

OMEGA-3 FATTY ACIDS REDUCE VIABILITY AND MODULATE MIR-34 IN DYSPLASTIC ORAL KERATINOCYTES

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Abstract

Introduction: This study aimed to evaluate whether diet-relevant polyunsaturated fatty acids modulate the tumor suppressor miR-34 family during early stages of oral carcinogenesis.

Materials and methods: Oral dysplastic keratinocytes (DOK) were incubated with arachidonic acid (AA, omega-6), eicosapentaenoic acid (EPA, omega-3), and docosahexaenoic acid (DHA, omega-3) at different concentrations ranging from 10 to 200 μ M, or with vehicle control (ethanol). Cell viability was assessed using the resazurin assay, cell migration through the wound healing assay, and membrane lipid composition was analyzed by gas chromatography. Genotyping of TP53 and pri-miR-34b/c was performed by polymerase chain reaction (PCR), and RT-qPCR quantified miR-34 expression levels.

Results: EPA and DHA reduced cell viability at all concentrations tested, while AA enhanced cell migration and increased the omega-6/omega-3 ratio. No significant changes were observed in TP53 and pri-miR-34b/c genotyping. Nevertheless, miR-34a expression was significantly upregulated in cells exposed to omega-3 fatty acids compared with control and omega-6 fatty acids.

Conclusion: Omega-3 fatty acids are associated with upregulation of the tumor suppressor miRNA-34 family in dysplastic oral keratinocytes, suggesting a biologically plausible protective molecular mechanism, consistent with epidemiological evidence linking omega-3 intake to a reduced risk of cancer.

Key words: miR-34, TP53, polyunsaturated fatty acids, epithelial dysplasia, DOK cells

Resumen

Los ácidos grasos omega-3 reducen la viabilidad celular y modulan miR-34 en queratinocitos orales displásicos

Introducción: El objetivo de este estudio fue evaluar si los ácidos grasos poliinsaturados de la dieta modulan la familia del supresor tumoral miR-34 durante las etapas tempranas de la carcinogénesis oral.

Materiales y métodos: La línea celular de queratinocitos orales displásicos (DOK) fue incubada con ácido araquidónico (AA, omega-6), ácido eicosapentaenoico (EPA, omega-3) y ácido docosahexaenoico (DHA, ome-

ga-3) a diferentes concentraciones (10 y 200 μM), o con un control vehicular (etanol). La viabilidad celular se evaluó mediante el ensayo de resazurina, la migración celular mediante el ensayo de la herida, y la composición lipídica de membrana se analizó por cromatografía gaseosa. El genotipado de TP53 y pri-miR-34b/c se realizó mediante reacción en cadena de la polimerasa (PCR), y los niveles de expresión de miR-34 se cuantificaron mediante RT-qPCR.

Resultados: EPA y DHA redujeron la viabilidad celular en todas las concentraciones evaluadas, mientras que el AA aumentó la migración celular e incrementó la relación omega-6/omega-3. No se observaron cambios significativos en el genotipo de TP53 ni de pri-miR-34b/c. Sin embargo, la expresión de miR-34a se encontró significativamente aumentada en células tratadas con ácidos grasos omega-3, en comparación con el control y el tratamiento con ácidos grasos omega-6.

Conclusión: Los ácidos grasos omega-3 se asocian con una regulación positiva de la familia de miR-34 en queratinocitos orales displásicos, lo que sugiere un mecanismo molecular protector en la carcinogénesis oral, consistente con la evidencia epidemiológica que vincula la ingesta de omega-3 con una reducción del riesgo de cáncer.

Palabras clave: miR-34, TP53, ácidos grasos poliinsaturados, displasia epitelial, células DOK

KEY POINTS

Current knowledge

- Diet influences the risk of oral cancer development, yet the underlying molecular mechanisms remain incompletely understood. Polyunsaturated fatty acids can modulate epigenetic pathways, including microRNA regulation, in several cancers. However, their role during the early stages of oral carcinogenesis, such as epithelial dysplasia, is still poorly characterized.

Contribution of the article to current knowledge

- In dysplastic oral keratinocytes, omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) reduced cell viability and significantly upregulated the tumor suppressor miR-34a. In contrast, the omega-6 fatty acid arachidonic acid

increased cell migration and downregulated miR-34 family members. These findings provide evidence linking dietary fatty acids to early molecular events in oral carcinogenesis.

Oral squamous cell carcinoma typically develops on the tongue, lips, and floor of the mouth, representing the predominant type of malignant squamous cell carcinoma of the head and neck¹. Moreover, it negatively impacts the quality of life of patients with this pathology.

Carcinogenesis in the oral cavity begins with the transformation of a limited number of normal keratinocytes. These alterations occur via direct DNA damage or failures in the DNA repair process, resulting in unstable keratinization and the generation of a precancerous field. This field can then progress to malignant neoplastic changes that are clonally propagated to subsequent cell generations².

