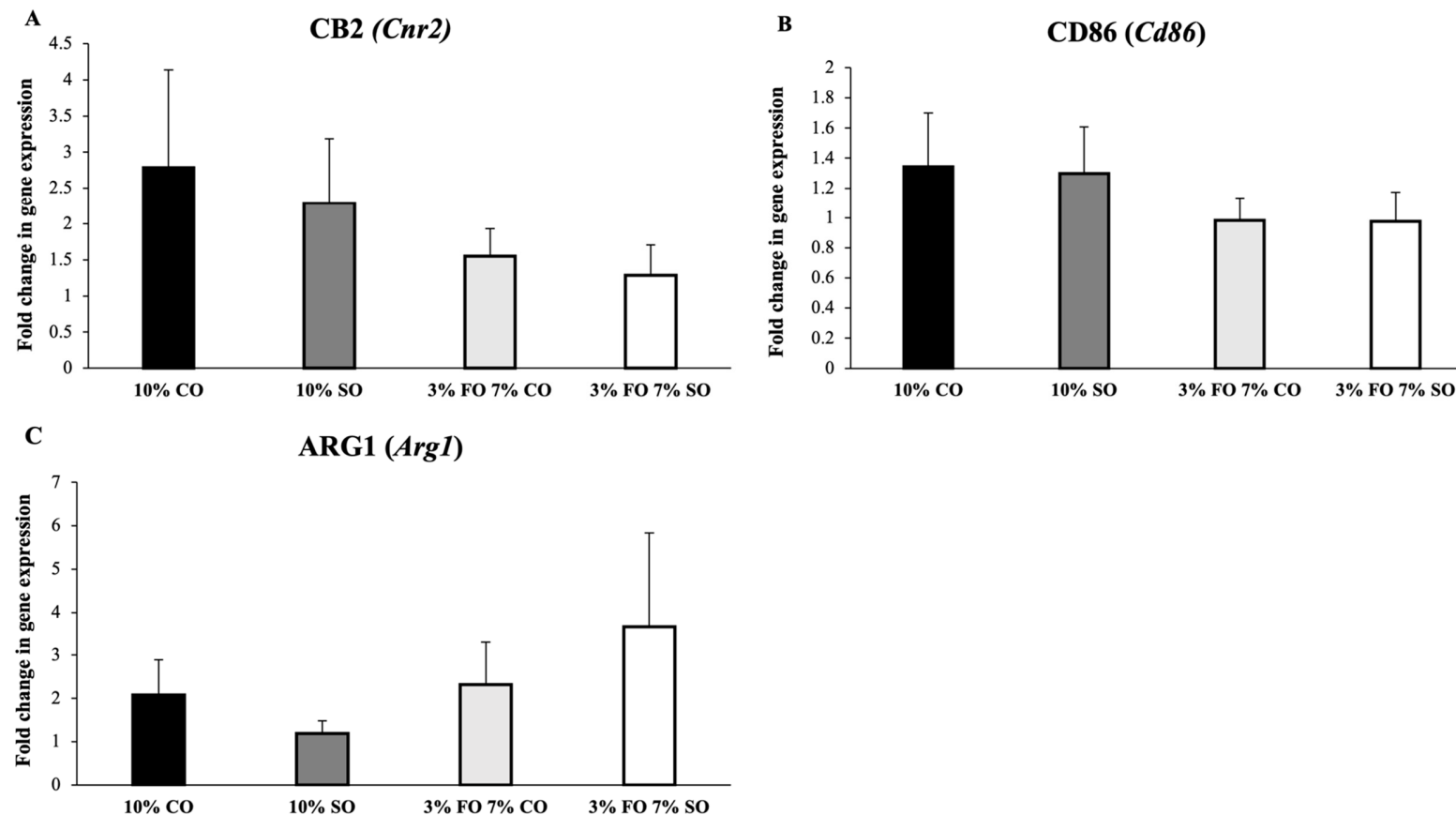


Figure S2: Gene expression in mammary glands of female MMTV mice at the time of termination (20 weeks of age). Bars represent mean values $\pm$ SEM ($n = 10$ /dietary group). Cycle threshold (C_t) values were normalized to the housekeeping gene *eEF2*, and $2^{-\Delta\Delta C_t}$ was used to calculate fold-change in relative mRNA expression of the n-3 PUFA group (3% FO 7% CO; 3% FO 7% SO) compared to the respective n-6 PUFA (10% CO; 10% SO) group. Normalized fold-change expression levels are displayed as means $\pm$ SEM of at least two replicates. Data was analyzed by one-way ANOVA or Kruskal–Wallis test. No significant differences in expression between CB2 (A), CD86 (B) and ARG1 (C) genes between the groups. 10% CO, 10% corn oil; 10% SO, 10% safflower oil; 3% FO 7% CO, 7% corn oil plus 3% menhaden oil; 3% FO 7% SO, 7% safflower oil plus 3% menhaden oil; *eEF2*, eukaryotic translation elongation factor 2; CB2 (*Cnr2*), Cannabinoid receptor 2; ARG1 (*Arg1*), Arginase 1; CD86 (*Cd86*), Cluster of Differentiation 86.

