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Fatty acids and antioxidants in older adults with visual impairment: A contribution to Healthy Ageing

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Fatty acids and antioxidants in older adults with visual impairment: A contribution to Healthy Ageing

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Abbreviation

AA	Arachidonic acid
AMD	Age macular degeneration
ALA	**α** -Linolenic acid
AREDS	Age related eye disease study
AT	Adipose tissue
BCVA	Best-corrected visual acuity
BMI	Body max index
CC	Choriocapillaris
CHD	Coronary heart disease
CS	Complement system
CNV	Choroidal neovascularization
COX	Cyclooxygenase
CRM	Complement regulatory molecules
CVD	Cardiovascular disease
DGLA	Dihomo-**γ**-linoleic
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
ETDRS	Early treatment diabetic retinopathy study

etOH	Ethanol
EVA1	Epithelial V-like antigen 1
FA	Fatty acid
FFA	Free fatty acids
FADS	Fatty acids desaturases
GA	Geographic atrophy
GLA	**γ**-Linoleic
GPR	G protein-coupled receptors
IUPAC	International union of pure and applied chemistry
LA	Linoleic acid
LCPUFA	Long-chain polyunsaturated fatty acids
LPL	Lipoprotein lipase
MUFA	Monounsaturated fatty acids
NAT2	Nutritional AMD treatment-2
NCD	Non-communicable diseases
NO	Nitric oxide
NRF2	Nuclear factor-erythroid 2 related factor 2
N-3	Omega-3
N-6	Omega-6
LOX	Lipoxygenase
OCT	Optical coherence tomography

O3I	Omega-3 index
PC	Phosphatidylcholine
PEA	Phosphatidylethanolamine
PGI2	Prostacyclin
PI	Phosphatidylinositol
PS	Phosphatidylserine
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SFA	Saturated fatty acids
SN2	Stereospecific numbering 2
SOD	Superoxide dismutases
TG	Triacylglycerol
VA	Visual acuity
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoproteins
WHO	World health organization

1 ABSTRACT

The growing older population comes with a rise in the high mortality and morbidity risk of non-communicable diseases (NCDs), in which cardiovascular and eye disease are two of the most frequent conditions. Optimised nutrition and a healthy and active lifestyle have proven to play a role in preventing such diseases; however, nutritional preventive strategies still need to be strongly practised. This research aims to study the effect of supplementation with docosahexaenoic acid (DHA) and antioxidants in older adults diagnosed with age-related macular degeneration (AMD) or cataracts to contribute to preventive nutritional care and healthy ageing.

This study involves a randomised and observed-blinded trial that included 109 patients >50 years of age with a previous diagnosis of AMD and was conducted in nine sites in Spain and Portugal between November 2014 and April 2018. We study the effect of a 2-year intervention with a nutritional supplement. Plasma samples were analysed at baseline and after 12 and 24 months to determine the fatty acid (FA) status. The omega-3 index (O3I) was also calculated to assess the mortality risk of coronary heart disease (CHD). Additionally, a review of another visual impairment and the effects on antioxidants was made to support the nutrition strategies as an essential factor for healthy ageing.

The FA levels at baseline showed no differences between groups. However, at month 12 and 24-month follow-up, the mean changes in the polyunsaturated fatty acids (PUFAs) were statistically different between the intervention and control groups, except for the total omega-6 (n-6) long-chain (LC) PUFAs. Thus, DHA, total omega-3 (n-3) PUFAs and total n-3 LCPUFAs showed a greater increase with the intervention than with the control treatment, with an effect size that was moderate to large. In contrast, the total n-6 PUFAs, total n-6 LCPUFAs and the ratios of n-6/n-3 PUFAs and LCPUFAs showed a greater decrease in the intervention group than in the control group.

On the primary visual acuity assessment after a year, ETDRS letters had decreased with the intervention (*N* = 45; mean change −1.73, 95% CI −3.28 to −0.19) and in the control group (*N* = 48; mean change −0.10, 95% CI −2.03 to 1.83). Although there were no

significant differences in terms of visual acuity, in the group with DHA supplementation it was possible to observe a tendency to delay the progression of the loss of visual acuity.

The O3I at baseline exhibited both study groups at the second highest cardiovascular risk category (O3I >2.9 - 4.0). Nonetheless, by the 2-year intervention, the supplemented patients presented an O3I of 4.16%, reaching the second-best category of low risk for cardiovascular diseases (O3I >4.0 – 5.2), while the control group remained the same.

Additionally, a review was conducted on the role of the antioxidant vitamin C in cataracts, another common eye disease, to support nutritional strategies as an essential factor in healthy ageing. While dietary intake of vitamin C might have a positive effect on cataracts, its supplementation does not show the same effect. The protective effects of vitamin C in cataracts still need to be clarified; further assessments are encouraged.

The collection of analyses performed in this research allows us to confirm:

1. The link between FAs and health.
2. That DHA supplementation is promising to slow the progression of visual acuity loss in the older adult population with a previous AMD diagnosis.
3. The need to balance n-6 and n-3 proportions.
4. That DHA supplementation optimises two markers of health, the n6-n3 ratio and the O3I.
5. The antioxidant effect of vitamin C in cataracts remains unclear.

Altogether, these results endorse the role of nutrition in healthy ageing, decreasing the risk for NCDs, which are responsible for poor health and quality of life. This line of research deserves further attention, given its potential to culminate in actionable, tangible, and feasible dietary recommendations regarding DHA and vitamin C intake to promote healthy ageing.

Keywords: healthy ageing, n-3, n-6, old adults, AMD, cataracts, cardiovascular health, DHA, NCD, wellbeing, quality of life.

2 BACKGROUND

2. BACKGROUND

2.1 AGE-RELATED MACULAR DEGENERATION DEFINITION

Age-related macular degeneration (AMD) is a multifactorial eye disease influenced by a combination of environmental factors and genetic variants (1). AMD affects the central part of the retina, known as the macula lutea, a small but essential component in the visualisation of fine details and image resolution. The macula (**Figure 1**) is 5–6 mm in diameter, and at its centre is the fovea, which allows the ability to see "20/20" and provides the best colour vision (2).

The most common characteristics of AMD can be recognised by the formation of drusen (aggregates of extracellular material) and the growth of the choroidal vessels (choroidal neovascularisation). A few small hard drusen (about the size of < 65 μm) can be found at partially 96% of the aged population. Numerous larger (> 125 μm) hard drusen, and especially large soft drusen (125-250 μm) present in the macula, are considered notably when accompanied by pigment irregularities or depigmentation being a higher risk factor for the advanced form of AMD (3). Also, the appearance of drusen and neovascularisation is the result of chronic changes of the macula and, in particular of the retinal pigment epithelium (RPE), the choriocapillaris (CC), the photoreceptors (rods and cones) and Bruch's membrane (4–8).

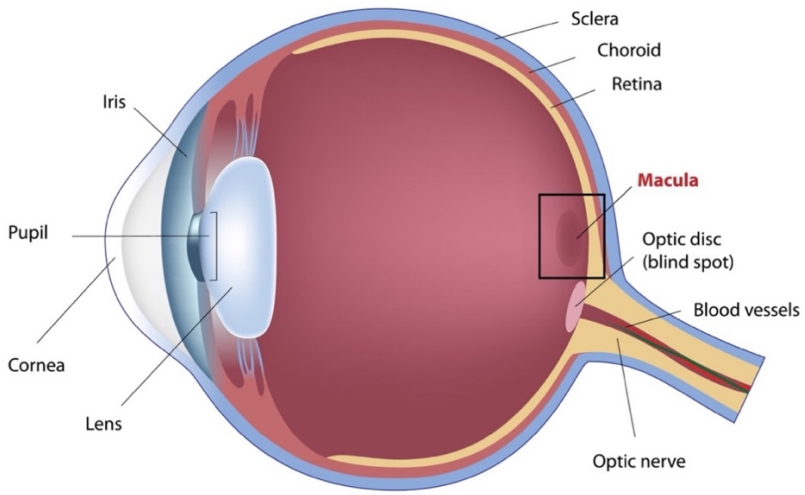


Figure 1. Healthy eye. Location of the macula at the back of the eye and centre of the retina, the region that subserves central, detailed and colour vision (9).

The onset of the pathology is clinically asymptomatic, therefore, difficult to diagnose. The initial stages are clinically characterised by extracellular yellow lipofuscin deposits, a pigment derived from lipid oxidation (drusen), and changes in the RPE. In the intermediate stage (**Figure 2**), the drusen shift to a larger size and become more concurrent. The later stages are called either neovascular (wet AMD) or geographic atrophy (GA), also known as dry AMD (10). Hence, AMD alters clinically into two different types: wet and dry. Both are progressive, differing in their clinical manifestations, prognosis, and treatment (11).

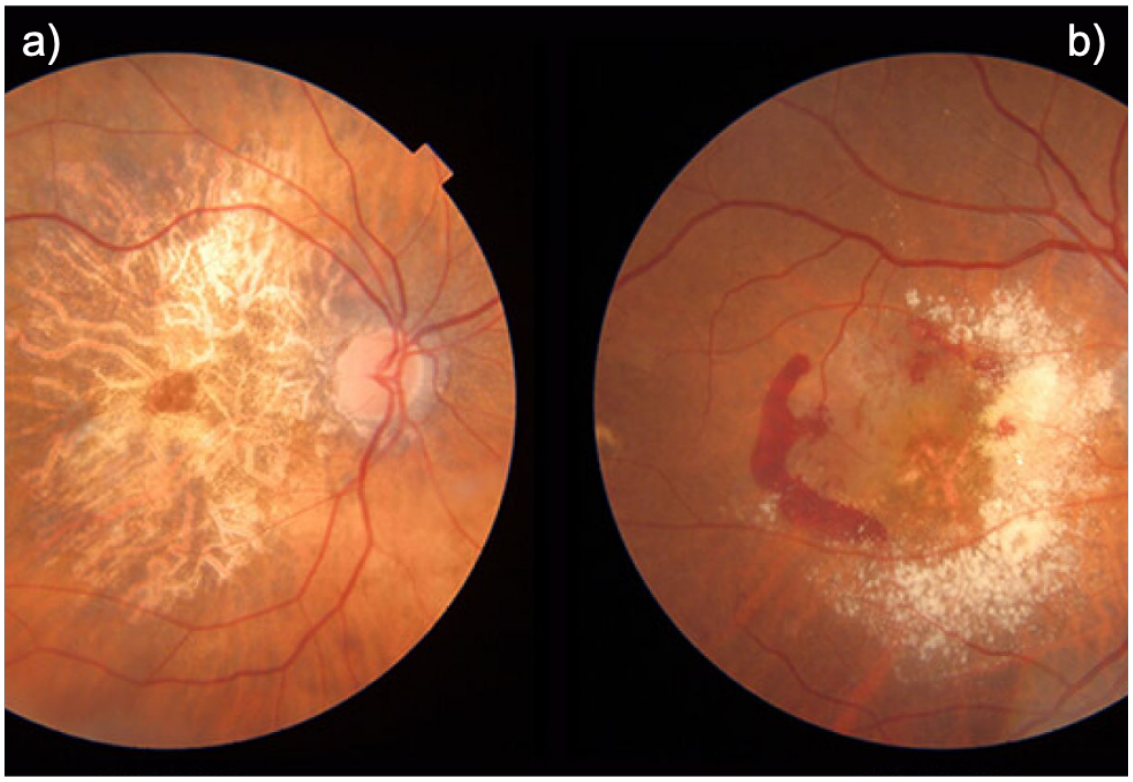


Figure 2. Different stages of AMD progression. (a) Large and intermediate drusen in the intermediate stage of AMD. (b) Neovascular AMD - eye with evidence of sub-retinal fluid, haemorrhage, and exudate in the presence of choroidal neovascularisation (12).

Over time, numerous AMD classification schemes, grading systems, and severity scales have been developed to provide standards to assist clinicians and researchers in

diagnosing and managing AMD. The late classification for clinical use proposed for AMD is summarised in **table 1** (13,14).

Table 1. Clinical classification for AMD (13).

Classification		Definition
Without pathology	No apparent aging changes	No drusen and no AMD pigmentary abnormalities
	Normal ageing changes	Only drupelets (small drusen (≤63 µm),) and no AMD pigmentary abnormalities
With AMD	Early AMD	Medium drusen (>63 y ≤125 µm) and no AMD pigmentary abnormalities
	Intermediate AMD	Large drusen (>125 µm) and/or Any AMD pigmentary abnormalities
	Late AMD	Neovascular AMD and/or any geographic atrophy

The clinical symptoms are a gradual loss of central vision, distortion of images and straight lines, and blurred and dark areas of central vision (4,12). Treatment is effective for wet AMD, while no therapy is successful still for dry AMD (14).

2.1.1 PREVALENCE AND EPIDEMIOLOGY

Worldwide, AMD is one of the most common causes of blindness in adults > 55 years of age, with a prevalence of 0.05% before age 50, rising to 30% after 74 years of age (15). The first causes of visual impairment are uncorrected refractive errors, followed by cataracts. Then there is AMD, with a population of around 170 million individuals affected, thus being the third leading cause of vision loss worldwide and the leading cause of irreversible central vision loss in the Western hemisphere (16–20).

In 2020 the number of people affected with AMD was estimated to reach 196 million and up to 288 million by 2040, of which 113 million are in Asia, the continent predominating compared to Europe or Africa. In Eastern Europe and Central Europe, AMD alone is responsible for 19.5% and 15.9% of all blindness cases reported and is expected to affect 77 million Europeans by 2050 (21–23).

This eye disease is a significant global economic and clinical concern, potentially leading to vision loss and hurting patients' quality of life with lost productivity, welfare, and informal care. The international annual cost of visual impairment was estimated to be 3000 billion US dollars (563 billion US dollars for Europe) (24). In countries such as the United States, the health costs of AMD are estimated at 343,000 million dollars (8,16). Where more than 4.2 million Americans aged >40 years are either legally blind, having best-corrected visual acuity (VA) (of 6/60 or worse (=20/200) in the better-seeing eye), or with low vision (having best-corrected VA less than 6/12 (<20/40) in the better-seeing eye, excluding those who were categorised as being blind (26).

2.1.2 PATHOPHYSIOLOGY

Research studies on AMD have identified multiple genetic loci implicated in critical biological pathways in the pathogenesis of the disease, such as complement and lipid pathway, oxidative pathway, stress pathway and angiogenesis signalling pathway that allow understanding of the mechanism of this pathology (14,27).

Among the inflammation markers are cytokines IL-1β, IL-18, IL-17, the complement system (CS), macrophages and, recently, inflammasomes of the innate immune system (28). However, it is still unknown whether the disease is a consequence of inflammation and whether it is due to changes in metabolic abnormalities, hypoxia, and oxidative stress (29).

Macrophages are innate immune cells associated with phagocytosis and antigen presentation in acute and chronic inflammation and wound healing. These are mainly seen in or near drusen, Bruch's membrane, etc. (27,30).

Cells corresponding to the non-specific response secrete numerous molecules to regulate inflammation, phagocytosis, cell growth, and death (31). They play an essential role in innate immunity, and this involvement in immune disorders supports their role in AMD. It is important to note that there are two main macrophage phenotypes: M1 (classically activated) and M2 (alternatively activated). M1 macrophages are pro-inflammatory, microbicidal, and antitumor. M2 macrophages are anti-inflammatory, pro-tumour, immunoregulatory, and proangiogenic, the latter participating in tissue remodelling (32–34).

The macrophage population differs in the eyes of healthy subjects compared to the eyes of AMD. The eyes of patients with advanced AMD show significantly higher levels of M1 macrophages than 2 and higher M1 / M2 ratios in the spot. Also, multiple studies have suggested an association between the classical activation of M1 macrophages and the development of AMD in the eye (35,36).

The phagocytic microglial cells in AMD accumulate in the affected areas of the macula and can contribute to inflammation in the pathogenesis of this disease (37). In addition, the accumulation of subretinal microglial cells is associated with migratory defects that can lead to drusen formation, choroidal neovascularisation, and retinal degeneration (37,38). Regarding the complement system, it is essential to mention that part of the innate immune system is involved in tissue inflammation, cell opsonisation, and cytolysis. The classical and alternative pathways form the cytolytic membrane attack complex capable of generating perforations in the cell membrane, thus promoting cell lysis and eliminating unnecessary cells (35,37).

Different studies show a strong association between genetic polymorphisms encoding complement regulatory proteins and the development of AMD. Complement activation is controlled effectively through membrane-associated complement regulatory molecules (CRMs) coordinated action. The basal activation of CS increases the synthesis of these endogenous molecules as genetic potentials. Membrane-binding CRMs are up-regulated by inflammatory cytokines such as INF**γ**, TNF-**α**, and IL-1**β**, by repeated non-lethal exposure to oxidative stress. This phenomenon is essential as a protective mechanism in

the natural ageing of the retina related to age. However, the same mediators known for the protective role of CS are also responsible for the pathological effects. Activation of this unregulated system can damage host tissue through an active complement cascade (39,40).

It is also evidenced that vascular endothelial growth factor (VEGF) is involved in vasculogenesis and angiogenesis of the individual, a key component of AMD since it favours angiogenesis through endothelial cell migration, proliferation, and vasculogenesis. Furthermore, vascular endothelial growth factor polymorphism is associated with many disorders, such as retinopathy of prematurity, diabetic retinopathy, and AMD (27,41–43).