In recent years, epigenetic studies of chromatin modifications and non-coding RNAs such as microRNAs (miRNAs) have revealed mechanisms that regulate gene expression independently of genetic alterations. It has now been demonstrated that an epigenetic language is dysregulated in several types of cancer³.

Our previous research established that dietary omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) modulate the development of 7,12-dimethylbenz[a]anthracene (DMBA)-induced premalignant lesions in mouse tongue tissue⁴.

Dietary components can influence gene expression through epigenetic modifications in oral cancer⁵. The interplay between epigenetic alterations and metabolic reprogramming is critical to cancer cell aggressiveness.

Additionally, miRNAs can modulate the expression of key enzymes in lipid biosynthesis and degradation, influencing lipid availability and membrane synthesis⁶.

Approximately 25% of all known miRNAs are significantly dysregulated in various cancers, modulating inflammation processes, proliferation, and cell differentiation, which are involved in tumorigenesis⁷.

Among the miRNAs dysregulated in cancer, the miR-34 family has received particular attention. It is known as a type of tumor suppres-

sor miRNA, and its three members are reduced in several cancers, including lung and breast cancer⁸. The deregulation of miRNAs, particularly the miR-34 family, has been closely linked to the progression of carcinogenesis due to its function as a tumor suppressor and its relation to TP53⁹. Additionally, Don et al. have shown an association between high-fat dietary intake and the presence of mutant alleles of TP53-R72P and *pri-miR 34b/c*-rs4938723 in patients with premalignant and malignant oral lesions¹⁰. However, the underlying biological and molecular mechanisms have not yet been fully elucidated.

Therefore, the objective of this work was to evaluate whether dietary-relevant PUFAs modulate the tumor suppressor miR-34 family during early stages of oral carcinogenesis in dysplastic oral keratinocytes (DOK).

Materials and methods

Cell culture and PUFA treatment

Human-derived dysplastic oral keratinocytes (DOK) were kindly provided by Dr. Vicente A. Torres (University of Chile). DOK were isolated from a 57-year-old man diagnosed with squamous-cell carcinoma in the dorsal tongue. Remnant dysplasia was graded as mild to moderate, removed, and used to initiate primary cultures that originated from DOK (European Collection of Authenticated Cell Cultures, ECACC General Collection #94122104).

DOK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 5 µg/ml hydrocortisone^{11,12} and were randomly divided into the following treatments: arachidonic acid (AA; 20:4 omega-6), eicosapentaenoic acid (EPA; 20:5 omega-3), docosahexaenoic acid (DHA; 22:6 omega-3), or ethanol (ETOH) as the vehicle control (Fig. 1A and B). Fatty acids were dissolved in ethanol to achieve the final concentrations, following protocols previously established in our laboratory. Hence, ethanol was used as a vehicle control in all experiments to specifically control for solvent-related effects and isolate PUFA-specific effects¹³. A number and letter code were assigned to each treatment in order to avoid bias in the analyses.

Viability assay

Cell viability was assessed using a colorimetric and fluorometric resazurin assay, as an initial functional readout to determine whether PUFAs exert dose-dependent effects on DOK and to establish biologically relevant concentrations for subsequent assays.

Briefly, cells were seeded in 96-well plates (1×10^4 cells/well) and treated for 48 h with increasing concentrations of AA, EPA, or DHA (10–200 µM), or ETOH. Subsequently, cells were incubated at 37°C with resazurin (1 mg/ml) (Sigma-Aldrich, St. Louis, MO), and fluorescence was measured at excitation/emission wavelengths of 560/590 nm using a GloMax Multi-Detection System (Promega), as previously described¹⁴. Viability results were expressed as absorbance values (optical density) to compare raw metabolic activity across treatments. Three independent biological replicates were performed, each carried out in technical triplicate.

Migration assay

Cell migration was assessed using a wound closure assay to evaluate changes in cell motility, a hallmark associated with early malignant progression. Migration results were interpreted in light of viability data to distinguish true motility effects from potential differences in cell survival.

DOK cells were seeded in 12-well plates (2×10^4 cells/well) and grown until 95% confluence. A longitudinal scratch was made in the monolayer using a sterile 200 µL pipette tip with a constant angle and pressure. The consistency of the initial wound was verified by microscopic observation. Cells were incubated with AA, EPA, or DHA (50 µM), DMEM, or ETOH. After 24 hours, cells were fixed with 4% formaldehyde and stained with 10% methylene blue. Microphotographs were taken at 0 and 24 hours after wounding using an inverted microscope equipped with a camera (Zeiss Axiovert 135). ImageJ, RRID: SCR_003070 (<https://imagej.net/>) was used to quantify the wound area, and migration was calculated as follows: migration area (%) = $(T_0 - T_1) / T_0 \times 100$, where T_0 represents the initial wound area and T_1 the remaining wound area at the measurement point¹⁵.