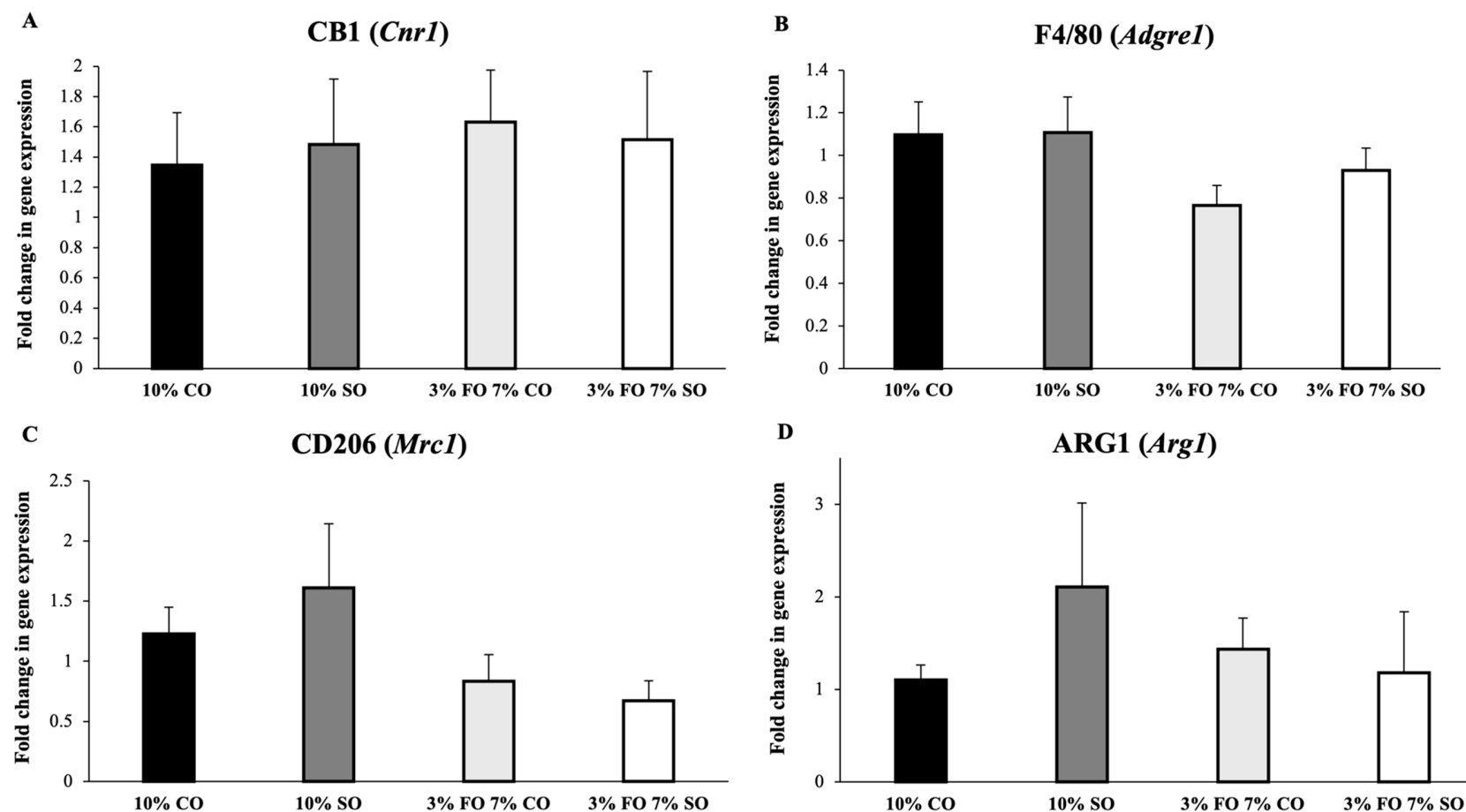


Figure S3: Gene expression in tumours of female MMTV mice at the time of termination (20 weeks of age). Bars represent mean values $\pm$ SEM ($n = 10$ /dietary group). Cycle threshold (C_t) values were normalized to the housekeeping gene *eEF2*, and $2^{-\Delta\Delta C_t}$ was used to calculate fold-change in relative mRNA expression of the n-3 PUFA group (3% FO 7% CO; 3% FO 7% SO) compared to the respective n-6 PUFA (10% CO; 10% SO) group. Normalized fold-change expression levels are displayed as means $\pm$ SEM of at least two replicates. Data was analyzed by one-way ANOVA or Kruskal–Wallis test. No significant differences in expression between CB1 (**A**), F4/80 (**B**), CD206 (**C**) and ARG1 (**D**) genes between the groups. 10% CO, 10% corn oil; 10% SO, 10% safflower oil; 3% FO 7% CO, 7% corn oil plus 3% menhaden oil; 3% FO 7% SO, 7% safflower oil plus 3% menhaden oil; *eEF2*, eukaryotic translation elongation factor 2; CB1 (*Cnr1*), Cannabinoid receptor 1; F4/80 (*Adgre1*), Adhesion G Protein-Coupled Receptor E1; CD206 (*Mrc1*), Mannose receptor C-type 1; ARG1 (*Arg1*), Arginase 1.