2.1.3 DRY-TYPE AGE-RELATED MACULAR DEGENERATION

They are also known as non-neovascular, non-exudative or atrophic. About 90% of all people with AMD have the "dry" type. This condition occurs when the macula's light-sensitive cells gradually deteriorate, causing central vision to blur in the affected eye (45,46). As dry macular degeneration worsens, a blurred spot may be noticed in the centre of vision, and central vision may be progressively lost in the affected eye (12,44). Patients suffering from dry AMD have a significant anomaly of drusen in the RPE layer, leading to the formation of drusen to a thinning and drying out of the macula. Eventually, if not treated results in the loss of macular function (45).

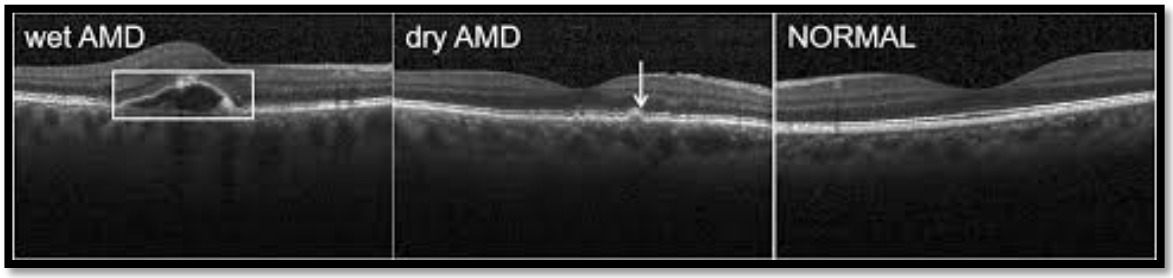


Figure 3. Retinal optical coherence tomography (OCT) image of normal, dry and wet AMD. Sample images: belongs to the UCSD dataset. The white arrows point to druses in the dry AMD, and the white box shows the irregular blood vessel under the macula in the wet AMD (46).

In **figure 3,** the difference in the macular can be seen by a novel automatic detection method of AMD from optical coherence tomography (OCT).

2.1.4 WET-TYPE AGE-RELATED MACULAR DEGENERATION

Wet, neovascular, or exudative AMD occurs when abnormal blood vessels behind the retina grow beneath the macula (44). These new blood vessels tend to be very fragile and frequently exudate, raising the macula from its usual place in the fundus. This choroidal neovascularisation (CNV) may lead to macular swelling with reversible vision loss or haemorrhage, which can be profoundly toxic and frequently cause irreversible vision loss. Once CNV has developed in one eye, the other eye is in a high-risk state (46).

In wet AMD, damage to the macula occurs rapidly, unlike the dry type and 10% to 20% of people with dry AMD progress to the wet type (**Figure 4**) (6,12,47).

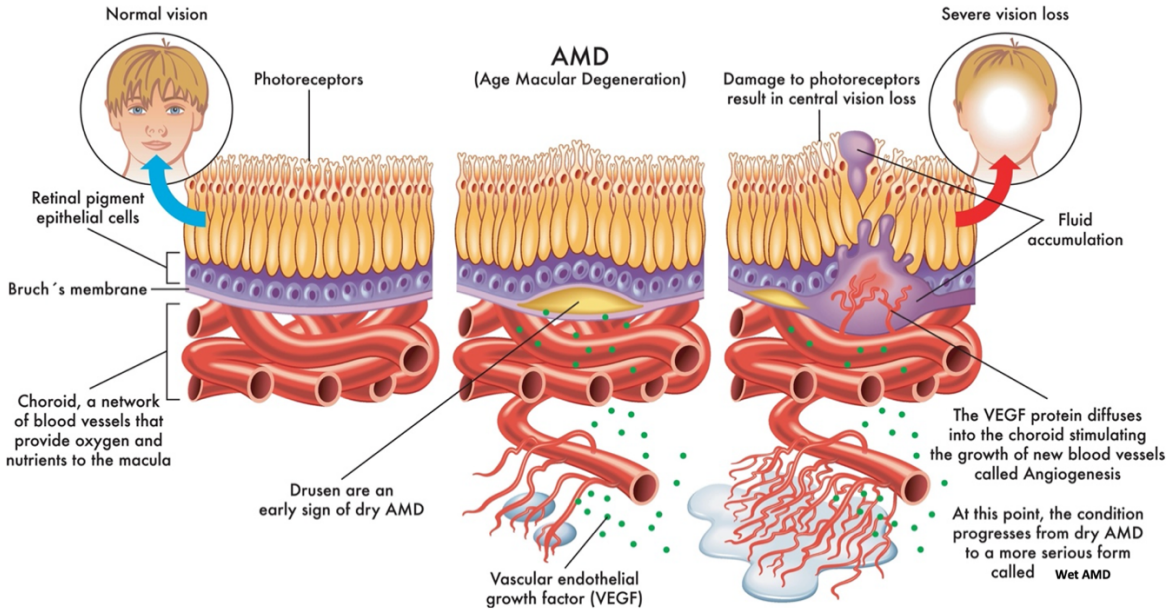
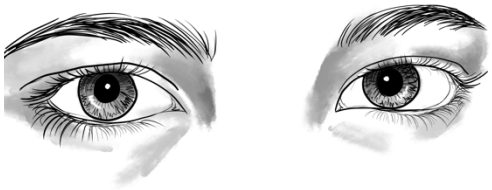


Figure 4. Retinal pigment epithelium (RPE) layer first in a normal eye, then dry and wet age-related macular degeneration (AMD) (48).

2.1.5 RISK FACTORS

AMD is a complex disorder with multiple risk factors related to genetics, age, and environment (49). While it can affect men and women, some reports present a higher risk in women by 1.3 times more than in men with AMD (50).



As non-modifiable factors, age and genetics are the two most potent risk factors for AMD (50,51). Generally caused by many genetic variants, there are about three dozen genes that affect 4 points in vision (photoreceptors, RPE, Bruch membrane, choroidal capillaries), leading to destabilisation of key physiological factors such as homeostasis and stress response, phagocytosis, extracellular remodelling, and related inflammation with the immune complement system (52). Around 50% or more of the heritability of AMD can be explained by two primary loci harbouring coding and non-coding variation: at chromosomes 1q (*CFH*) and 10q (*ARMS2/HTRA1*), making AMD the most genetically defined complex disorder (50,53).

One of the factors associated with age is the focal deposition of acellular detritus (drusen), located between the RPE and the Bruch's membrane. For retinal function, it is fundamental that phagocytosis of shed photoreceptor rod outer segments by the RPE, but with age, it becomes less effective with the accumulation of residual bodies that may cause loss of RPE cells. Age can also be associated with changes in the thickness or composition of the Bruch's membrane that determine an essential reduction of fluid and nutrient transport, vital for the function of photoreceptors. Age is accountable for a 50% reduction in the thickness of choroidal vessels and an alteration of the sinusoid structure; together with the thickness of the Bruch's membrane, they can cause hypoxia, leading to vascular endothelial growth factors secretion, which is partly responsible for to the development of neovessels (54,55).

Concerning modifiable risk factors, smoking is the consistently high factor related to AMD. Smokers are exposed to two to three times higher risk than non-smokers due to

increases in oxidative stress, platelet aggregation, fibrinogen levels and a reduction in high-density lipoprotein levels and antioxidants in the blood (56,57).

It is essential to understand the bases of the disease concerning all the risk factors: age, smoking, obesity, arterial hypertension, history family (genetics) and polymorphisms (5,21,47). Most of these risk factors are linked to the induction of oxidative stress.

2.1.6 SIGNS AND SYMPTOMS

In dry or wet AMD, the utmost common symptoms are blurred vision and impaired recognition of people's faces due to drusen formation. It usually affects both eyes, but vision can be lost in one eye while the other appears unaffected (12,58).

Another important point when talking about AMD is that it is a bilateral disease on many occasions, and its consequences are even more dramatic. Several population studies have analysed the risk of involvement of the second eye in patients with advanced AMD in the first eye, and it is quantified that it is around 40-50% in the first five years 23-26 and up to 100% in 10 years (59). The risk of the second eye being affected by the disease is associated with the presence and degree of early forms of AMD (60).

Dry-type AMD is classified into three subtypes:

- **Early macular degeneration:** Small drusen (<63µm) or some medium drusen. At this stage, there are no symptoms or loss of vision.
- **Intermediate macular degeneration:** Medium-sized drusen (63-124µm) or one or more large drusen. There is a blurred spot in the centre of your vision. Those affected may need more light to read and perform other tasks.
- **Advanced macular degeneration**: Large drusen (> 25µm). In addition to the formation of these drusen, there is a deterioration of the light-sensitive cells and the supporting tissue in the central area of the retina. This deterioration can cause a blurry spot in the centre of your vision. Over time, the blurred site may enlarge and darken, making the central vision opaquer (difficulty reading or recognising people who are too close).

On the other hand, in wet or neovascular AMD, there is a loss of central vision, and it can happen very quickly. It is also considered an advanced form of AMD and is more severe than the dry form. It does not have stages like dry macular degeneration. One of the first signs of wet AMD is that straight lines appear wavy (**Figure 5**) (58,61).

a) b)

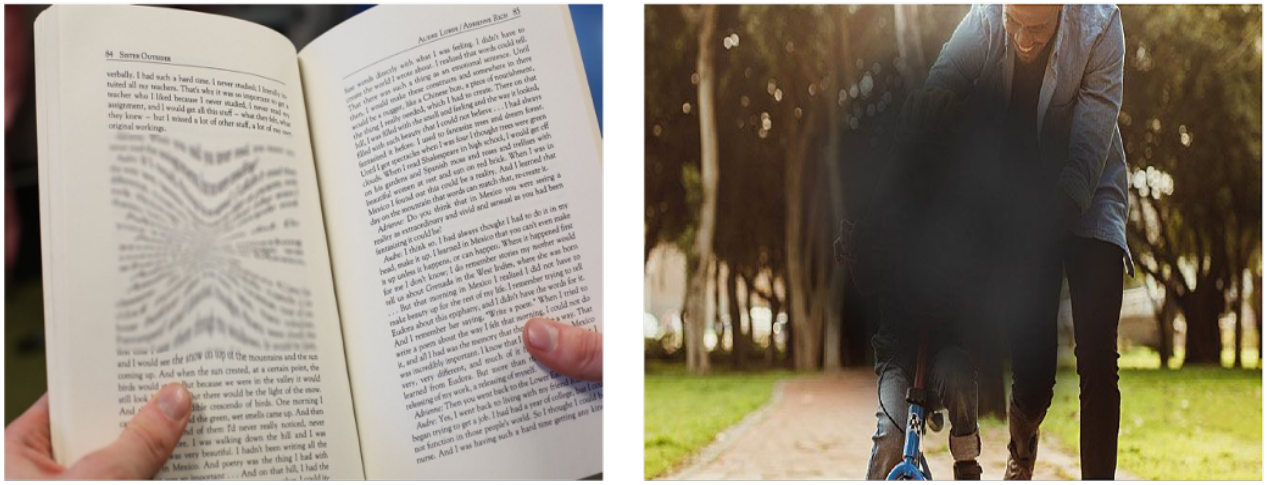


Figure 5. Different symptoms of AMD progression. (a) The first symptom is a blurred or distorted area in the central vision. (b) Advanced AMD, the centre of the field of vision, can include a blind spot. This blank area could be grey, red, or black (62,63).

2.1.7 DIETARY SUPPLEMENTS & AGE-RELATED MACULAR DEGENERATION

In recent years, numerous studies have related components of the diet and their protective role in AMD (64,65). For instance, antioxidants that, combined with increasing physical activity and adopting a healthy dietary pattern, can modify the risk of AMD. Also, AMD and maculopathies are partly the results of a photo-oxidative attack. Therefore, nutrients with antioxidant properties, as seen in supplementation with vitamins C and E, Carotenoids (such as lutein, zeaxanthin), and minerals (for example, zinc), have been shown to slow the progression of atrophic macular degeneration (4,25,47).

Antioxidants have been proposed to limit damage to photo-receptors at the macular level by protecting against the cumulative effects of oxidative stress, which would be a mechanism of cellular injury caused by ROS. The antioxidant system, composed of

enzymes and non-enzymatic molecules, endorses the appropriate levels of ROS. Vitamins C and E, β-carotene, and glutathione (low-molecular-weight compounds) are non-enzymatic antioxidants that aid against ROS. On the other hand, the enzymatic antioxidant defences of Superoxide dismutases (SODs), catalase, and enzymes are responsible for glutathione metabolism, which are regulated at transcriptional levels by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (66–68).

One of the most extensive multicentre clinical studies to assess the safety and efficacy of antioxidants was the Age-Related Eye Disease Study (AREDS) which aimed to evaluate the effect of high doses of vitamins C and E, β-carotene, and zinc in 3,640 participants aged 55 to 80 years (Table 2). This study showed a significant reduction in the probability of developing advanced AMD when taking a supplement food with antioxidants and zinc instead of "just antioxidants or just zinc." Therefore, the authors suggest that patients with intermediate-sized drusen and at least one large drusen in one or both eyes, or advanced AMD or vision loss due to this disease, should take this type of supplement without contraindications, such as smoking. The study results show risk reductions when taking antioxidants alone or zinc alone of 17% and 21%, respectively. When taking antioxidants plus zinc, the removal is 25% in the probability of developing advanced AMD in 5 years (69).

Table 2. Components and a daily dose of the AREDS study (64).

Study groups	Capsule content
1. Antioxidants	Vitamin C 500mg Vitamin E 400UI Beta carotene 15mg.
2. Zinc + Copper	Zinc Oxide 80mg Cupric Oxide 2mg
3. Antioxidants + Zinc+ Vitamin C	Vitamin C 500mg Vitamin E 400UI Betacarotene 15 mg Zinc Oxide 80mg
4. Placebo	-

In contrast, the AREDS-2 study (4,203 participants), a randomised, double-blind clinical trial, was designed to evaluate whether the addition of lutein, zeaxanthin, and long-chain polyunsaturated fatty acids (LCPUFAs) omega-3 (n-3), to the original formula AREDS (Table 3), affects the rates of progression to advanced AMD (70).

The study concludes that the addition of lutein + zeaxanthin, docosahexaenoic acid (DHA)+ eicosapentaenoic acid (EPA), or both to the AREDS formulation in the primary analyses does not further reduce the risk of progression to advanced AMD (12,70). Thus, the results of this clinical study did not support the prevailing view based on several lines of evidence from epidemiological, laboratory, and other clinical trials that suggested that the intake of LCPUFA n-3 could play a protective role in the progression of AMD.

However, a more detailed analysis suggests that the design of the AREDS-2 study may not have demonstrated the prophylactic potential of n-3 fatty acids (FAs). This study did not include a placebo group for DHA / EPA treatment, as the control group took the AREDS1 formulation. Additionally, 11% of control subjects took DHA / EPA nutritional supplements and were not removed from the study. In this context, it is worth noting that the serum level of DHA in the control group had increased by 14% at the end of the study. Consequently, these factors could have influenced the final result and made it difficult to demonstrate the effect of DHA / EPA (71,72).

Table 3. Components and a daily dose of the AREDS-2 study (69).

Study groups	Capsule content
1. Lutein + Zeaxanthin	Lutein 10mg Zeaxanthin 2mg
2. EPA + DHA	EPA 650mg DHA 350mg
3. Lutein+ Zeaxanthin + EPA + DHA	Lutein 10mg Zeaxanthin 2mg EPA 650mg/DHA 350mg
4. Placebo	-

In controversy with the AREDS-2 study, the authors Arnold. et al. (2013) (73) found a different result using LCPUFAS. Working with an intervention clinical trial (172 participants), randomised, double-blind and with a placebo group, after the ingestion of xanthophylls and n-3 LCPUFAs, (**Table 4**) for 12 months. They observed that the functionality of the macula lutea depends on the nutritional absorption of lutein and zeaxanthin, which is inversely associated with the risk of AMD. Exhibiting that LCPUFAs n-3 can also be protective (7,73).

Plasma carotenoid concentrations and macular pigment optical density increased significantly in the randomised groups when receiving xanthophylls and n-3 LCPUFAs after one month of intervention. The double dose was a beneficial modification in the plasma FA profile of AMD patients compared to the amount in group one. The lipophilic antioxidant capacity in plasma was significantly higher. According to the above, the authors find that a supplement of lutein, zeaxanthin and LCPUFAs n-3 for 12 months can significantly improve the antioxidant capacity in plasma, the levels of xanthophylls and the optical density of the macular pigment (7,74).

Table 4. Components and a daily dose of the study by Arnold, Winter et al. (73).

Study groups	Capsule content
1. Lutein + Zeaxanthin + DHA + EPA	Lutein 10mg Zeaxanthin 1mg DHA 100mg EPA 30mg
2. Lutein + Zeaxanthin + DHA + EPA	Lutein 20mg Zeaxanthin 2mg DHA 200mg EPA 60mg
3. Placebo	-

Another important study is the Nutritional AMD treatment-2 (NAT2), a randomised, double-blind intervention. The objective was to evaluate the efficacy of oral

supplementation with DHA as a prevention mechanism for AMD in 263 patients aged 55-85 with age-related maculopathy lesions. The patients received fish oil capsules of 840 mg/day of DHA and 270 mg/day of EPA or those in the placebo group (olive oil capsules) for three years (75). The incidence of CNV in the study eye was not significantly different between the DHA and placebo groups. At first glance, the NAT2 results are not different from the AREDS2 study. However, the authors observed poor compliance in the two study groups. Analysing the FAs of the red blood cell membrane, a higher consumption of n-3 FA was demonstrated in the placebo group, with an increase of 24% after three years, hence the lack of discrimination between the two groups (74). Still, it was revealed that in the intervention group, participants with higher levels of DHA and EPA in their red blood cells were significantly more protected against AMD than those with lower levels of n-3 FA, which is consistent with what was observed in epidemiological studies (71,76).