Determination of fatty acid profiles in dysplastic cell membranes

Membrane fatty acid profiling was performed to verify cellular incorporation of PUFAs and to evaluate whether functional effects correlated with changes in lipid composition. Fatty acid profiles were determined by gas chromatography (GC) of PUFA-treated cells, following the routine methodology of our laboratory¹⁴. A capillary column was used in a Perkin-Elmer gas chromatograph at 180°C. Peak identification was performed using standards of known composition (Sigma-tau; RRID: SCR_000488) (<http://www.sigma-tau.it/eng/>). Results were expressed as the relative percentage of total identified fatty acids.

Genotyping of TP53 and hsa-pri-miR-34b/c: DNA isolation and polymerase chain reaction (PCR)

Genotyping was conducted to assess whether PUFAs treatment was associated with changes in polymorphic variants of TP53 and pri-miR-34b/c. DNA was obtained from DOK cells seeded in 6-well plates (5×10^4 cells/well) and treated for 24 and 48 hours with AA, EPA, or DHA (50 μ M) or ETOH. DNA extraction was performed following our laboratory's protocols¹⁶. The concentration and purity of extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific), ensuring an A260/A280 ratio between 1.8 and 2.0 before amplification.

Genomic DNA (150 ng) was amplified by allele-specific PCR for TP53 rs1042522 and restriction fragment length polymerase (RFLP) for hsa-pri-miR 34b/c rs4938723 in a final reaction volume of 50 μ L.

The amplification protocol was as follows: 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at the annealing temperature specific to each gene's primers, and 45 seconds at 72°C, with a final elongation step of 5 minutes at 72°C.

The primers used were: TP53 rs1042522; G/C: Pro-line form: Forward GCCAGAGGCTGCTCCCC; Reverse CGTGCAAGTCACAGACTT. Arginine form: Forward TCCCCCTTGCCGTCCCAA; Reverse CTGGTG-CAGGGGCCACGC¹⁷. hsa-pri-miR-34b/c rs4938723: Forward CCTCTGGGAACCTTCTTTGACCTGT; Reverse CCTGGGCCTTCTAGTCAAATAGTGA¹⁸. PCR products were separated on a 2% agarose gel stained with GelRed® (Biotium, Genbiotech), using a DNA ladder (GeneRuler 100 bp, Thermo Scientific, Buenos Aires) to determine DNA fragment size.

Analysis of miR-34 family expression: RNA Isolation, cDNA synthesis, and real-time PCR (RT-qPCR)

Given the central role of the miR-34 family in p53-dependent tumor suppression, we next evaluated miRNA expression to explore epigenetic mechanisms underlying PUFA-induced functional changes.

Total RNA was isolated using Trizol (Invitrogen) from DOK cells (1×10^4) incubated with different PUFAs (50 μ M) or ETOH for 24 hours. miRNAs were reverse transcribed using the stem-loop method¹⁹. Briefly, 200 ng of total RNA and 1 mM of stem-loop primer were preheated at 70°C for 5 minutes. Reverse transcription (RT) was performed using M-MLV reverse transcriptase (FLYE-Ultra, InBio Highway S.A., Buenos Aires, Argentina) and incubated in an Ivema T18 thermocycler (30 minutes at 16°C, 60 minutes

at 42°C, and 2 minutes at 70°C). qPCR reactions were carried out in a final volume of 10 μ L containing 1 mM of each primer and 5 μ L of GreenLight qPCR Master Mix (InBio Highway S.A., Buenos Aires, Argentina) using a QuantStudio 3 Real-Time PCR System (Applied Biosystems) under the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 65°C for 15 seconds, and 60°C for 1 minute²⁰. All reactions were performed in triplicate. miRNA expression levels were calculated using the $\Delta\Delta$ CT method and normalized to cel-miR-54-3p as an exogenous control, selected after preliminary optimization at different concentrations. Therefore, 0.1 μ M was determined to provide the most stable and reproducible amplification. Primer sequences used for the amplification of miR-34 are listed in Supplementary Table S1.

Statistical analysis

The data were described using mean values and ranges. Comparisons between treatments for cell viability, migration, miRNA expression levels, and membrane fatty acid profiles were performed using the Kruskal-Wallis test (after corroborating the non-normality of the data using the Shapiro-Wilk test). Post hoc analyses were conducted using Wilcoxon test. To evaluate differences in migration between time points, paired t-tests were applied. The sample size was formed by three independent biological replicates, and each reaction was run in technical triplicate, as is generally considered the minimum for statistical significance in experimental studies. Statistical significance was established at $p < 0.05$ ^{21, 22}. Data analyses were conducted using R (R Core Team, 2024; www.r-project.org) and Infostat Professional 2020 (<https://www.infostat.com.ar/>)²³.

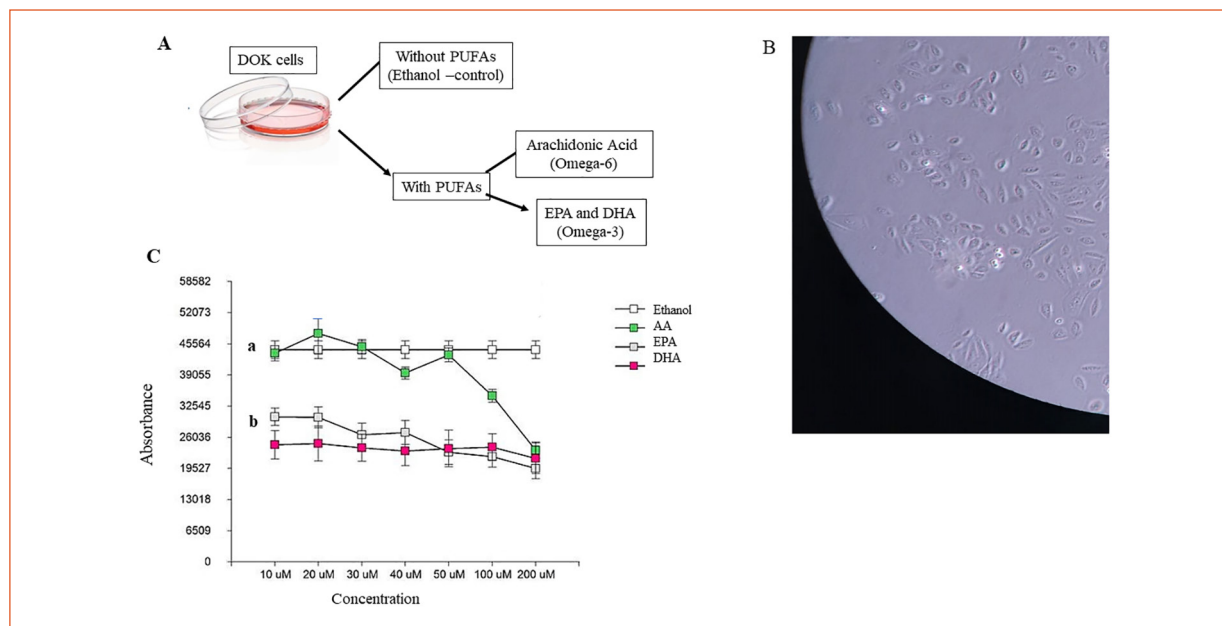
Results

Omega-3 PUFAs decrease cell viability in dysplastic oral keratinocytes

Treatment of DOK cells for 48 h with the omega-3 fatty acids EPA and DHA (10–200 μ M) significantly reduced cell viability at all tested concentrations compared with ETOH ($p < 0.0001$). In contrast, no significant differences were observed between AA-treated cells and control (Fig. 1C).

In this context, this result could indicate a cytotoxic or citostatic effect of both omega-3 PUFAs on dysplastic oral keratinocytes. Regarding AA at concentrations ranging from 10 to 100 μ M, increased cell viability compared with DHA- and

Figure 1 | A: Experimental design. B: Representative micrographs of the DOK (Oral dysplastic keratinocytes) cell line (Magnification: 10X). C: Viability curves of the different treatments applied to DOK cells; dot plots show mean $\pm$ standard error. PUFAs (polyunsaturated fatty acids) were added at different doses (10–200 μ M) for 48 hours. Ethanol was used as a control. Different letters indicate significant differences between treatments ($p_{\text{Kruskal-Wallis}} < 0.0001$), $n=4$



EPA-treated cells at equivalent concentrations. However, exposure to high concentrations of AA (200 μ M) significantly reduced cell viability ($p_{\text{Kruskal-Wallis}} < 0.0001$) (Fig. 1C).

Omega-6 PUFAs (AA) enhance migration in DOK cells

A cell migration assay revealed a significant reduction in wound area after 24 hours in all experimental groups, including ethanol (Fig. 2). Treatment with AA induced a significantly higher migration rate compared with control ($p_{\text{Kruskal-Wallis}} = 0.0007$), suggesting that this omega-6 PUFA may enhance cell motility. AA treatment increased wound closure by 49.63% relative to ETOH. In contrast, EPA and DHA showed no significant differences compared with the control.

Omega-6/Omega-3 ratio in membrane fatty acid composition: differential effects of PUFAs in DOK cells

Lipid profile analysis revealed no significant differences in the composition of individual fatty acids within the cell membranes between fatty acid treatments and control