In another more recent prospective, double-blind, placebo-controlled study, 80 AMD patients were randomised (2:1) to receive one tablet/day of a nutritional supplement containing a mixture of carotenoids, vitamins, and n-3 FAs or placebo. At the end of the study, a progression of AMD was observed in 2.1% of the patients in the intervention group and 15.4% of the patients in the placebo group, concluding that a clinically significant stabilisation of macular degeneration can be obtained from age-related intermediate over two years treating patients with a mixture of carotenoids, vitamins, and n-3 FAs (77). Nonetheless, more studies are needed to confirm the beneficial role of n-3 LCPUFA in patients with AMD.

For years, epidemiological studies have associated treating modifiable risk factors, for example, switching an unhealthy diet to a healthy diet to help lower rates of early AMD. Many studies examine the effect of diets rich in n-3 on the progression of AMD and found a high degree of coherence in concerning the preventive effect of n-3 in AMD (64,71). In general, n-3 dietary variables (such as fish consumption), are estimated by food frequency questionnaires. Also, in the studies that evaluate the consumption of fatty fish, higher consumption of this food was associated with a lower risk of AMD (57,78–82). Other findings estimate the intake of DHA and EPA and obtain similar results concerning AMD (57,79–87).

In the study by Merle et al. (2013) (88), the levels of n-3 FAs in plasma were measured instead of using a food frequency questionnaire to estimate the intake, and in the same way, the protective effects of n-3 FAs in the AMD were confirmed (88).

On the other hand, in studies where genetics were considered, Reynolds et al. (89) observed that homozygous subjects for the ARMS2 / HTRA1 genotype have a high risk of AMD and were protected against atrophy by increasing the intake of DHA (89). Ho et al. (87) identified a significant interaction between the genetic variants CFH Y402H and LOC387715 A69S with the intake of EPA / DHA, concluding that the genetic predispositions to the development of AMD can be counteracted by the dietary intake of n-3 FAs (89).

The importance of n-3 FAs in the different studies is due to their fundamental role in the body. The n-3 and omega-6 (n-6) FAs are essential components of tissue lipids, particularly cell membrane phospholipids. One of the primary membranes of LCPUFA is DHA, and with dietary intake, it is incorporated into the circulating lipids and cells of the body, as well as EPA (76). DHA is present in the retina, an important structural lipid and therefore affects the permeability, fluidity, and thickness of the lipid phase and the properties of the photoreceptor membrane and can also be involved in signalling cascades, acting to improve membrane-bound retinal proteins regenerating rhodopsin (73). Likewise, the n-3 FAs have been shown to reduce the inflammatory response. By competition with arachidonic acid (AA, C20: 4 n-6), since the latter acts as a precursor for pro-inflammatory processes by activating eicosanoids of pro-inflammatory series (leukotrienes series 4, prostaglandins series 2 and thromboxanes series 2) on the contrary, n-3 FAs change this pattern, activating eicosanoids with no inflammatory activity (leukotrienes series 5, prostaglandins series 3 and thromboxanes series 3).

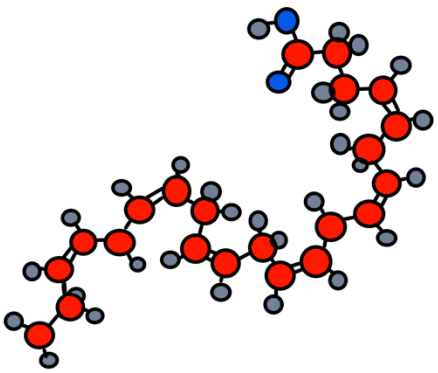
More recently, bioactive lipid mediators derived from DHA, such as resolvins (series D), protectins, and maresins with anti-inflammatory, antioxidant, anti-angiogenic, and neuroprotective properties, have been discovered (90). Correspondingly, EPA and DHA are known to decrease the reactivity of lymphocyte cells CD4 + T and make the inflammatory environment change from a pro-inflammatory to an anti-inflammatory;

another mechanism by which n-3 FAs can alter the signalling and gene expression cascades related to inflammation (74,91).

Therefore, incorporating DHA and the sum of n-3 FAs may have a beneficial effect in patients with AMD, mainly due to the anti-inflammatory effect of these FAs. However, more studies are needed (7,73–75,91,92).

2.2 POLYUNSATURATED FATTY ACIDS

There is a long list of FAs (**Table 5**); what is more, dietary fats are needed for normal metabolism and good health when balanced. They are necessary for the proper absorption, transportation, and function of the fat-soluble vitamins A, D, E, and K. They are used by the body to produce cellular components, hormones, and other compounds that are essential to the proper functioning of the body. Still, the most important FAs are those that the body cannot create and thus must derive from the food we eat. The human body can form all but two of the FAs: Linoleic acid (LA, C18:2n-6) (precursor from the n-6 series FAs) and **α**-linolenic acid (ALA, C18:3n-3) precursor from the n-3 series FAs. Each is the leading member of their PUFAs group and is marked as essential FAs (93).



Cell membranes contain ALA<EPA<DPA<DHA; consequently, cell membrane properties and functions are highly influenced by the presence of n3 PUFAs, especially DHA. The n-3 is a group of long-chain and very long-chain PUFAs. These FAs are essential since the human body cannot produce them on its own; they must be obtained from foods and beverages rich in this nutrient. However, the body can convert some ALA into EPA and then to DHA in very small amounts. Therefore, getting EPA and DHA from foods or dietary supplements is the practical way to elevate these n-3 FAs in the body (94).

Table 5. List of different types of fatty acids (95).

Fatty acid	Serie	Common name	Systematic name (IUPAC)
C14:0		Myristic	Tetradecanoico
C16:0		Palmitic	Hexadecanoico
C18:0		Stearic	Octadecanoico
C20:0		Arachic	Eicosanoico
C22:0		Behenic	Docosanoico
C24:0		Lignoceric	Tetracosanoico
MUFAS			
C16:1	n-7	Palmitoleic	cis-9-hexadecaenoico
C16:1	n-7	Palmythelaidic	trans-9-hexadecaenoico
C18:1	n-9	Oleic	cis-9-octadecaenoico
C18:1	n-9	Elaidic	trans-9-octadecaenoico
C18:1	n-7	t-vaccenic	trans-11-octadecaenoico
C20:1	n-11	Gadoleic	cis-9-eicosaenoico
C20:1	n-9	Gondoic	cis-11-eicosaenoico
C22:1	n-9	Erucic	cis-13-docosaenoico
C24:1	n-9	Nervonic	cis-9-tetracosaenoico
PUFAS			
C18:2	n-6	Linoleic (LA)	cis-9, 12-octadecadienoico
C18:2	n-6	Rumenic (RA/ CLA)	cis-9, trans-11-octadecadienoico
C18:3	n-3	α -linolenic acid (ALA)	cis-12,15-octadecatrienoico
C18:3	n-6	γ -linolenic acid (GLA)	cis-6, 9,12-octadecatrienoico
C18:4	n-3	Stearidonic (SA)	cis-6, 9,12,15-octadecatetraenoico
C20:2	n-6	Eicosadienoic (EDA)	cis-11,14-eicosadienoico
C20:3	n-6	Dihomo- γ -linolenic acid (DGLA)	cis-8, 11,14-eicosatrienoico
C20:3	n-9	Mead	cis-5, 8,11-eicosatrienoico
C20:4	n-6	Arachidonic (AA)	cis-5, 8,11,14-eicosatetraenoico
C20:4	n-3	Eicosatetraenoic (ETA)	cis-8,11,14,17-eicosatetraenoico
C20:5	n-3	Eicosapentaenoic (EPA)	cis-5, 8,11,14,17-eicosapentaenoico
C22:4	n-6	Adrenic (A/DTA)	cis-7, 10,13,16-docosatetraenoico
C22:5	n-6	Osbond	cis-5, 8,11,14-eicosatetraenoico
C22:5	n-3	Clupanodonicus (DPA)	cis-7,10,13,16,19-docosapentaenoico
C22:6	n-3	Cervonic (DHA)	cis-4,7,10,13,16,19-docosahexaenoico

Monounsaturated (MUFAs), Polyunsaturated (PUFAs), International Union of Pure and Applied Chemistry (IUPAC) (96).



The n-3 FAs are vital constituents of the membranes surrounding each cell in the human body, and DHA levels are found to be exceptionally high in the retina, brain, and sperm cells. Also, they provide the body with energy and have many functions in the heart, blood vessels, lungs, immune system, and endocrine system (97–99).

Functional effects of EPA and DHA in the body involve reducing inflammation, insulin resistance, blood lipids, blood pressure, atherosclerosis, and cancer, which could be used as prevention or as part of treating existing diseases. Some of these functional effects and mechanisms are the following (98,100–103):

- Reduce inflammation and insulin resistance: These FAs can partially inhibit several aspects of inflammation. For instance, replacing AA with EPA and DHA decreases the production of inflammatory eicosanoids and increases the production of resolvins and protectins. As a result, there is a consequent decrease in inflammation markers such as cytokines, adhesion molecules and acute phase protein. Therefore, decrease the risk of cardiovascular disease (CVD) and metabolic and inflammatory diseases, and have beneficial effects on immunity, allergies, coagulation, vasoconstriction, cancer, etc. Also, the G protein-coupled receptors (GPRs) have high specificity for n3 PUFAs, where GPR120 is found vastly in inflammatory macrophages and adipocytes. By binding DHA, GPR120 influences anti-inflammatory effects on macrophages and adipocyte insulin sensitivity.
- Reduce blood pressure: EPA and DHA can lower blood pressure by producing eicosanoids with vasoactive effects, aldosterone, nitric oxide (NO), vascular reactivity and cardiac hemodynamics.
- Prevent atherosclerosis: These FAs can reduce cholesterol plaques by producing the anti-inflammatory NO and prostacyclin (PGI2).
- Influence tumour cell proliferation and viability: These 2 FAs have been associated with preventing and slowing prostate, colorectal and breast cancers and improving cancer patients' quality of life and physical function. DHA induces tumour cell apoptosis, and EPA+DHA reduce prostaglandin E2 (which promotes tumour growth and proliferation).
- Reduce blood lipids: N-3 PUFAs modify blood lipids. EPA and DHA have been shown to lower Triacylglycerol (TG) concentrations, possibly by lowering hepatic synthesis

and secretion of very low-density lipoproteins (VLDLs) main lipoproteins carrying TG, upregulating the lipoprotein lipase (LPL) in adipose tissue, and therefore promoting TG clearance, and decreasing the release (from adipocytes) of free fatty acids (FFAs) that function as substrates to synthesise TG in the liver. Also, there is increasing **β**-oxidation in skeletal and cardiac muscle, hence limiting FFAs for the liver.

- **Weight loss and maintenance**: Since n-3 PUFAs stimulate lipid oxidation, they reduce fat mass. In contrast to the n-6 PUFAs, which exhibit pro-inflammatory properties. Moreover, the n-3 PUFAs are associated with regulating appetite and satiety, possibly by lowering leptin levels, known as the satiety hormone.

2.2.1 STRUCTURE AND NOMENCLATURE

Synthesis of FAs is made through the condensation of malonyl coenzyme A units by a FA synthase complex. ALA and LA are essential FAs, and LCPUFAs contain a carboxyl head group and an even-numbered carbon chain with two or more methylene-interrupted double (unsaturated) bonds. Essential FAs and LCPUFAs are structurally classified by the number of carbons, double bonds, and proximity of the first double bond to the methyl (omega) terminal of the FA acyl chain (**Figure 6**). The composition of natural PUFAs at room temperature is liquid, which results from the double bonds (cis) in their molecular structure (7,104).

Classification of FA based on two main criteria:

- **Depending on the number of carbon atoms that make up its chain**. Classified as short chain FA (2-4 carbons), medium chain (6-12 carbons), long chain (14-22 carbons) and very long chain (24 or more atoms).
- **Depending on their degree of unsaturation**. FAs are distinguished as a Saturated (SFA) molecule containing no double bonds, acids monounsaturated (MUFA) with one double bond and PUFA that have two or more double bonds (**Figure 7**).

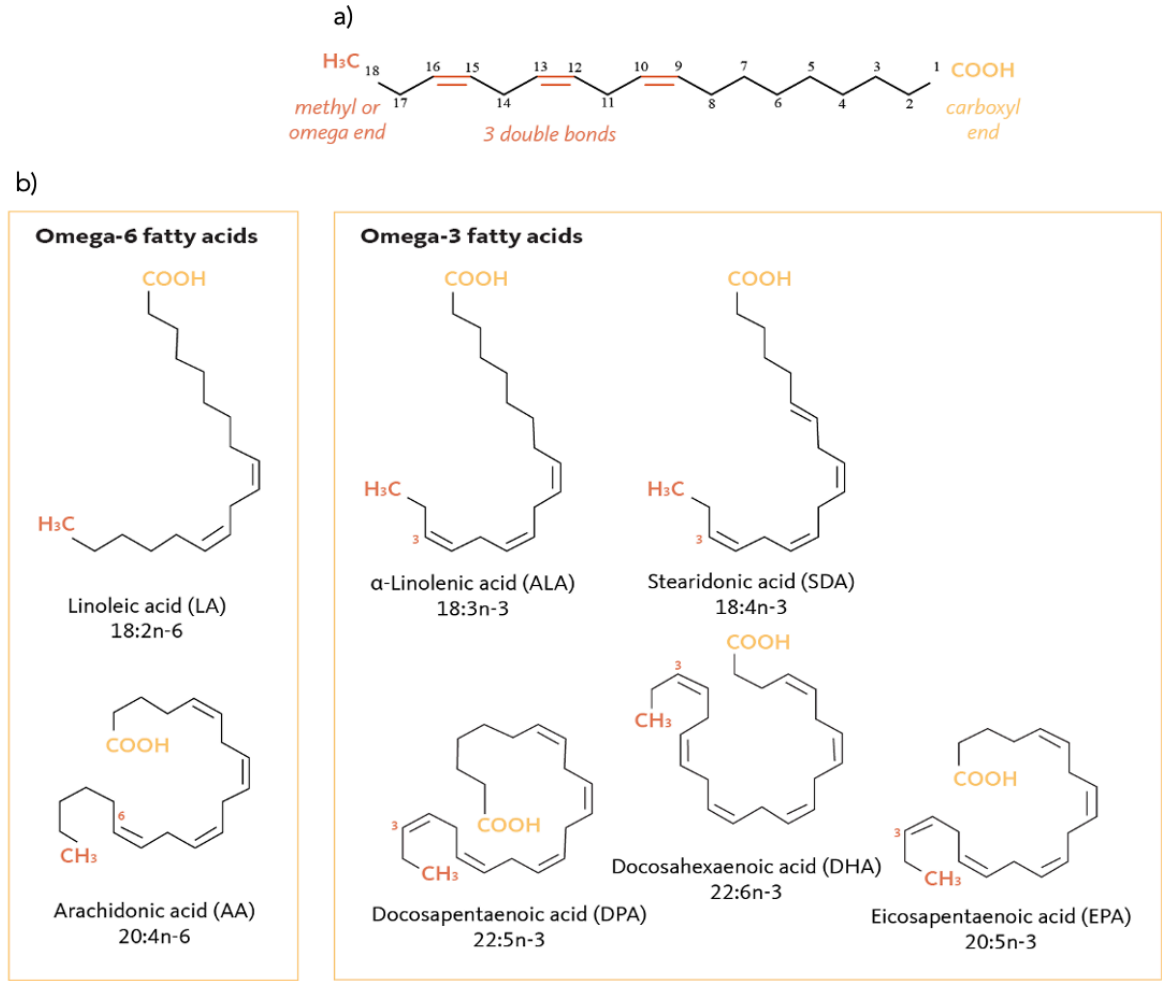


Figure 6. a) Chemical structure of **α**-Linolenic acid (ALA) 18:3n-3. The first part (18:3) tells that ALA is an 18-carbon fatty acid with three double bonds, while the second part (omega-3) tells them that the first double bond is in the omega-3 position, which defines this fatty acid as an omega-3. b) Molecular structure of an omega-3 and omega-6 (104).

The main two components in the diet of n6 PUFAs, are LA (C18:2n6) and AA, with different biological characteristics. For instance, LA is a vital FA for the production of n6 LC-PUFAs, such as **γ**-Linoleic (GLA), dihomo-**γ**-linoleic (DGLA) and AA (93). On the other hand, the precursor to the n-3 LC-PUFAs, ALA, is transformed to EPA: (C20:5n3), docosapentaenoic acid (DPA n-3: C22:5n3) and DHA (C22:6n-3).

DHA (D4,7,10,13,16,19-DHA; C22H32O2) has a molecular weight of 328.488 with 22 carbon chains, 6 cis double bonds and anti-inflammatory effects. It can be biosynthesised from ALA or commercially manufactured from microalgae. It is n-3 FA and

a major structural component of the human brain, cerebral cortex, skin, and retina, playing an essential role in their development and function (105). EPA (D5,8,11,14,17-EPA; C20H30O2) with a 20-carbon backbone and five double bonds, potential supplementing, anti-inflammatory, anti-thrombotic, immunomodulating, anti-angiogenic and chemopreventive activities and a molecular weight of 302.451(106). These FAs are of physiologic significance, as they act as constituents of lipid-protein complexes, substrates for bioactive eicosanoids or endocannabinoids, and natural ligands to nuclear transcription factors. And AA (D5,8,11,14-eicosatetraenoic acid; C20H32O2) a n-6 is a C20, PUFA having four (Z)-double bonds at positions 5, 8, 11 and 14 with a molecular weight of 304.467 (107–110).

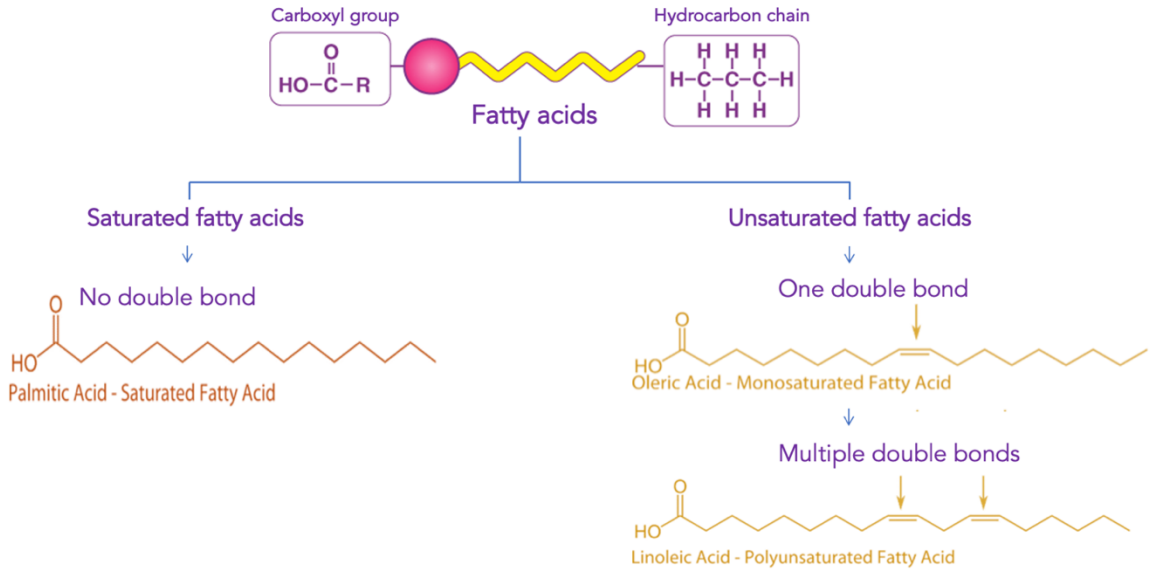


Figure 7. Fatty acid's structure. Most saturated fatty acids in nature have unbranched structures with an even number of carbon atoms. An unsaturated fatty acid with a double bond can have two possible configurations, either cis or trans, depending on the relative positions of the alkyl groups. The alkyl group refers to the carbon-hydrogen chain of fatty acids (107).

2.2.2 FOOD SOURCES (VEGETABLE, ANIMAL, OILS)

Different food sources contain different amounts of FAs, which may be affected by processing, storage, and cooking methods. Still, n-3 LCPUFA-rich foods are few and less frequently consumed than others in Western diets (111).

The predominant n-3 PUFA in the diet is ALA unless EPA and DHA supplements are being consumed. Some food sources of ALA are seeds such as flaxseed, nuts like walnuts and rapeseeds, and plants such as soybean and vegetable oils. In the n-3 PUFA series, while the ALA derives from plant oils, EPA, DPA, and DHA derive from marine lipids. EPA, especially DHA, are the most important n-3 PUFAs found in mackerel fish, salmon, sardine, herring and smelt and fish oils, including cod liver oil (**Table 6**) ((97,112–115).

Table 6. PUFA content of dietary components.

FATS	LA	ALA	AA	EPA + DHA
SATURATED				
Butter fat	2300	1400		
Coconut oil	1400			
Beef tallow	80			
UNSATURATED				
MUFAs				
Peanut oil	23900			
Pecans	20600	1000		
Almonds	9860	260		
Olive oil	8000	950		
Avocado	1970			
PUFAs				
n-6				
Safflower oil	74000	470		
Sunflower oil	60200	500		
Soybean oil	53400	7600		
Corn oil	50000	900		
Cotton seed oil	47800	1000		
Walnut	34100	6800	590	
Brazil nut	24900			
n-3				
Canola oil	19100	8600		
Salmon	440	550	300	1200
Tuna	260	270	280	400
Herring	150	62	37	1700
Trout	74		30	500
Cod	4	2	3	300

Linoleic (LA), α-linolenic acid (ALA), arachidonic (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), monounsaturated (MUFAs) omega-6 (n-6), polyunsaturated (PUFAs), omega-3 (n-3). Data are expressed as mg/100 g. The content of fatty acids may vary slightly according to species, sources, and analytical factors (93).

Nowadays, there is an increase in consumption of saturated fats, n-6 PUFAS (LA rich vegetable oils associated with western diets), and an overall decrease in n-3 PUFAs intake (116). In Europe, FAs represent around 28–42% of total energy consumed by its population, in contrast to ancestral nutrition, where FA intake was approximately 20–30% of total energy (117). Optimal n-6 : n-3 ratio dietary intakes should be around 1–4 : 1; still, according to the diet characteristics described above from western diets, the ratio has changed, increasing to a range of 10 : 1 to 20 : 1 (111,116,118).

2.2.3 DAILY RECOMMENDATIONS

Experts have yet to establish an exact recommended amount for n-3 FAs, except for ALA. Average daily recommended amounts for ALA are listed below (**Table 7**) in grams (g). The amount a person needs depends on the age and sex (97) :

Table 7. National Institute of Health Life stage recommended amount of ALA (97).

Life Stage Recommended	Amount of ALA
Birth to 12 months	*0.5 g
Children 1–3 years	0.7 g
Children 4–8 years	0.9 g
Boys 9–13 years	1.2 g
Girls 9–13 years	1.0 g
Teen boys 14–18 years	1.6 g
Teen girls 14–18 years	1.1 g
Men	1.6 g
Women	1.1 g
Pregnant teens and women	1.4 g
Breastfeeding teens and women	1.3 g

*As total omega-3s. All other values are for **α**-linolenic acid alone.

However, the overall fat dietary intake recommendation should be up to 35% of the total energy intake, including all plants or animals consumed in the diet that contain lipids(112).

Dietary fats may come as solids or liquids, and once they are in the body, TGs are hydrolysed by lipases to liberate FFAs for their absorption. Thus consumed FAs can be found in the bloodstream of healthy people after digestion and absorption (115).

Some of the dietary recommendations regarding FA intake include (112):

- ↓ SFA intake (maximum 10% of total energy intake)
- ↑ MUFA intake
- ↑ PUFA intake to a minimum of 6-11% of total energy
- ↑ n3 PUFA intake
- Assure LA and ALA in diet (at least 2.5% of LA and 0.5% of ALA of total energy intake)
- ↓ n6: n3 dietary ratio
- Avoid trans FA intake

2.2.4 METABOLISM OF THE INTERACTION OF OMEGA 3 WITH THE EYE

N-3 LCPUFAs have demonstrated the capacity to modulate bioactive molecules' production, activation, and potency (119). Now and then, these LCPUFAs serve as lipid-protein complexes via signalling cascades in nuclear and cytosolic compartments. In others, they affect substrate pools or the availability of biosynthetic enzymes (120). They influence gene expression as ligands to several transcription factors and act as endocannabinoid autocoids. Metabolic and dietary DHA insufficiency is associated with visual system structure and function alterations. DHA and its substrate, EPA, influence eicosanoid metabolism by reducing n-6 LCPUFA levels (mainly AA) and competing for enzymes, such as cyclooxygenase (COX) and Lipoxygenase (LOX), used to produce AA-based angiogenic and proinflammatory series 2-and 4-eicosanoids (121).

However, the body does not have the enzymatic capacity to meet tissue demands for LCPUFA through biosynthesis. Therefore, tissue status is modifiable and dependent on intake. Then the body stocks LCPUFAs mainly as esterified complexes in the sn2 (stereospecific numbering 2) position of phospholipids or TGS. Inside the neural retina, phospholipids represent the predominant LCPUFA-rich lipid class; these compounds are

stored primarily as structural elements of membranes. Retinal phospholipids are composed of 40–50% phosphatidylcholine (PC) and are localised mainly in the outer leaflet of the membrane. At the same time, phosphatidylethanolamine (PEA) exhibits 30–35% and phosphatidylserine (PS) a 5–10% of retinal phospholipids; both categories tend to orient within the cytoplasmic leaflet. Lastly, phosphatidylinositol (PI) composes 3–6% of retinal phospholipids, a constituent of membrane domains acting in signalling cascades (7,122).

The hepatocyte is the primary site of LCPUFA biosynthesis. They are esterified into TGS and phospholipids, integrated with chylomicrons or very low-density lipoproteins (VLDP) before being transported to the choriocapillaris. The LCPUFA-rich phospholipids are hydrolysed and taken up by a high affinity, receptor-mediated process at the choroid-RPE. They are then transported through the interphotoreceptor matrix to the photoreceptor inner segment and esterified DHA-phospholipid compounds are then hydrolysed, actively transferred to the cytosol of the inner segment and re-esterified into phospholipids. DHA is incorporated into photoreceptors and moved to the outer segment. Disks migrate to the apical tip of the photoreceptor with time; they are shed and phagocytised by RPE cells. DHA is then stored within oil droplets in the RPE and efficiently recycled to the inner segment via a receptor-mediated process. Also, LCPUFAs of cellular origin may be biosynthesised on neural (astrocytes, photoreceptors) and vascular retinal endoplasmic reticulum and peroxisomes (Figure 8).

A membrane with a higher concentration of PUFAs exhibit less rigid global properties compared to membranes concentrated in sterol esters or SFAs because of the multiple unsaturated bonds in PUFAs that do not allow dense packing of the hydrophobic FAs components. LCPUFAs, with their long-chain constitution, also supply a less-dense structure since a more fluid membrane allows a faster response to stimulation. For DHA, the position of the first unsaturated bond at the n-3 (between D-20 and D-19) carbon provides leverage in the efficiency of membrane dynamics over that observed in a differently structurally identical FA with the first double bond at the n-6 carbon.

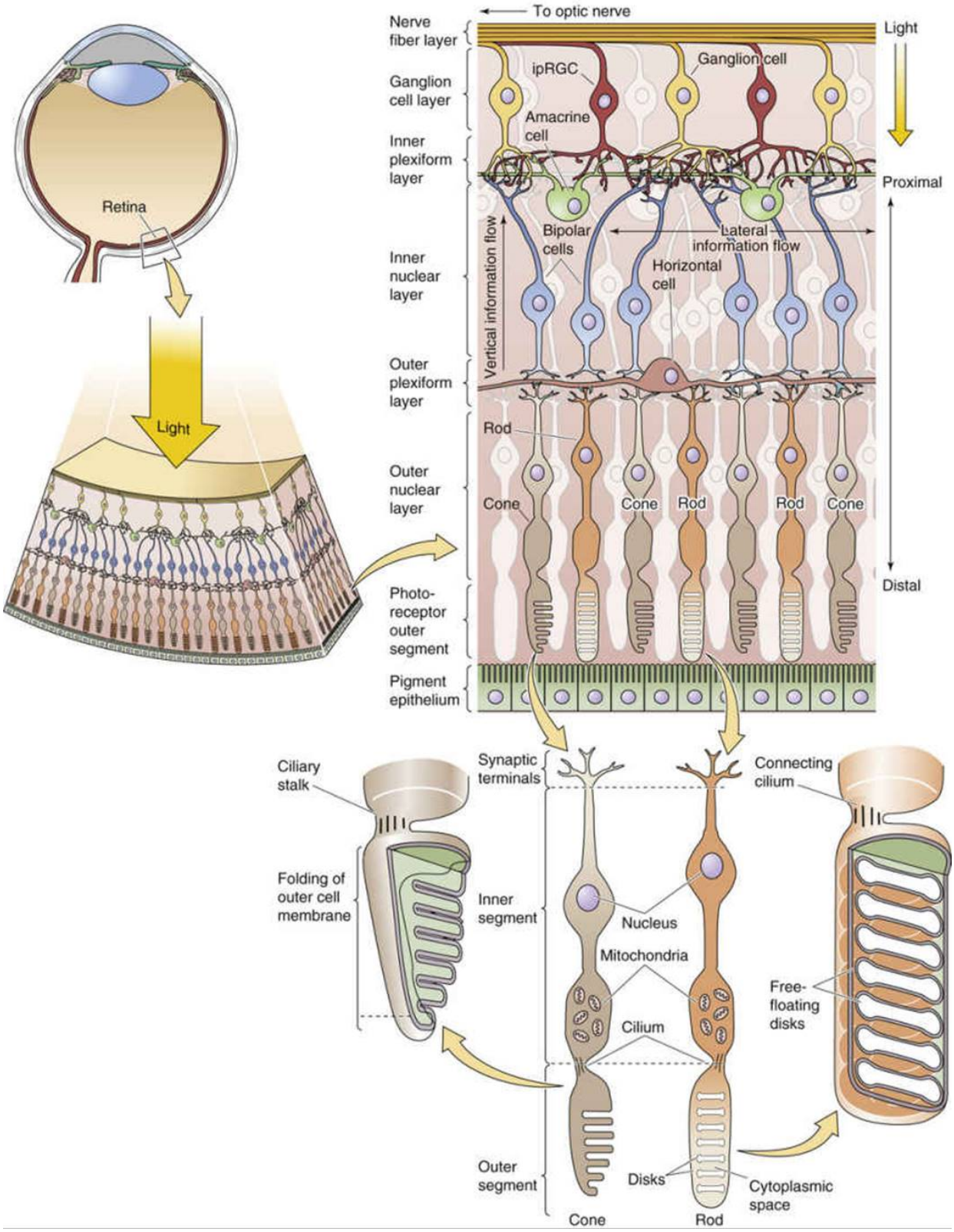


Figure 8. Neural circuits in the primate retina (123).

Biochemical characteristics of DHA may also explain why it is concentrated in the metabolically active retinal outer segment: FAs in membrane phospholipids are a primary source of signalling molecules that modulate intercellular communication and autocrine signalling from the plasma membrane. These processes also influence nuclear control of gene expression; however, esterified AA is more efficiently released from membrane stores than DHA; retinal astrocytes probably provide a readily mobilised source of DHA for such purposes (122,124).

2.2.5 ROLE IN EYE HEALTH

A major structural lipid of retinal photoreceptor outer segment membranes is DHA. Still, studies of the physiological importance of LCPUFAs, particularly DHA, remain a relatively uncharted field (113).

The retinal photoreceptor outer segment discs membrane contains rhodopsin, the photopigment necessary for initiating visual sensation; DHA is efficiently incorporated and selectively retained in disc membranes. The highest body concentrations of DHA per unit area are found in the disc membranes and the overall percent of DHA, which is 30% of total retinal FAs. The composition of retinal photoreceptor outer segments is unique in that 80–90% of structural lipids are glycerophospholipids, and 8–10% are neutral lipids. Neutral lipid species are mainly cholesterol, with a lower concentration of FFAs. A phospholipid is a polar molecule with a hydrophilic phosphate head group and two hydrophobic FA tails on a glycerol backbone (**Figure 9**). Retinal phospholipids are particular because many are polyenoic in nature. Polyenoic phospholipids contain PUFAs in the molecules' glycerol backbone's C1 (sn-1) or C2 (sn-2) positions.

Biophysical and biochemical properties of DHA may affect photoreceptor membrane function by altering permeability, fluidity, thickness, and lipid phase properties. The DHA status in the tissue affects retinal cell signalling mechanisms involved in phototransduction. Also, DHA may operate in signalling cascades to enhance the

activation of membrane-bound retinal proteins and may be involved in rhodopsin regeneration.

The stereochemical structure of DHA, with its 22 carbons and six double bonds, allows an efficient conformational change of the transmembrane protein rhodopsin in response to light absorption (photon capture). Furthermore, when tissue DHA insufficiency is present, it is associated with alterations in retinal function, and in some cases, visual processing deficits have been ameliorated with DHA supplementation (7,125,126).