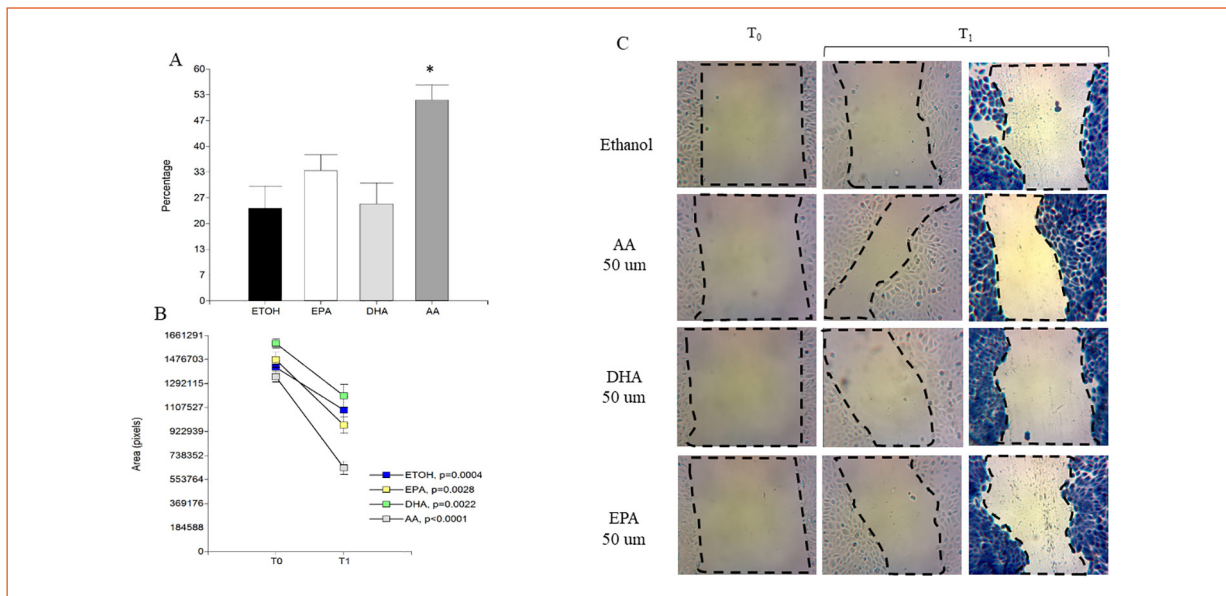
(Supplementary Table S2 and S3). However, the omega-6/omega-3 ratio was significantly elevated in AA-treated cells (6.29 ± 2.8) compared with those treated with EPA (4.31 ± 2.9) or DHA (3.46 ± 1.6) ($p_{\text{Kruskal-Wallis}} = 0.0250$) (Fig. 3A).

DOK cells exhibit heterozygous risk polymorphic variants

Genotyping analysis by PCR revealed that DOK cells treated for 24 hours with AA, EPA, DHA, or ETOH consistently displayed a heterozygous CT genotype for the *hsa-pri-miR-34b/c* gene. Similarly, all groups exhibited a heterozygous GC genotype for the *TP53* gene. Since no differences were observed, an additional time point (48 hours) was analyzed to determine whether prolonged exposure could induce detectable genotypic alterations. However, no modifications in the allelic patterns were found.

These findings indicate that under the experimental conditions tested, PUFA treatment does not alter the genetic variants studied. All DOK cells presented heterozygous variants for *TP53* and *pri-miR-34b/c*, both of which are considered risk-associated genotypes.

Figure 2 | A: Cell migration (mean $\pm$ Standard Error) with the different treatments applied to DOK (Oral dysplastic keratinocytes) cells at 24 hours using the wound technique. PUFA (polyunsaturated fatty acids) treatment was performed at a concentration of 50 μ M. Ethanol was used as a control. *Indicates significant differences found between treatments ($p_{\text{Kruskal-Wallis}} = 0.0307$). n=4. B: Wound area according to time and treatment ($p_{\text{paired Wilcoxon-test}} < 0.05$). C: The image represents the wound assay from time 0 hours (T0) to time 24 hours (T1) with methylene blue staining for the different treatments. Magnification: 40X



Omega-3 upregulates hsa-miR-34a expression in DOK cells

Relative expression analysis of the miR-34 family in DOK cells revealed distinct effects of the different PUFAs. Treatment with DHA and EPA significantly increased hsa-miR-34a expression. DHA induced the highest upregulation, approximately 3.5-fold relative to the control (Fig. 3B, $p_{\text{Kruskal-Wallis}} = 0.0036$).

For hsa-miR-34b, EPA induced a marked downregulation, with expression reduced to approximately 0.1-fold relative to the control (Fig. 3C, $p_{\text{Kruskal-Wallis}} = 0.0400$). AA treatment reduced hsa-miR-34a expression by nearly 70% compared to DHA and EPA.

Regarding hsa-miR-34c, AA treatment caused the strongest reduction in expression, with levels approximately 0.2-fold of the control (Fig. 3D, $p_{\text{Kruskal-Wallis}} = 0.0165$).

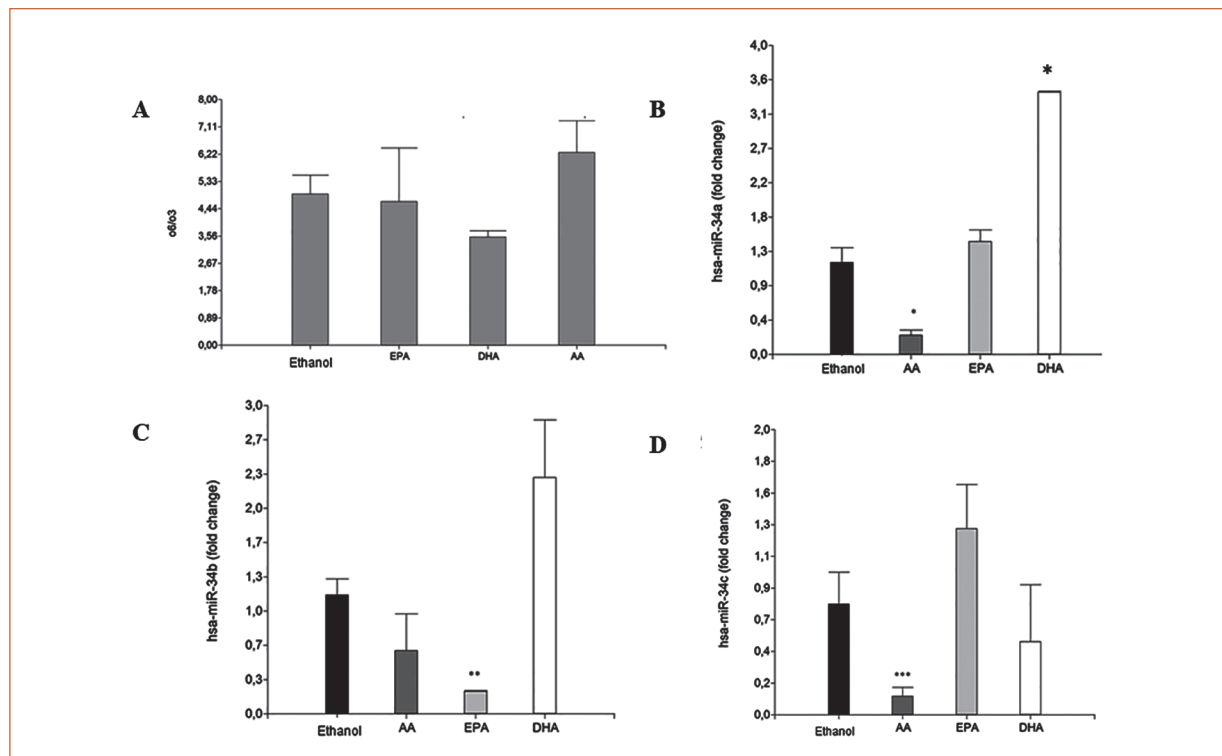
These results indicate that omega-3 PUFAs selectively upregulate hsa-miR-34a, while omega-6 predominantly downregulates miR-34 family members, reflecting differential modulation of pro-apoptotic pathways in dysplastic oral keratinocytes.

Discussion

Oral epithelial dysplasia is a key histopathological feature associated with the risk of malignant transformation²⁴. In this study, a human-derived dysplastic oral keratinocyte (DOK) cell line was used as a model to assess whether diet-relevant PUFAs modulate the miR-34 tumor suppressor family during the early stages of oral carcinogenesis. This knowledge would allow the implementation of preventive strategies in the population.

PUFAs exerted differential effects on DOK cell viability depending on both the type and concentration of the fatty acid. EPA and DHA significantly reduced cell viability at all tested concentrations compared with control. AA reduced cell viability at supramaximal concentrations (200 μ M) respect to control. These findings are consistent with previous studies showing that omega-3 PUFAs possess anticancer properties by inducing apoptosis and inhibiting proliferation²², while AA may act either as a pro- or antiproliferative agent depending on cell type and concentration; it may also exhibit antiproliferative effects^{25, 26}.

Figure 3 | A: Omega-6/omega-3 ratio in DOK cells treated with ETHANOL, EPA, DHA, and AA. Values are presented as mean $\pm$ SE of three independent samples. * Indicates significant differences in the omega-6/omega-3 ratio between DHA-treated vs. AA-treated conditions, and between EPA-treated vs. AA-treated conditions ($p_{\text{Kruskal-Wallis}} = 0.0250$). B-D: Relative expression levels of *hsa-miR-34a* (B), *hsa-miR-34b* (C), and *hsa-miR-34c* (D) in DOK (Oral dysplastic keratinocytes) cells incubated with ETOH (ethanol), EPA (eicosapentaenoic acid), DHA (docosahexaenoic acid), and AA (arachidonic acid). Values are presented as the relative expression change (fold change) normalized to the exogenous control, calculated using the $\Delta\Delta\text{CT}$ method. Data represent the mean $\pm$ SD of three independent replicates. *Indicates significant differences in miR-34a between ETOH/AA and ETOH/DHA ($p_{\text{Kruskal-Wallis}} = 0.0241$). C: **Indicates significant differences in miR-34b between ETOH/EPA ($p_{\text{Kruskal-Wallis}} = 0.0373$). D: ***indicates significant differences in miR-34c between ETOH/EPA ($p_{\text{Kruskal-Wallis}} = 0.0254$).