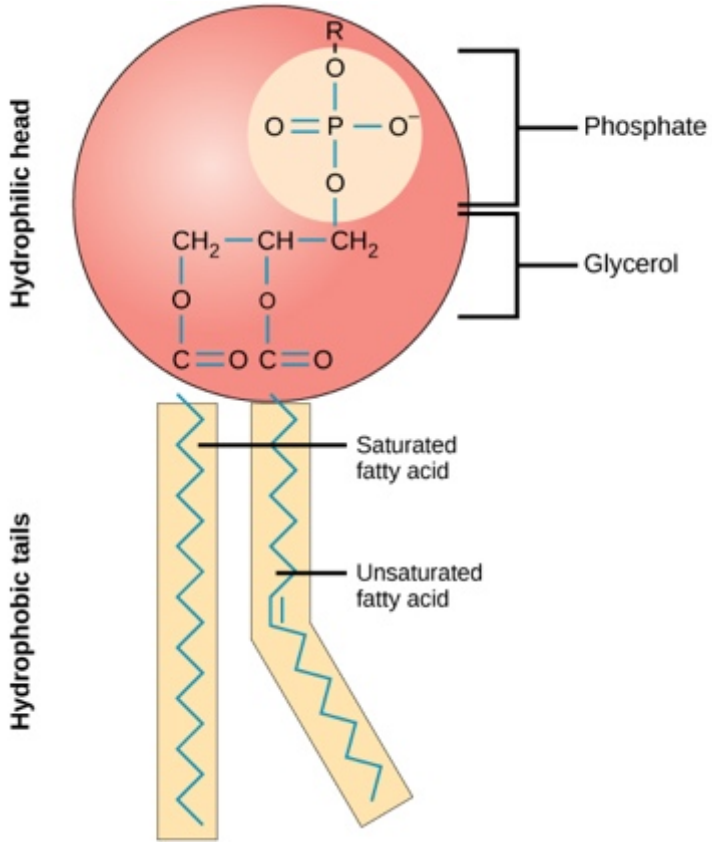


Figure 9. Phospholipid structure (127).

Many retinal diseases of public health significance manifest tissue and cellular dysfunction in abnormal angiogenesis, proliferative neovascularisation, excessive vascular permeability, immunoregulatory dysfunction, alterations in physiologic reduction-oxidation (redox) balance, or neuronal/RPE cell degeneration (41).

2.3 OLDER ADULTS

Over the past 150 years, life expectancy has increased dramatically over the years, although not all gained are healthy. The analysis of the Global Burden of Disease dataset (2020) suggests that the proportion of life in good health has remained broadly constant, suggesting increasing years in poor health (128–130).

Human ageing over time, at the biological level, is the result of the accumulation of a wide variety of molecular and cellular damage throughout life. This causes a gradual decrease in physical and mental capacity, a growing risk of disease and eventually death for every living being. However, these changes are neither linear nor consistent and are only relatively associated with a person's age in years. The heterogeneity seen in older adults is not random. Above biological changes, ageing is often associated with other life shifts, for example retirement, relocation to a home for the elderly and the death of friends or partners (131).

As time passes, typical conditions in older adults may include hearing loss, eye diseases such as cataracts and refractive errors, osteoarthritis, chronic obstructive pulmonary disease, diabetes, depression, and dementia. Some people are even likely to experience several conditions simultaneously if their health isn't taken care of in their younger years (132).

Geriatric syndrome is the term that highlights the unique features of common health conditions in older adults. They are commonly the repercussions of multiple underlying factors, including frailty, urinary incontinence, falls, delirium, and pressure ulcer, and are associated with substantial morbidity and poor health outcomes (133).

Some of the variations in older people's health are genetic. Still, most are due to people's physical and social environments; this includes their homes, neighbourhoods, and communities, as well as their characteristics such as (128,130):

- Sex
- Ethnicity
- Socioeconomic status

The long-term effects on how people age start with their environments as children and even as developing fetuses, all of which combine with their personal characteristics (134).

It is crucial to maintain healthy behaviours throughout life to diminish the risk of non-communicable diseases while ageing. Mainly eating a nutritious, balanced diet, engaging in regular physical activity, and avoiding tobacco use all contribute to reducing the risk of diseases as well as improving physical and mental capacity, delaying care dependency (129,131).

If people, while ageing, can experience these extra years of life in good health and a supportive environment, their ability to do the things they value will be improved and therefore have a better impact on society. If these added years are dominated by declines in physical and mental capacity, the implications for older adults and society are more negative (129).

2.3.1 HEALTHY AGEING

Ageing is often equated with chronological age. The United Nations define an older adult as a person over 60 years. Although society often uses other sociocultural referents to determine age, including family status (grandparents), deteriorating physical appearance, or age-related health conditions. The psychological and psychosocial toll of traumatic experiences, combined with poor nutrition and exposure to disease, can cause people to 'age' faster than settled populations. Consequently, numerous health challenges associated with old age will appear in people under 60 (135,136).

The World Health Organization (WHO) describes healthy ageing as "the process of developing and maintaining the functional ability that enables well-being in older age" (137). Healthy ageing is a related concept involving the essence of physical and cognitive functional preservation but without the requirement of disease avoidance. Healthy ageing is a more inclusive concept that accurately describes more individuals as the population ages (138).

An ageing population is a significant important medical and social demographic problem globally. Currently, Japan, Finland, and Italy are the countries with the oldest people. But the countries classified as the fastest ageing are Greece, Korea, Poland, Portugal, Slovenia, and Spain, according to the OECD (the Organization for Economic Co-operation and Development). Regarding non-OECD countries, the fastest ageing countries are Brazil, China, and Saudi Arabia (138,139).

2.3.2 PREVALENCE, LIFE EXPECTANCY

Globally people are living longer. Nowadays, most people can expect to live into their sixties and beyond. Every country in the world is experiencing growth in the proportion of older adults in the population (135).

It is estimated that by 2030, 1 in 6 people worldwide will be aged 60 years or over. The share of the population aged 60 years and over will increase from 1 billion in 2020 to 1.4 billion by 2050. The world's population of people aged 60 years and older will double (2.1 billion), and the number of persons aged 80 years or older is expected to triple between 2020 and 2050 to reach 426 million (**Figure 10**) (135,140,141).

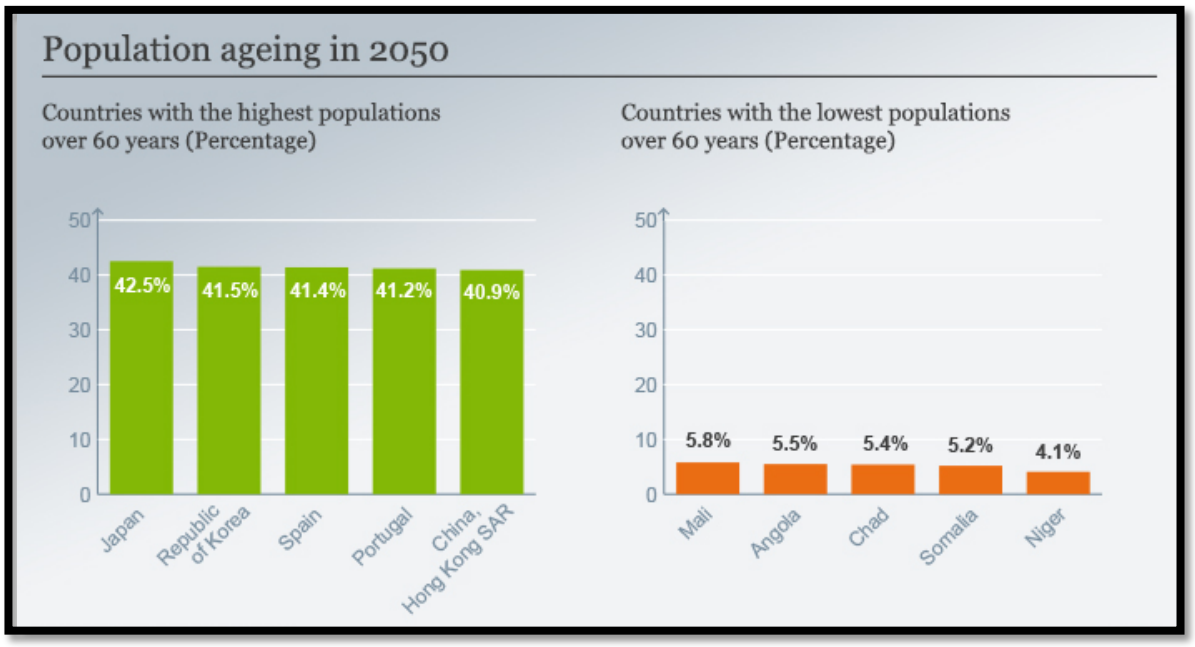


Figure 10. Population ageing in different countries (140).

While rising life expectancy and decreasing birth rates are considered significant achievements in modern science and healthcare, they will also significantly impact future generations, a negative one if they are not ageing healthy enough. The Organization for Economic Co-operation and Development (OECD) data demonstrates how the old-age-to-working ratio will change by 2060, highlighting some of the world's fastest-ageing countries (**Figure 11**). All over the world, countries face tremendous pressure to manage their ageing populations effectively. Preparing for this demographic shift early and aiming for healthy aged people will contribute to countries' economic advancement, allowing populations to live long and prosper (142,143).

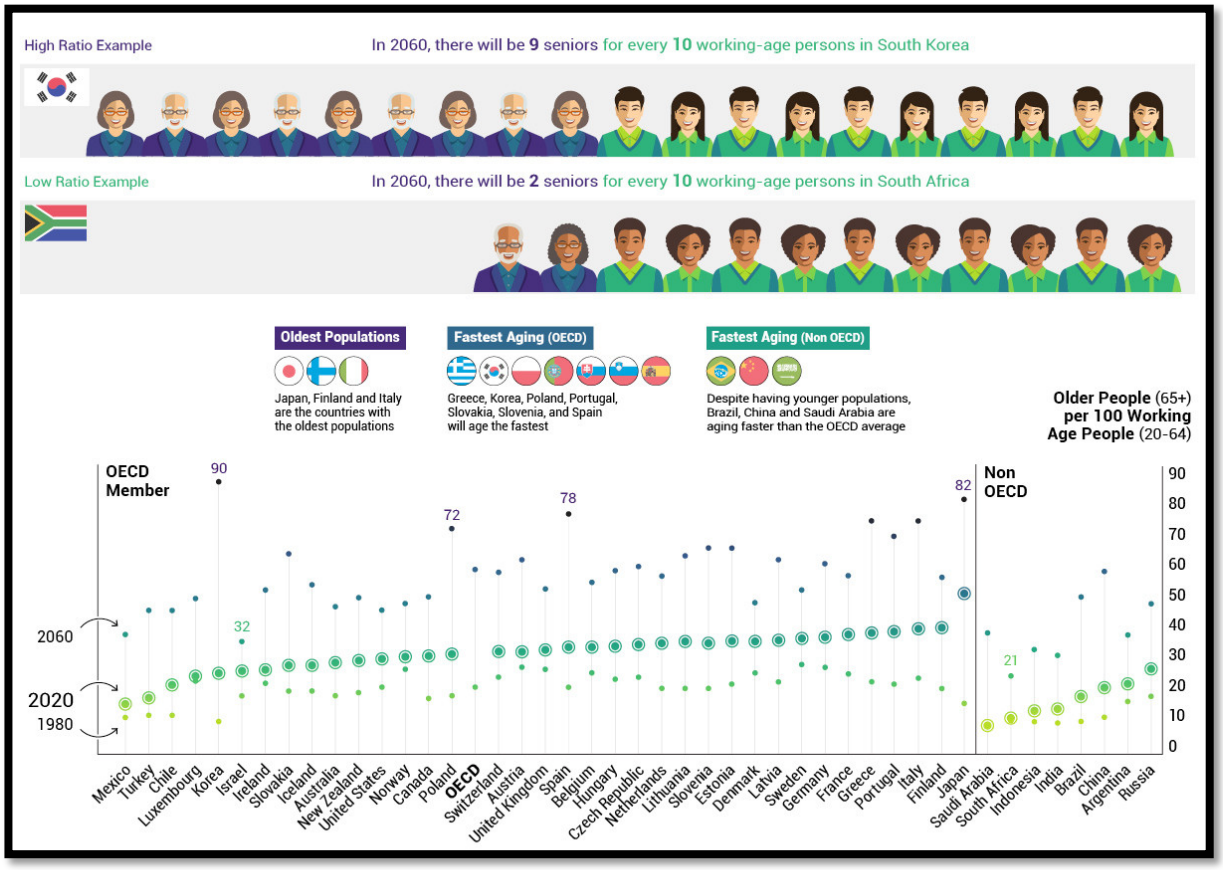


Figure 11. The rising ratio. In numerous countries, the old age to working age ratio will almost double in the next 40 years (143).

2.3.3 OTHER AGE-RELATED PATHOLOGIES

Since the unfolding of human civilisation, the aspiration for a longer life has been a prevailing pursuit. Due to improved living conditions and the continuous development of medical technology, the human lifespan has increased dramatically over the last century (144). Ageing is a direct factor in various age-related diseases, including neurodegenerative diseases, cardiovascular diseases (CVD), immune system disorders, eye diseases, musculoskeletal disorders, and cancer (145).

2.3.3.1 CARDIOVASCULAR DISEASES IN OLDER ADULTS

Adults aged 65 and older are more likely to suffer from CVD; by 2030, approximately 20% of the population will be 65 or older. CVD will result in 40% of all deaths in this age group and ranks as the leading cause. Furthermore, the cost to treat CVD will triple in that time (146).

According to the National Heart, Lung, and blood institute at the national institutes of Health, the risks factors for heart disease are the following:

- High blood pressure
- High blood cholesterol
- Diabetes and prediabetes
- Smoking
- Being overweight or obese
- Being physically inactive
- Having a family history of early heart disease
- Having a history of preeclampsia during pregnancy
- Unhealthy diet
- Age (55 or older for women)

Ageing can cause heart and blood vessels changes that may increase a person's risk of developing CVD. Still, age is an independent risk factor for CVD in adults, but these risks are compounded by added factors, along with frailty, obesity, and diabetes. These combined factors complicate and enhance cardiac risk factors associated with the onset of advanced age (147).

Gender is a potential non-modifiable risk factor in ageing adults, given that older females are reported to be at a greater risk for CVD than age-matched men. Still, in both men and women, the risks associated with CVD increase with age, which correspond to an overall decline in sex hormones, primarily oestrogen and testosterone (148).

Unbalance diet (exceptionally high in saturated fats) over a prolonged period is one of the major modifiable risk factors associated with the development of CVD, and thus a balanced diet towards macronutrients (proteins, carbohydrates, and lipids) and micronutrients (vitamins and minerals) are essential for maintaining cardiovascular health (144)

A healthy lifestyle influences the "rate of ageing" in the healthy heart and arteries. The ageing of other organ systems, including the muscles, kidneys, and lungs, also likely contributes to heart disease (149). Research is ongoing to unravel how these ageing systems influence each other, which may reveal new targets for treatments.

Several recent studies have interconnected higher blood levels and/or dietary intakes of the long-chain n-3LCPUFAs with greater longevity. In the Cardiovascular Health Study (150) the plasma phospholipid n-3 PUFA levels were inversely linked with total mortality rates. Similar associations were perceived for this endpoint with the red blood cell content of EPA plus DHA in the Heart and Soul StudyClick or tap here to enter text.(152). This metric, called for simplicity, is the Omega-3 Index (O3I) (**Figure 12**) and has been proposed as a risk factor for death from CVD.

In another recently completed Framingham study, each 1% increment in the O3I was associated with a 15% decrease in cardiovascular events and a 10% decrease in total mortality (151).

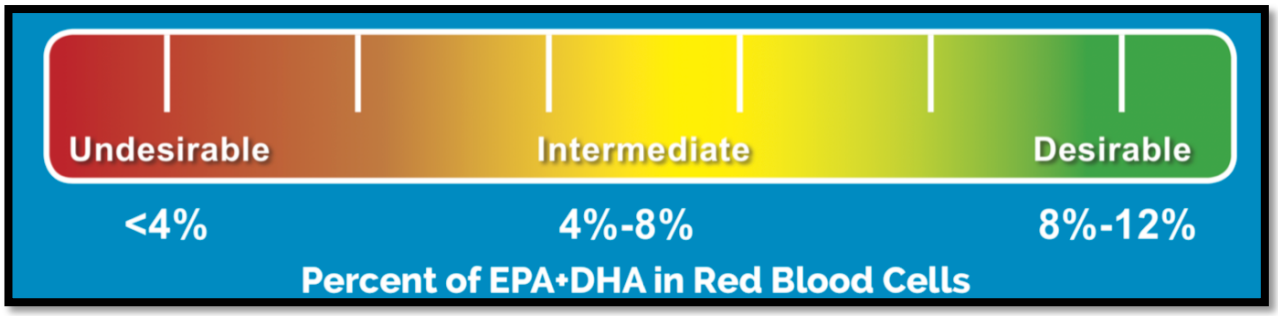


Figure 12. Omega-3 index risk zones (153).

Coherent with these observations, there is an inverse relationship between the O3I and the rate of telomere attrition, a marker of cellular ageing. Early randomised controlled trials with n-3 PUFAs found reduced overall mortality; however, other more recent studies have not confirmed such a protective effect. Still, some potential reasons why recent studies may have generated null results for an intervention involving n-3 PUFAs are linked to background use of statins, short follow-up periods, low n-3 PUFA doses, and improvements in acute care, to name a few (152).

2.3.3.2 CATARACTS

Cataracts are the leading cause of blindness and are responsible for 51% of global blindness. About one in five adults over the age of 65 has a cataract. Cataracts are a clouding and darkening of the eye lens, which blocks vision and can affect one eye at a time or both simultaneously (**Figure 13**). Stronger lighting and eyeglass adjustments can help when cataracts are small (154,155).