AA-treated cells also exhibited significantly enhanced migratory activity compared with the control, as evidenced by increased wound closure. Notably, this enhancement occurred independently of metabolic viability, suggesting a specific effect on motility-related pathways rather than a secondary consequence of enhanced cell survival. This effect may be related to the ability of AA to activate inflammatory and intracellular signaling cascades involved in cell migration and cytoskeletal reorganization, such as Akt/PI3K and EGFR pathways, which promote migration and invasion in breast cancer cells²⁷. Similar effects of omega-6 fatty acids on migration have been observed in other tumor models, reporting that linoleic acid increases migration in ovar-

ian cancer cells through cytoskeletal rearrangements essential for mobility²⁸. Furthermore, Jiang et al. found that enhanced conversion of glucose into de novo-synthesized fatty acids (palmitate, stearic acid, and oleic acid) was rapidly incorporated into complex cellular structures, suggesting a lipid-mediated oncogenic signaling mechanism that promotes metastatic behavior in OSCC (oral squamous cell carcinoma) cells²⁹.

Regarding lipid membrane composition, although individual fatty acids remained largely unchanged, the omega-6/omega-3 ratio emerged as a sensitive indicator of PUFA-driven membrane remodeling. This shift in lipid balance may underlie downstream signaling events linked to inflammation, migration, and epigen-

etic regulation. Based on these findings, future studies combining both fatty acid types at varying concentrations could help identify the optimal ratio at which omega-3 PUFAs counteract the pro-carcinogenic effects of omega-6 in vitro.

Genotypic analysis revealed no significant differences in TP53 and *hsa-pri-miR-34b/c* polymorphisms among PUFA-treated DOK cells, indicating that observed functional and molecular changes are not attributable to genotypic differences induced by treatment. All groups exhibited the heterozygous risk variants GC (TP53) and CT (*hsa-pri-miR-34b/c*), consistent with their dysplastic phenotype. These results align with our previous case-control study, in which patients carrying the GC TP53 allele had a fourfold higher risk of developing OSCC, and those carrying the T allele of *hsa-pri-miR-34b/c* exhibited a three- to eight-fold increased risk of developing OSCC and oral potentially malignant disorders, respectively¹⁰.

MicroRNAs have been identified as capable of distinguishing between progressive oral potentially malignant disorders and non-progressive leukoplakias, as well as normal tissues³⁰. In our study, analysis of the effects of PUFAs on mature members of the miR-34 family revealed a differential response in DOK cells depending on the fatty acid type. Omega-3 PUFAs, particularly DHA, appeared to induce a strong upregulation of *hsa-miR-34a*, suggesting a pro-apoptotic role, whereas AA downregulated all miR-34 family members, both related to control treatment. Interestingly, EPA induced lower expression of *hsa-miR-34b* relative to control, indicating a selective capacity of this omega-3 PUFA to modulate individual miR-34 family members. This pattern may reflect subtle molecular differences in the signaling pathways activated by different omega-3 PUFAs, as also reported in colorectal cancer cells³¹.

Variations in mature miR-34 expression observed in our study may explain the effects of PUFAs on proliferation, viability, and migration in DOK cells, highlighting their relevance in early malignant transformation and tumor progression. The mechanisms underlying PUFA-mediated miRNA expression could involve epigenetic modulation through histone acetylation and DNA methylation³² or interaction with transcription factors, such as peroxisome pro-

liferator-activated receptors (PPARs), to regulate gene repression³³. In this manner, DHA-treated colorectal cancer cells have shown decreased DNA methylation at the miR-126 locus while concomitantly increasing mature miR-126 expression³¹. Conversely, elevated PGE2 levels, derived from omega-6 PUFAs, were shown to reduce Dicer expression, a key enzyme in miRNA processing, in prostate cancer cells³⁴. Likewise, other omega-6-derived metabolites such as PGE2, LTB4, and 15-HETE have also been shown to alter miRNA expression patterns related to inflammation and tumor progression³⁵.

Although miR-34a is classically described as a tumor suppressor, it may play an anti-inflammatory role during early pre-neoplastic stages. Hidalgo-Sastre et al. reported that miR-34a ablation triggers a cell-autonomous inflammatory response characterized by increased TNF- α and IL-6 expression in pancreatic acinar cells³⁶. Moreover, miR-34 regulates cellular metabolism by limiting the activation of pathways that drive uncontrolled proliferation⁶. Evidence suggests that miR-34 functions as a negative regulator of MYC, an oncogene that promotes cell growth through glycolytic and biosynthetic activation. Thus, MYC-mediated regulation of cell size, proliferation, and metabolism may occur in a miR-34-dependent manner³⁷.

Overall, our findings highlight the potential of omega-3 fatty acids to modulate tumor-suppressive miRNA pathways in dysplastic oral keratinocytes. This represents a biologically plausible protective molecular mechanism, consistent with epidemiological evidence linking omega-3 intake to a reduced risk of cancer.

In contrast, omega-6 PUFAs exhibited pro-tumorigenic features at early stages, as evidenced by increased cell migration and downregulation of miR-34 members in the dysplastic cell line. Notably, these effects were accompanied by an increased omega-6/omega-3 membrane ratio, reinforcing the relevance of lipid balance in modulating cellular behavior.