The four main forms of lens cataracts that are clinically recognised are sub-capsular, cortical, nuclear, and mixed (nuclear and cortical). Of these types, diabetic cortical cataracts and age-related nuclear cataracts are the most common (156).

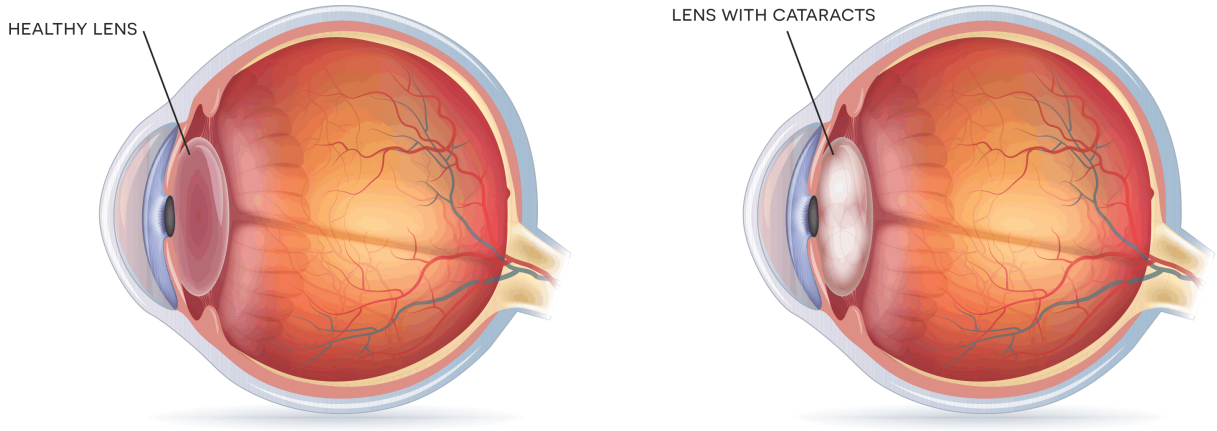


Figure 13. Standard eye lens and lens with cataracts (157).

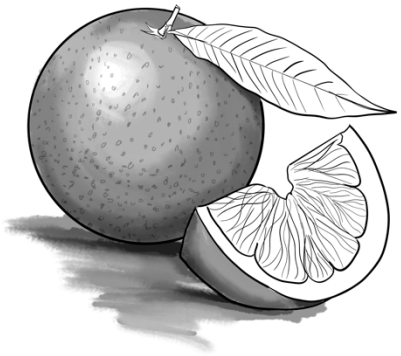
Age is a significant risk factor for cataracts, and the progression of the disease is gradual, appearing first in the fourth or fifth decade, but not affecting vision until typically the sixth decade. The lens is made up of water and proteins; the last one arranged precisely to keep the lens clear and let light pass through to the back of the eye. As people age, some of these proteins may clump together and cloud a small area of the lens (158).

Diabetes is another risk factor, with diabetic patients 2–5 times more at risk for developing cataracts and at an earlier age (159,160). Sedentary lifestyles, unhealthy diets and the increasing prevalence of obesity are raising the number of people with diabetes mellitus (161).

The only available treatment for cataracts is surgery, which involves the replacement of the cataractous lens with an artificial plastic lens which effectively restores sight. However, inadequate surgical facilities in poor and developing countries, and long waiting lists in developed countries, mean that alternatives to cataract surgery are required. It has been calculated that delaying the onset of cataracts by 10 years would halve its incidence and therefore reduce the need for and cost associated with, cataract surgery (158,162).

Because of the proven association between lens cataracts and oxidative damage, antioxidant supplementation has been promoted as a treatment strategy to slow the

progression of cataracts (163). Although the literature is mixed on how much to support the effectiveness of nutritional strategy, there seems to be a general consensus that a diet high in fruit and vegetables containing vitamin C, E, A and multivitamin-mineral supplements may be protective against cataracts (164,165). While most dietary intake studies show a reduction in the risk of disease or its progression, the most substantial decreases were achieved with diets high in vitamins C, E and A, with reduced effects for diets rich in carotenoids and selenium. Therefore, a healthy diet and a multivitamin supplement may offer protection against cataracts (156).



3 AIMS

3. AIMS

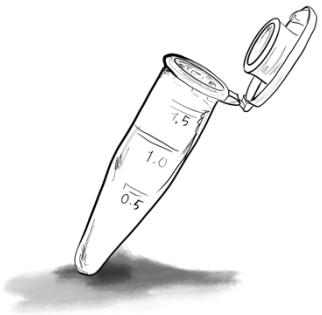
General aim:

Study the effect of supplementation with docosahexaenoic acid and antioxidants in ≥ 50-year-old patients diagnosed with age-related macular degeneration and cataracts to contribute to preventive nutritional care and healthy ageing.

Specific aims:

- To study the changes in plasma fatty acid profile in patients who receive a food supplement with docosahexaenoic acid *versus* patients who receive a food supplement without docosahexaenoic acid.
- To compare the visual acuity in patients that receive a food supplement with docosahexaenoic acid *versus* patients who receive a food supplement without docosahexaenoic acid.
- Analyse the coronary heart disease mortality risk in older adults before, during and after a 2-year supplementation with docosahexaenoic acid.
- Review the role of antioxidants as a therapy to prevent or delay cataracts.

4 METHODOLOGY



4. METHODOLOGY

4.1 ETHICS STATEMENT

This study was registered at ClinicalTrials.gov (NCT04756310). It was carried out by the ethical standards established by the Declaration of Helsinki (2004), the Good Clinical Practice recommendations of the EEC (document 111/3976/88 July 1990) and the current Spanish legislation governing clinical research in humans (Royal Decree 561/1993 on clinical trials). Additionally, the approval by Clínica Universidad de Navarra Committee was obtained, all participating sites endorsed this approval, and written informed consent was obtained from all the participants before any study procedure.

4.2 STUDY DESIGN AND POPULATION

The THEA-UNAV study was a randomised and observer-blinded trial **(Figure 14)** that included 109 adults aged 50 years or older with a previous diagnosis of AMD to study the effect of a 2-year intervention with a nutritional supplement. The study was conducted in nine sites in Spain and Portugal between November 2014 and April 2018.

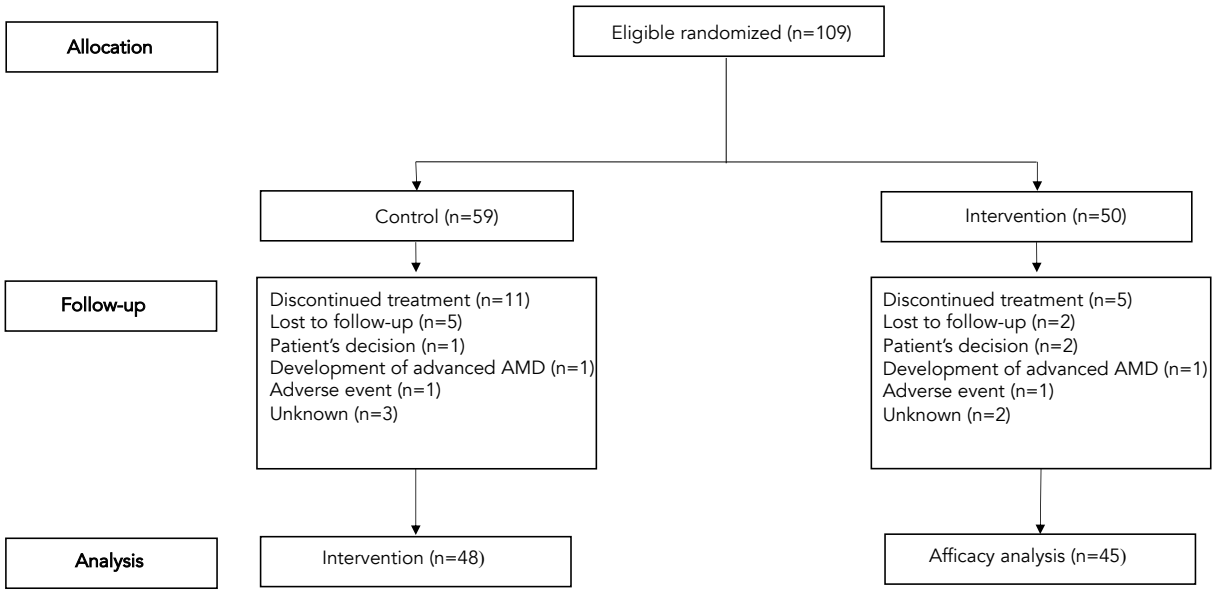


Figure 14. Patient disposition.

Inclusion criteria

Patients were included if they met the following criteria:

- Study population: male or female, ≥ 50 years of age.
- Presence, at a unilateral level, of choroidal neovascularisation secondary to AMD or any or any of its sequelae (i.e., disciform scar, pigment epithelium detachment secondary to subretinal fluid, and/or subretinal haemorrhage (stage V of the modified AREDS classification) with no exudative involvement in the contralateral eye (study eye).
- Patients who understood the conditions and particularities of the study and provided written informed consent.

Exclusion criteria

Patients were excluded if they met any of the following criteria:

- Myopia greater than six diopters.
- The presence of posterior pole abnormalities may cause choroidal neovascularisation: nevi, angioid striae, central serous choroidopathy, inherited degenerative retinal diseases, myopic choroiditis, diabetic retinopathy, and choroiditis.
- Coexisting media opacities that prevent an adequate assessment of the fundus.
- Based on the investigator's judgment, patients considered to be at risk of becoming lost to follow-up.
- Participated in any other therapeutic efficacy protocol in the last three months.
- Received any nutritional supplement within one month of the study entry.
- Suspected or confirmed diagnosis of substance use disorder (illegal drugs) and/or were not able to understand the study procedures.

Study groups:

- Group 1 (G1). Received the food supplement without DHA (control group).
- Group 2 (G2). Received the food supplement with DHA (intervention group).

Both subgroups take the treatment uninterruptedly throughout the study, using the exact dosage in each case. Blood plasma samples were collected from patients belonging to seven centres. Blood samples are obtained at the beginning of the study (T0), at 12 months (T12) and 24 months, respectively (T24).

4.3 DOSAGE AND TREATMENT OF PATIENTS

All patients received a supplement containing the components of the AREDS original formulation (i.e., vitamin C, vitamin E, beta-carotene, and zinc), manganese, selenium and refined soybean oil (control group) or the intervention supplement containing the AREDS original formulation, except for beta-carotene, plus copper, DHA from microalgae *Schizochytrium*, lutein, zeaxanthin, resveratrol, and hydroxytyrosol (**Table 8**). Both of the AREDS formulation doses complied with European requirements for these supplements. Each box containing the control or intervention products was identical in appearance and consecutively numbered according to the randomisation schedule. Regardless of the assigned group, all participants were instructed to receive two capsules daily, not allowing any change in the dosage or any other type of nutritional supplement concomitantly.

Table 8. Nutritional information of the supplements used.

Nutritional Information	Intervention supplement Retilut® (2 tablets)	Control supplement Theavit® (2 tablets)
Omega 3		
Docosahexaenoic (c22:6 n-3)	400 mg	
Vitamins		
Vitamin A		800 µg
β-carotene		800 µg
Vitamin C (ascorbic acid)	160 mg	120 mg
Vitamin E (d-**α**-tocopherol)	24 mg **α**- TE	20 mg TE
Others		
Resveratrol	30 mg	
Lutein	10 mg	
Zeaxanthin	2.6 mg	
Hydroxytyrosol	3 mg	
Trace elements		
Zinc (Zn)	20 mg	15 mg
Copper (Cu)	2 mg	
Manganese (Mn)		2 mg
Selenium (Se)		50 mcg

4.4 DATA AND SAMPLE COLLECTION

Information from each patient was collected and evaluated in the initial assessment. At baseline (T0), information on medical and ophthalmologic history was documented, and a brief nutritional questionnaire was applied; the best-corrected visual acuity (BCVA) was assessed in the study eye in a sitting position using Early Treatment Diabetic Retinopathy Study (ETDRS) testing charts; in addition, blood samples were obtained for biochemical analysis (described below). Throughout the follow-up, BCVA, was performed every six months, biochemical analyses were performed at baseline and 12 months (T12).

Blood samples were obtained at the beginning of the study T0, T12 and 24 months (T24), respectively for the biochemical analyses. Blood samples were collected by venipuncture, in a fasting state, and stored in tubes with EDTA-K3 as an anticoagulant. Plasma was then obtained by centrifugation at 1500g for 15 minutes at 4°C and stored at -80°C until analysis.

4.5 DETERMINATION OF FATTY ACIDS IN PLASMA

For FA analysis, blood samples were obtained. The method used to analyse the FA profile in plasma was based on the method developed by our research group and published elsewhere (166). The plasma was initially subjected to a saponification stage with sodium methylate and anhydrous methanol to obtain FAs in their free form. FAs methyl esters (or FAMEs) were obtained using boron trifluoride and methanol, and finally, the FAMEs were extracted with hexane and injected into the gas chromatograph. Quantification was done by normalisation, expressing the results in relative amounts (percentages). SFAs, MUFAs, n-3 PUFAs, n-3 LCPUFAs, n-6 PUFAs and n-6 LCPUFAs sums were created by adding the individual corresponding FAs. Additionally, the ratios n-6: n-3 PUFAs and n-6: n-3 LCPUFAs were calculated for the analysis and the O3I (EPA+DHA proportions) were created to assess the cardiovascular risk.

4.6 ANALYSIS OF THE EVIDENCE OF THE EFFECTS OF SUPPLEMENTAL OR DIETARY VITAMIN C ON CATARACTS

A search within the literature of human studies was done. Publications included two electronic databases, PubMed and Google Scholar, searched if data from epidemiological and clinical studies were collected within the last ten years (at that time) up to June 2020. The following data were identified: "human" "vitamin c'', either from "diet" or "supplement "in "cataracts". In the identification process, non-English languages and studies focusing solely on the use of vitamin c on other eye diseases or animal studies were excluded. Eligible articles were selected, of which three studies investigated the effect of vitamin C supplements on the development of cataracts and four studies investigated the impact of diets high in vitamin C on the development of cataracts.

4.7 STATISTIC ANALYSIS

SPSS statistical software package for Windows (version 20.0 and 23.0; SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses.

The primary outcome was the mean change from baseline in the BCVA in the non-affected eye by choroidal neovascularisation secondary to AMD (study eye). Secondary outcomes included the mean change from baseline in the evaluated cytokines and lipids and the frequency of adverse reactions, mainly the development of choroidal neovascularisation in the study eye.

To detect a mean difference between treatments in the BCVA of 4.6 letters (standard deviation 8.9), assuming a high correlation between the baseline assessment and the determination to be compared (minimum correlation of 0.8 between both), with a two-sided significance level of 5%, a power of 90%, and an anticipated number of dropouts of 10 to 20%, a sample size of 40 patients per treatment arm was required.

All efficacy outcomes were analysed in the intent-to-treat population using a visit-wise approach. The unpaired Student's t-test or the Mann–Whitney U test was used to compare the mean changes from baseline in the different efficacy outcomes. All tests

were two-sided and considered significant if $p < 0.05$. Effect sizes for the difference in mean changes between the intervention and control groups were calculated using Cohen's d. Cohen's d was considered of <0.20, 0.20 to 0.49, 0.50 to 0.79, and ≥0.80 to reflect trivial, small, moderate, and large effect sizes, respectively. Effect sizes that were at least moderate were interpreted as relevant changes.

A further test was conducted to analyse the visual acuity; we compared levels between the study groups using the General Linear Model. Values at baseline were corrected for sex and age, while values at 12 months were corrected for sex, age and plasma levels of DHA, trans-resveratrol, hydroxytyrosol, lutein and zeaxanthin. For this analysis, only patients with treatment compliance were considered. The plasma level of C22:5n6 was viewed as a control of supplement compliance. Therefore, participants with a level >0.25 were considered for treatment compliance.

For FAs analyses, data were tested for normality using the Kolmogorov-Smirnov test, and non-normal data were log-transformed. We evaluated the O3I and FA evolution and differences at T0, T12 and T24 of each study group. Since the O3I tool was initially created to assess the FAs from red blood cells, we used the adapted cut-offs proposed by Stark et al. to calculate the O3I from plasma samples. This classification has four categories of CHD mortality risk starting from high risk to low: <2.9, >2.9-4.0, >4.0-5.2 and >5.2% (153). The FA analysis was performed by the General Linear Model (GLM) with the Bonferroni post hoc correction and adjusted by dietary fish intake as a covariate, given the correlation with DHA plasma status (**Table 10**). The FA percentages were expressed as means ± standard deviations.

The n for analyses may variate according to data availability. The confidence level was established at 95% for all the tests. Thus, results obtaining a p-value below 0.05 were considered statistically significant and are highlighted in bold.

5 RESULTS

5. RESULTS

5.1 BASELINE CHARACTERISTICS OF THE POPULATION

The population (109) was assigned randomly to treatment where 50 patients received the intervention, and 59 received the control. The mean age of the participants was 77.1 years (standard deviation [SD]; 7.6) and was uniformly distributed concerning sex. Between the two groups, baseline characteristics were similar, except for the AMD status, which results were worse in patients from the intervention group (**Table 9**). The number of dropouts that left the treatment was 5 (10%) in the intervention treatment and 11 (18.6%) in the control treatment. A total of 93 patients completed the trial.

Table 9. Baseline characteristics.

Characteristic		Intervention *N* = 50	Control *N* = 59
Age (years), mean (SD)		78.4 (7.0)	76.0 (8.0)
Sex (women), *n* (%)		22 (44.0)	31 (52.5)
BCVA, mean (SD)		75.6 (11.1)	76.2 (11.7)
Corneal alterations (yes), *n* (%)		2 (4.0)	2 (3.4)
AMD status			
Presence of drusen, *n* (%)		41 (82.0)	42 (71.2)
Degree of drusen, *n* (%)			
	1	8 (16.0)	14 (23.7)
	2	17 (34.0)	15 (25.4)
	3	10 (20.0)	6 (10.2)
	4	6 (12.0)	7 (11.9)
Missing		8 (18.0)	17 (28.8)
Pigmentary alterations, *n* (%)			
Hyperpigmentation		15 (30.0)	13 (22.0)
Hypo-/hyperpigmentation		15 (30.0)	15 (25.4)
Hypopigmentation		3 (6.0)	5 (8.5)
No alterations		17 (34.0)	15 (42.4)
Geographic atrophy (yes), *n* (%)		9 (18.0)	4 (6.8)
LENS status			
Phakic		30 (60.0)	37 (62.7)
Pseudophakic		20 (40.0)	22 (37.3)

Age-related macular degeneration (AMD), best-corrected visual acuity (BCVA), standard deviation (SD).

5.2 COMPARISON OF PLASMA FATTY ACIDS AMONG STUDY GROUPS

At baseline, FA levels showed no differences between groups (Table 10). However, at month 12 and 24-month follow-up, the mean changes in the PUFAs were statistically significantly different between the intervention and control groups except for the total n-6 LCPUFAs; thus, DHA, total n-3 PUFAs and total n-3 LCPUFAs showed a greater increase with the intervention than with the control treatment, with an effect size that was moderate to large. In contrast, the total n-6 PUFAs, total n-6 LCPUFAs and the ratios of n-6/n-3 PUFAs and LCPUFAs showed a greater decrease in the intervention group than in the control group, also with moderate to large effect sizes.

The FAs of the intervention group had a higher impact overtime on the FA profile, with substantial changes ($p<0.001$) in C22:5n-6, DHA and the ratio n-6 LC: n-3 LC, but also impact in C22:4n-6, n-3 PUFAs, n-3 LC-PUFAs, and the n-6: n-3 ratio ($p<0.05$).

Table 10. Fatty acids in plasma per study group and time of the study.

	T0							T12							T24							p	p
	Control n=43			Intervention n=38			p	Control n=35			Intervention n=34			p	Control n=19			Intervention n=13			p	FA evolution of control group	FA evolution of intervention group
%	Mean	±	SE	Mean	±	SE		Mean	±	SE	Mean	±	SE		Mean	±	SE	Mean	±	SE			
C12:0	0.11	±	0.01	0.09	±	0.01	0.067	0.09	±	0.01	0.13	±	0.01	0.124	0.09	±	0.02	0.11	±	0.02	0.552	0.267	0.879
C14:0	0.82	±	0.05	0.72	±	0.06	0.445	0.68	±	0.05	0.77	±	0.05	0.216	0.82	±	0.09	0.71	±	0.10	0.537	0.997	0.782
C16:0	21.49	±	0.30	21.01	±	0.35	0.330	20.77	±	0.36	21.24	±	0.40	0.379	21.73	±	0.47	21.62	±	0.56	0.879	0.743	0.779
C16:1n-9	0.48	±	0.02	0.49	±	0.02	0.959	0.45	±	0.02	0.43	±	0.02	0.733	0.45	±	0.02	0.41	±	0.03	0.390	0.634	0.178
C16:1n-7	1.53	±	0.07	1.34	±	0.08	0.115	1.32	±	0.07	1.28	±	0.08	0.948	1.41	±	0.11	1.32	±	0.13	0.362	0.621	0.645
C17:0	0.26	±	0.01	0.26	±	0.01	0.762	0.25	±	0.01	0.27	±	0.01	0.163	0.25	±	0.01	0.26	±	0.01	0.443	0.731	0.986
C17:1	0.16	±	0.00	0.16	±	0.01	0.728	0.16	±	0.01	0.16	±	0.01	0.712	0.17	±	0.01	0.16	±	0.01	0.297	0.921	0.741
C18:0	6.74	±	0.09	6.54	±	0.11	0.163	6.72	±	0.12	6.75	±	0.13	0.858	6.52	±	0.12	6.85	±	0.15	0.124	0.385	0.171
C18:1t	0.18	±	0.01	0.16	±	0.01	0.142	0.18	±	0.01	0.19	±	0.01	0.366	0.17	±	0.01	0.14	±	0.01	0.297	0.668	0.371
C18:1n-9	24.06	±	0.69	25.07	±	0.79	0.462	23.64	±	0.77	24.15	±	0.86	0.669	24.45	±	1.04	24.40	±	1.24	0.782	0.976	0.928
C18:1n-7	1.81	±	0.09	2.00	±	0.10	0.121	1.88	±	0.08	1.96	±	0.09	0.585	2.33	±	0.19	2.55	±	0.22	0.563	**0.005**	0.172
C18:2n-6	28.51	±	0.80	28.95	±	0.92	0.733	30.10	±	0.96	28.78	±	1.07	0.376	27.99	±	1.14	26.43	±	1.36	0.254	0.981	0.276
C18:3n-6	0.45	±	0.02	0.41	±	0.03	0.099	0.43	±	0.03	0.38	±	0.03	0.083	0.45	±	0.03	0.35	±	0.03	**0.010**	0.961	0.552
C18:3n-3	0.31	±	0.02	0.28	±	0.02	0.661	0.29	±	0.02	0.32	±	0.02	0.218	0.29	±	0.01	0.25	±	0.01	0.068	0.969	0.607
C20:0	0.10	±	0.01	0.09	±	0.01	0.294	0.09	±	0.01	0.11	±	0.01	0.528	0.07	±	0.01	0.06	±	0.01	0.066	**0.036**	0.107
C20:1n-9	0.17	±	0.01	0.17	±	0.01	0.967	0.18	±	0.01	0.18	±	0.01	0.928	0.19	±	0.01	0.19	±	0.01	0.916	0.102	0.254
C20:2n-6	0.17	±	0.01	0.16	±	0.01	0.387	0.17	±	0.01	0.17	±	0.01	0.398	0.14	±	0.01	0.14	±	0.01	0.558	0.067	0.184
C20:3n-9	0.10	±	0.00	0.09	±	0.01	0.297	0.10	±	0.00	0.09	±	0.00	0.133	0.08	±	0.00	0.08	±	0.01	0.276	0.083	0.056
C20:3n-6	1.50	±	0.05	1.39	±	0.06	0.175	1.50	±	0.05	1.30	±	0.06	**0.022**	1.59	±	0.08	1.26	±	0.10	**0.021**	0.722	0.179
AA	6.87	±	0.24	6.64	±	0.27	0.519	7.02	±	0.24	6.29	±	0.27	**0.036**	6.91	±	0.34	6.17	±	0.41	0.217	0.999	0.629
EPA	0.71	±	0.07	0.75	±	0.08	0.733	0.70	±	0.09	0.86	±	0.10	0.252	0.77	±	0.11	0.94	±	0.13	0.154	0.880	0.093

C22:4n-6	0.17	±	0.01	0.17	±	0.01	0.574	0.17	±	0.01	0.15	±	0.01	**0.006**	0.18	±	0.01	0.14	±	0.01	**0.006**	0.970	**0.023**
C22:5n-6	0.15	±	0.01	0.14	±	0.01	0.681	0.15	±	0.01	0.31	±	0.01	**<0.001**	0.16	±	0.02	0.26	±	0.03	**0.002**	0.759	**<0.001**
C24:1	0.08	±	0.01	0.09	±	0.01	0.228	0.08	±	0.00	0.09	±	0.01	0.639	0.10	±	0.01	0.09	±	0.02	0.911	0.574	0.996
C22:5n-3	0.43	±	0.02	0.43	±	0.02	0.994	0.40	±	0.02	0.36	±	0.02	0.085	0.53	±	0.03	0.51	±	0.04	0.732	**0.005**	0.076
DHA	2.43	±	0.10	2.34	±	0.12	0.565	2.36	±	0.12	3.12	±	0.14	**<0.001**	2.30	±	0.16	3.22	±	0.19	**0.001**	0.457	**<0.001**
SFAs	29.52	±	0.36	28.70	±	0.41	0.157	28.60	±	0.44	29.27	±	0.49	0.300	29.49	±	0.52	29.61	±	0.63	0.876	0.962	0.533
MUFAs	28.29	±	0.73	29.31	±	0.84	0.451	27.71	±	0.80	28.25	±	0.90	0.634	29.09	±	1.13	29.12	±	1.35	0.837	0.824	0.987
n-6 PUFAs	37.82	±	0.81	37.86	±	0.93	0.925	39.55	±	0.99	37.36	±	1.11	0.138	37.41	±	1.23	34.75	±	1.47	0.125	0.956	0.212
n-3 PUFAs	3.87	±	0.17	3.79	±	0.19	0.566	3.75	±	0.21	4.66	±	0.23	**0.003**	3.89	±	0.25	4.92	±	0.30	**0.014**	0.899	**0.003**
n-6 LC-PUFAs	8.86	±	0.26	8.49	±	0.30	0.364	9.02	±	0.26	8.21	±	0.29	**0.034**	8.97	±	0.37	7.97	±	0.45	0.110	0.991	0.563
n-3 LC-PUFAs	3.56	±	0.17	3.52	±	0.19	0.657	3.47	±	0.21	4.34	±	0.23	**0.005**	3.60	±	0.25	4.67	±	0.29	**0.011**	0.934	**0.002**
n-6 : n-3	10.45	±	0.51	10.97	±	0.59	0.667	11.41	±	0.54	8.69	±	0.60	**0.002**	10.53	±	0.67	7.49	±	0.80	**0.006**	0.970	**0.001**
n-6 LC: n-3 LC	2.63	±	0.12	2.65	±	0.14	0.888	2.81	±	0.12	2.04	±	0.13	**0.002**	2.70	±	0.17	1.81	±	0.21	**0.006**	**<0.001**	**<0.001**

The data were analysed using the univariate general linear model, adding dietary fishy intake as a covariate. The presented values are means of the percentages from total FAs.

p-values < 0.05 (level of significance) are highlighted in bold.

5.3 BEST CORRECTED VISUAL ACUITY

At month 12, ETDRS letters had decreased with the intervention (*N* = 45; mean change −1.73, 95% CI −3.28 to −0.19), and in the control group (*N* = 48; mean change −0.10, 95% CI −2.03 to 1.83), for an estimated treatment difference between the intervention and control groups of –1.63 (95% CI –0.83 to 4.09; *p* = 0.192).

Furthermore, a comparison of visual acuity among study groups considering different covariants was also made. Although not statistically significant, the patients in the intervention group that complied with the treatment showed a tendency to improve their visual acuity (Table 11). In contrast, the patients in the control group show a progression of visual acuity loss. Comparing the control to the intervention group at month 12 of DHA supplementation, the latter group nominally improved their visual acuity.

Table 11. Comparison of visual acuity levels between study groups.

	Control				Intervention				
Visual acuity	n	Mean	±	SE	n	Mean	±	SE	*P*
0 months	40	78.922	±	1.333	26	78.074	±	1.648	0.648
12 months	20	81.380	±	1.671	20	75.483	±	1.740	0.052
% of Change		+3.11				-3.32			

Comparisons of visual acuity among study groups were determined with the General Linear Model. Values at 0 months were corrected for sex and age, while values at 12 months were corrected for sex, age and plasma levels of polyphenols, lutein and zeaxanthin. Only patients with treatment compliance were considered.

5.4 THE OMEGA-3 INDEX AND CORONARY HEART DISEASE MORTALITY RISK.

Table 12 presents the cardiovascular risk in the two study groups, from baseline to the 24-month follow-up visit. At baseline, both study groups were in the second-highest cardiovascular risk category (O3I >2.9 - 4.0). Nonetheless, by the 2-year intervention, the supplemented patients presented an O3I of 4.16%, reaching the second-best category of low risk for cardiovascular diseases (O3I >4.0 – 5.2), while the control group remained the same.

Table 12. Omega-3 Index and cardiovascular risk.

	Control				Intervention				*p*
Omega-3 Index	n	Mean	±	SE	n	Mean	±	SE	
At baseline	43	3.14	±	0.16	38	3.09	±	0.18	0.654
At 12 mo follow-up	35	3.07	±	0.20	34	3.98	±	0.22	**0.032**
At 24 mo follow-up	19	3.07	±	0.24	13	4.16	±	0.29	**0.026**
p		**0.709**				**0.009**			

Comparisons determined by the univariate general linear model with fish consumption as a covariate. Risk categories for cardiovascular diseases start from high risk to low: <2.9, >2.9-4.0, >4.0-5.2 and >5.2%.

5.5 ASSOCIATION OF DIETARY FISH INTAKE WITH DOCOSAHEXAENOIC ACID AND OMEGA-6/OMEGA-3 RATIO IN PLASMA

Table 13 shows the association of dietary fish intake with DHA and n-6: n-3 ratio in plasma at the baseline of all included patients. The dietary fish intake was positively associated with DHA in plasma, whereas the n-3: the n-6 ratio was negatively associated with it.

Table 13. Association of dietary fish intake and DHA and n-6: n-3 ratio in plasma at baseline.

Plasma FAs	Fish dietary intake n= 81	
	β	*p*
DHA	0.248	**0.033**
n-6:n-3	-0.257	**0.015**

Associations were determined using the linear regression analysis.

5.6 THE EFFECT OF VITAMIN C SUPPLEMENTS OR DIET ON THE DEVELOPMENT OF CATARACTS

While some studies generally support the association between an increased intake of supplements with vitamin C and other antioxidant nutrients with a decreased risk of cataracts, longer-term clinical trials do not tend to support this conclusion (**Table 14**), indicating that vitamin C had little or no benefit for treatment durations up to 6.5 years from studies in the last 12 years. One study even found that, at high doses, the supplementation may in fact exacerbate cataract progression.

Table 14. Human studies investigate vitamin C supplement's effect on the development of cataracts.

Study, Type	Nutrients	Population	Disease Outcome	Results
Age-related cataract in a randomized trial of vitamins E and C in men. Eight years of treatment and follow-up RCT (167).	Vitamin E 400 IU or placebo on alternate days and vitamin C 500 mg of or placebo daily	Participants: 11,545 United States male ≥ 50 years	Incidence of age-related cataract	No significant beneficial or harmful effect on the risk of cataract. HR 1.02; 95% confidence interval, 0.91–1.14
The Swedish mammography cohort study follow up. 8.2 years of follow-up Population-based, prospective cohort of women (168).	Vitamin C (approximately 1 g) Vitamin c within a multivitamin supplement (approximately 60 mg)	Participants: 24,593 Sweden female 49–83 years	Incidence of age-related cataracts	The use of vitamin C supplements may be associated with a higher risk of age-related cataract among women. The multivariable HR for vitamin C supplement vs. nonusers was 1.25 (95% CI: 1.05, 1.50). The HR for the duration of 10 y of use before baseline was 1.46 (95% CI: 0.93, 2.31). The HR for the use of multivitamins containing vitamin C was 1.09 (95% CI: 0.94, 1.25).
High-dose Supplements of Vitamins C and E, Low-Dose Multivitamins, and the Risk of Age-Related Cataract Follow-up of 8.4 years Cohort (169).	Vitamin C and vitamin E as single supplements was estimated to be 1 g and 100 mg, respectively. Multivitamins were estimated to contain 60 mg of vitamin C and 9 mg of vitamin E	Participants: 31,120 Sweden male 45–79 years	Risk of age-related cataract	Use of high-dose (but not low-dose) single vitamin C supplements increased the risk of age-related cataract. The multivariable adjusted HR for men using vitamin C supplements only was 1.21 (95% confidence interval (CI): 1.04, 1.41) in a comparison with that of non-supplement users. The HR for long-term vitamin C users (≥10 years before baseline) was 1.36 (95% CI: 1.02, 1.81). The risk of cataract with vitamin C use was stronger among older men (>65 years) (HR = 1.92, 95% CI: 1.41, 2.60) and corticosteroid users (HR = 2.11, 95% CI: 1.48, 3.02)

Randomized Control Trials (RCT), Hazard Ratio (HR), Odd Ration (OR), Hormone Replacement Therapy (HRT).

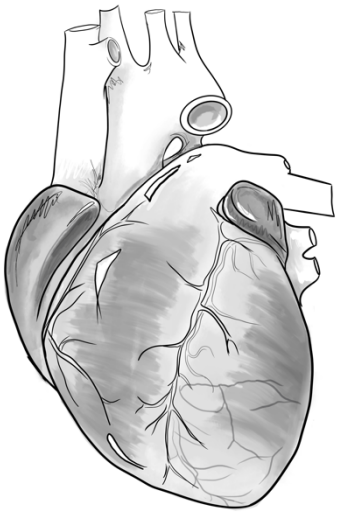
Nonetheless, outlined in **table 15,** literature within the last 12 years shows evidence that a healthy diet enriched in vitamin C was associated to the reduced risk of cataracts.

Table 15. Human studies investigating the effect of diets high in vitamin C on the development of cataracts.