Collectively, these findings support the concept that maintaining an appropriate omega-6/omega-3 balance may influence early molecular and functional events involved in oral carcinogenesis, and may constitute a key preventive strategy, particularly in developing countries and in populations exposed to other risk factors.

Future studies should address the translational relevance of these findings by exploring dietary modulation and omega-3 supplementation as adjunct preventive approaches in patients with potentially malignant oral disorders, aimed at reducing the risk of progression to oral squamous cell carcinoma.

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Supplemental Table 1S. Primer sequences used for the amplification of miR-34 by RT-qPCR

miRNA	Accession	Stem-loop	Primer sequences (5' - 3')	
			Forward	Reverse
hsa-miR-34-5p	MIMAT0000255	GTCTCCTCTGGTGCAGG GTCCGAGGTATTTCGCAC CAGAGGAGACACAACC	GGCGGTGGCAGTGTCT TAGC	
hsa-miR-34b-5p	MIMAT0000685	GTCTCCTCTGGTGCAGG GTCCGAGGTATTTCGCAC CAGAGGAGACCAATCA	CCGCGTAGGCAGTGTCT ATTAGC	
hsa-miR-34c-5p	MIMAT0000686	GTCTCCTCTGGTGCAGG GTCCGAGGTATTTCGCAC CAGAGGAGACGCAATC	CGGCGAGGCAGTGTAG TTAGC	TGGTGCAGGGTCCGA GGTATT
hsa-miR-103a-3p	MIMAT0000101	GTCTCCTCTGGTGCAGG GTCCGAGGTATTTCGCAC CAGAGGAGACTCATAG	CGGGAGCAGCATTGTA CAGGG	
cel-miR-54-3p	MIMAT0000025	GTCTCCTCTGGTGCAGG GTCCGAGGTATTTCGCAC CAGAGGAGACCTCGGA	GGGGGCCTACCCGTAAT CTTCA	

Supplemental Table 2S. Mean % and standard error (SE) values of saturated fatty acids from treated DOK cells

Treatment	SFA (Average $\pm$ SE)			
	C14:0	C16:0	C18:0	C20:0
Ethanol	6.75 $\pm$ 1.23	20.05 $\pm$ 3.72	12.47 $\pm$ 3.36	0.04 $\pm$ 0.03
EPA	3.29 $\pm$ 0.37	26.98 $\pm$ 3.05	17.6 $\pm$ 9.68	0.08 $\pm$ 0.07
DHA	1.17 $\pm$.62	35.87 $\pm$ 1.5	17.06 $\pm$ 3.7	0.07 $\pm$ 0.03
AA	1.64 $\pm$ 1.64	25.33 $\pm$ 4.86	8.87 $\pm$ 3.61	0.08 $\pm$ 0.07

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; AA: arachidonic acid
Each value is the result of three repetitions

Supplemental Table 3S. Mean % and standard error (SE) values of unsaturated fatty acids from treated DOK cells

Treatment	PUFAs (Average $\pm$ SE)						
			n-3			n-6	
	C18:3	C20:3	C20:5	C22:5	C22:6	C18:2	C20:4
Ethanol	0.55 $\pm$ 0.35	0 $\pm$ 0	0.06 $\pm$ 0.06	0.27 $\pm$ 0.14	0.63 $\pm$ 0.02	3.98 $\pm$ 0.31	3.26 $\pm$ 0.48
EPA	0.21 $\pm$ 0.11	0.04 $\pm$ 0.04	0.16 $\pm$ 0.16	0.42 $\pm$ 0.22	0.71 $\pm$ 0.06	2.64 $\pm$ 0.4	3.99 $\pm$ 1.34
DHA	0.25 $\pm$ 0.04	0.04 $\pm$ 0.04	0.11 $\pm$ 0.08	0.4 $\pm$ 0.01	0.82 $\pm$ 0.31	2.12 $\pm$ 0.15	3.49 $\pm$ 0.65
AA	0.88 $\pm$ 0.73	0.06 $\pm$ 0.04	0.17 $\pm$ 0.09	0.21 $\pm$ 0.18	0.41 $\pm$ 0.25	6.82 $\pm$ 3.3	4.06 $\pm$ 0.32
Treatment	n-7		n-9			Other Fatty Acids	
	C16:1	C18:1	C22:1				
Ethanol	3.06 $\pm$ 1.86	7.74 $\pm$ 2.62	0.43 $\pm$ 0.37			40.71 $\pm$ 7.82	
EPA	4.8 $\pm$ 1.71	9.35 $\pm$ 3.97	0.15 $\pm$ 0.15			29.57 $\pm$ 6.59	
DHA	5.17 $\pm$ 0.14	7.47 $\pm$ 2.11	0.22 $\pm$ 0.06			25.74 $\pm$ 4.97	
AA	3.8 $\pm$ 1.84	7.38 $\pm$ 4.22	0.1 $\pm$ 0.1			40.23 $\pm$ 8.3	

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; AA: arachidonic acid
Each value is the result of three repetitions