Study, Type	Nutrients Studied	Population	Disease Outcome	Results
The India Study of Age-related Eye Disease (INDEYE study) a population-based study. Cross-sectional analytic study (170).	Vitamin C and inclusion of other antioxidants (lutein, zeaxanthin, retinol, **β**-carotene, and **α**-tocopherol)	Participants: 5638 North and South India Male and female ≥60 years	Incidence of cataract in the Indian setting	Vitamin C was inversely associated with cataract (adjusted OR for highest to lowest quartile = 0.61; 95% confidence interval (CI), 0.51–0.74; p = 1.1 × 10−6). Similar results were seen by type of cataract: nuclear cataract (adjusted OR 0.66; CI, 0.54–0.80; p = 0.0001), cortical cataract (adjusted OR 0.70; CI, 0.54–0.90; p = 0.002), and PSC (adjusted OR 0.58; CI, 0.45–0.74; p = 0.00003)
Healthy Diets and the Subsequent Prevalence of Nuclear Cataract in Women. Participated in the Carotenoids in Age-Related Eye Disease Study—7 years follow up (171).	Vitamin C (40 vs. 207 mg/d); vitamin E (3 vs. 11 mg/d)	Participants: 1808 United States female 50–79 years	Prevalence of nuclear cataract in women.	Adjustment of the OR for nuclear cataract among women with high vs. low HEI-95 scores, for vitamin C intake from foods attenuated the ORs (Multivariate OR (95%CI) = 0.76 (0.50–1.15), suggesting that higher vitamin C intakes partly explained the associations with HEI-95 dietary assessment. There was a significant linear trend for a protective association of vitamin C intake from foods
The European Eye Study (EUREYE study). Recruited during 1-year period. Multi-center cross-sectional population-based study (172).	Carotenoids, vitamins C (107 mg/d) and E	Participants: 599 Spain Male/female ≥ 65 years	Prevalence of cataract with fruit and vegetable intake	High daily intakes of fruit and vegetables and vitamin c were associated with a significantly decreased prevalence of cataract or cataract surgery (p for trend = 0.008). Increasing quartiles of dietary intakes from 107 mg/d of vitamin C showed a significant decreasing association with prevalence of cataract or cataract extraction (p for trend = 0.047)
Diet and cataract. Case-control study (173).	Carbohydrates carotene vitamins C and E	Participants: 314 cataract cases and 314 controls Greece Male/Female 45–85 years	Association between diet and risk of cataract in Athens	There was a protective association between cataract risk and intake of vitamin c (OR = 0.50, p \ 0.001 for cataract overall; OR = 0.55, p \ 0.001 for nuclear cataract; OR = 0.30, p\0.001 for PSC)

The European Eye Study (EUREYE), Hazard Ratio (HR), Odd Ratio (OR).

6 DISCUSSION



6. DISCUSSION

This thesis approaches one of the many ways nutrition is linked to healthy ageing. FAs are some of the nutritional components that have been extensively shown to be essential for optimal health and chronic disease prevention throughout the lifespan (174). And yet, ongoing and practical efforts are required to tackle the most prevalent disease in older adults, NCDs, which are not only the leading cause of death but also of poor well-being and quality of life of our population (175). Aiming to contribute to healthy ageing by developing feasible nutritional strategies, this research shows:

1. A report of the FA status in the older population diagnosed with AMD.
2. The impact of a DHA supplementation in their FA proportions in plasma, visual acuity and CHD mortality risk using the O3I.
3. The evidence regarding the use of the antioxidant vitamin C as a nutritional strategy for the improvement of cataracts.

Concerning the PUFAs, our population evidenced a poor n-6:n-3 ratio and O3I. As already known, PUFAs are the FAs that have extensively proven to be associated with health outcomes with a recommendation of a balanced n-6:n-3 ratio of 1-2:1 for the prevention of chronic diseases and optimal health (176). Before the supplementation, our population presented an n-6:n-3 ratio in plasma of 10:1, which is considerably higher compared to the general recommendation of 1-2:1. Nonetheless, after the daily supplementation with 400 mg of DHA, our population presented an improved n-6:n-3 ratio (7.49:1) reflecting also a general and positive influence over the rest of FAs in the n-6 and the n-3 series, for instance increasing the anti-inflammatory DHA and nominally reducing the proportion of the proinflammatory AA.

The O3I is interpreted according to four CHD mortality risk categories, starting from high risk to low: <2.9, >2.9-4.0, >4.0-5.2 and >5.2%(177). Similar to the n-6:n-3 ratio, our population at baseline showed a poor O3I falling into the second highest category of CHD mortality risk (O3I >2.9-4.0%). This was not surprising since previous studies have shown that the population has a mean O3I of high or intermediate CHD mortality risk (178–180). Also, in line with the n-6:n-3 ratio, over the course of the DHA supplementation, the intervention group

gradually improved the O3I, reaching 4.16%, allocating them to a lower risk category (O3I >4.0-5.2%).

In general, at baseline, the older adults of our study did not show n-6 and n-3 proportions that favour protection against NCDs; however, those proportions were improved concerning NCDs prevention with the 400 mg/day DHA supplementation for 12 and 24 months. Dietary intake is one of the influencing factors in NCDs, the less healthy the diet, the more exposure to fragility, CHD, AMD and other NCDs (181). As confirmed in our study, dietary intake, such as fish consumption, is a possible and feasible recommendation to improve the n-6:n-3 ratio and the proportions of the n-3 FAs, such as DHA, which compose the O3I and are related to preventing the development or progression of conditions such as cardiovascular and eye disease (178,182).

The DHA source of the intervention group came from the algae *Schizochytrium sp.* Taking into account that within the composition of the algae *Schizochytrium sp.,* it should be noted that apart from containing large amounts of DHA, it is also rich in C22:5n6 (183). As a result, our population showed an increase in the C22:5n6 as well.

DHA has been widely implicated in healthy ageing. Benefits in multiple patient outcomes have been seen by improving function and clinical courses, such as cognitive health, mass muscle decline, cancer treatment, surgical patients, and critical illness (184). Overall, n-3 LC-PUFAs reduce the inflammatory response by competing against the n-6 AA that mainly activates eicosanoids of the pro-inflammatory series (185). Additionally, FAs are an essential structural component of cell membranes, which impacts their physicochemical properties, naturally influencing organ functions (186).

In our population study, SFAs and MUFAs did not have significant changes between groups nor in the evolution from baseline to 12 months. Dietary SFAs are mainly provided by animal fats and some tropical oils, such as palm, coconut and peanut oil. The most predominant SFA in the diet is palmitic (C16:0), followed by stearic (C18:0) and myristic acid (C14:0) (187). In human health, there is strong evidence that the even-numbered SFAs raise total and low-density lipoprotein (LDL) cholesterol concentrations. Also, there is some evidence that SFAs increase coagulation, inflammation, and insulin resistance.

Consequently, high consumption of these FAs is associated with a higher risk of CVD and type 2 diabetes. Regarding the MUFAs, oleic (C18:1n9) and palmitoleic (C16:1) are the most common FA in diet, both derived from animal and vegetable oils. These FAs, along with the major dietary n-6 PUFA linoleic acid (C18:2), can help lower LDL cholesterol concentrations when they replace dietary SFAs. This seems to be associated with lower cardiovascular risk (187).

Compared with a study performed in a population of healthy adults and adults diagnosed with Behcet Disease (a systematic inflammatory disease), our population showed SFAs more similar to those of patients with Behcet Disease, who showed higher proportions than the healthy adults. In vitro studies have reported that SFA, mainly palmitic acid, activate inflammatory signalling, including the Nuclear Factor κB pathway and enhances the synthesis of the inflammatory cytokines in the vasculature and leukocytes. Moreover, SFAs may modulate cytokines production through epigenetic modifications of their DNA promoter. More data related to inflammatory disease suggests that SFA are associated with chronic inflammatory conditions such as Crohn's disease and psoriasis (188). These findings in the literature could indicate the risk to our population, given their presumably high proportions of SFAs.

MUFAs in several studies reported inverse associations or no relation between cardiovascular risk and some inflammatory diseases (189). Our population shows apparent similar levels of MUFAs to those found in healthy adults and adults with insulin sensitivity who participated in a study that found no differences in MUFA proportions among groups. At first, the study indicated that the replacement of dietary SFAs with MUFAs as a result of a dietary intervention did not result in a significant improvement in insulin sensitivity (190). After a second assessment contemplating how changes in the proportion of individual FAs and subclasses may relate to changes in insulin sensitivity, the patients who consumed a diet high in MUFAs showed an improved balance comprising MUFAS (most notably oleic acid) and a modest improvement in insulin sensitivity—suggesting a potential advantage of increasing the proportion of dietary MUFAs on insulin sensitivity (191). This could suggest that our population shows average proportions of MUFAs compared to healthy adults or adults with

insulin sensitivity. However, an increased dietary intake of MUFAs could still optimise the proportions of those FAs.

One of the main objectives of the present study was to assess the impact of DHA supplementation on the visual acuity of the participants with previous AMD. A first analysis was made from baseline to a year evaluating BCVA using the ETDRS letters between the study groups. The impact was not significant, indicating that for patients with unilateral wet age-related macular degeneration, treatment with the supplemented AREDS formulation at doses approved in Europe (supplemented with DHA, lutein, zeaxanthin, resveratrol, and hydroxytyrosol) has no significant effect on visual acuity compared with the original AREDS formulation. Later, a second analysis was performed to explore results considering different factors to only study the impact of the supplemented DHA: only participants with treatment compliance were included, and correction for potential confounders such as sex, age, and plasma levels of DHA, resveratrol, hydroxytyrosol, lutein, and zeaxanthin was performed. As the results showed, after one year of supplementation, the visual acuity of the supplemented group tended to improve. While it was not a statistically significant improvement, the control group exhibited the opposite behaviour progressing the visual acuity loss, meaning that the DHA supplementation might significantly lower the progression of the visual loss. Even though the results were not statistically significant, they are worth studying and, importantly, the impact on the quality of life of people with AMD.

Further analyses are needed to establish the specific role of DHA and the other compounds in the supplements. The results add to the evidence of previous studies with a positive impact. A recent prospective, double-blind, placebo-controlled study in Italy in 2019 reported that the intervention group had a better outcome in visual acuity after a two-year supplementation with n-3 and a mixture of carotenoids, vitamins, or placebo (77). Another study supporting the role of n-3 FAs, this time assessing dietary oily fish, was in the NAT2 study (192).

After reviewing the role of vitamin C in cataracts through clinical studies from the last 12 years, different results were found depending on whether the source of vitamin C was from a supplement or the diet. In general, clinical trials, by large, have failed to show convincing

beneficial effects of vitamin C supplementation on cataract incidence, except in those cases in which patients may have had low vitamin C levels to begin with. Unfortunately, only a few studies included plasma vitamin C levels. There is no basis for supplementing with high doses since vitamin C over 250 mg/day is excreted in the urine.

Christen et al. (193) reported on the first randomised trial the individual effect of vitamin C supplementation on preventing cataracts. In this study, daily supplementation of 500 mg of vitamin C in healthy US male physicians 50 years or older revealed no effect on the risk of cataract or cataract extraction after eight years of treatment. Whereas the second study concerning findings from the Swedish Mammography Cohort of women aged 49–83 years showed that vitamin C supplementation for longer than ten years was associated with a 25% increase in the risk of cataract extraction. Among women 60 years and older, supplementation with vitamin C was associated with a 38% increased risk of cataract extraction (194). Then, in the third study, a follow-up study, the risk of age-related cataracts was investigated in Swedish men and revealed that the use of multiple supplements in combination with vitamin C was not associated with cataract risk but that the use of a high dose of vitamin C may increase the risk of cataract (195). While these supplements are taken for various health reasons, based on findings from the above clinical trials, the long-term value of vitamin C supplementation in decreasing the risk of cataract progression is questionable. It may exacerbate cataract progression at high doses.

Instead, the studies with the intake of vitamin C through the diet showed a better scenario for this antioxidant. The consensus of studies evaluating a well-balanced diet rich in fruit and vegetables suggests that intake via a healthy diet enriched with vitamin C is a more optimal approach towards slowing down the progression of age-related cataracts. The literature search within the last 12 years found studies supporting evidence for a healthy diet enriched in vitamin C and the reduced risk of cataracts. In the India Study of Age-related Eye Disease (INDEYE), which examined the association between vitamin C and age-related cataracts in the Indian population, plasma vitamin C and dietary vitamin C were inversely associated with cataracts. The authors highlighted that this strong association between vitamin C and cataracts in a vitamin C-depleted population might partly explain the high levels of cataracts in India (196). In the United States, a study assessing the association between healthy diet scores and the

prevalence of nuclear cataracts in women showed that having a high healthy eating index score was the strongest modifiable predictor of the low prevalence of nuclear cataracts. Women with higher healthy eating index scores had higher vitamin C intakes than those with lower scores, with a trend for a protective association of vitamin C intake from foods alone but not from a combination of foods and supplements, suggesting that vitamin C–containing foods rather than vitamin C itself may afford protection from nuclear cataract (197). Also, the European Eye Study (EUREYE) study investigated the relationship between cataracts, fruit and vegetable intake, and dietary and blood levels of carotenoids plus vitamins C and E in a Spanish population. High daily intakes of fruit and vegetables and vitamins C and E were associated with a significantly decreased prevalence of cataracts or cataract surgery. For instance, daily dietary vitamin C intakes above 107 mg were inversely associated with reduced odds of cataracts (198). And lastly, Theodoropoulou and colleagues conducted a case-control study to assess the association between diet and the risk of cataracts in Greece. The results showed a protective association between cataract risk and intake of vitamins C, E and carotene. An increase of 185 mg of vitamin C intake/day reduced, at least by half, the risk of cataracts overall, as well as nuclear and posterior subcapsular cataracts (199).

While it seems clear that a healthy diet rich in fruits and vegetables and healthy lifestyles can help reduce the risk factors for age-related cataracts, it is estimated that over 2 billion people do not have regular access to safe, nutritious, and sufficient food. Therefore, further work is still required to find alternatives to delay the cataract epidemic caused by our increasing ageing and diabetic population.

Knowing the current health status of our population can help identify and develop preventive strategies to contribute to better lives. This thesis, voting for nutrition as a critical player in NCDs prevention and healthy ageing, contributes with information on the implication of FAs in visual acuity and CHD mortality risk before, during and after a DHA supplementation, in older adults. We acknowledge and identify limitations, extending from the sample size to dropouts, treatment compliance and the lack of complete dietary, lifestyle, and subjective data of participants. Although risk factors, such as socio-demographic information and diet, allowed us to adjust our statistical models for potential confounders, we cannot rule out residual confounding, given the complexity and nature of this data collection.

7 CONCLUSIONS

7. CONCLUSIONS

The results of this thesis endorse the role of nutrition in healthy ageing and can be concluded in the following sentences:

- Docosahexaenoic acid supplementation improved the balance between omega-6 and omega-3 fatty acids, reflecting a benefit in individual fatty acids, such as an increase in the anti-inflammatory docosahexaenoic acid and a decrease in the proinflammatory arachidonic acid.
- The current fatty acid status of our study population is poor regarding the general recommendation to balance omega-6 and omega-3 proportions, evidencing the need to optimise dietary intake of such.
- In these patients, although there were no significant differences in terms of visual acuity, in the group with docosahexaenoic acid supplementation it was possible to observe a tendency to delay the progression of the loss of visual acuity after 1 year.
- The current coronary heart disease mortality risk of the older population between the study groups is high according to the omega-3 index and can be decreased with docosahexaenoic acid supplementation.
- Clinical trials have failed to show convincing beneficial effects of vitamin C supplementation on cataract incidence, except in those cases where patients may have had low vitamin C levels, to begin with.
- A healthy diet rich in fruits and vegetables helps reduce the risk factors for age-related cataracts.
- Docosahexaenoic acid supplementation is confirmed to influence the omega fatty acid plasma status, increasing docosahexaenoic acid and balancing n-6 and n-3 proportions, emphasising the importance and need to reinforce dietary recommendations for health and disease prevention.

This research supports the need to promote and indeed implement the frequently overlooked preventive healthcare and shows the potential of docosahexaenoic acid and vitamin C dietary recommendations in culminating in an actionable, tangible, and feasible small step towards healthy ageing and non-communicable diseases prevention.

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9 APPENDIX

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Review

Vitamin C and the Lens: New Insights into Delaying the Onset of Cataract

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Abstract: Cataracts or clouding of the lens is the leading cause of blindness in the world. Age and diabetes are major risk factors, and with an increasing aging and diabetic population, the burden of cataracts will grow. Cataract surgery is an effective way to restore vision; however, alternatives to cataract surgery are required to reduce the looming cataract epidemic. Since it is well established that oxidative damage plays a major role in the etiology of cataracts, antioxidants have been promoted as therapies to delay and/or prevent cataracts. However, many antioxidant interventions including vitamin C have produced mixed results as anti-cataract therapies. Progress has been made towards our understanding of lens physiology and the mechanisms involved in the delivery and uptake of antioxidants to the lens which may guide future studies aimed at addressing some of the inconsistencies seen in previous animal and human studies. Of interest is the potential for vitamin C based supplements in delaying the onset of cataracts post vitrectomy which occurs in up to 80% of patients within two years. These targeted approaches are required to reduce the burden of cataract on hospitals and improve the quality of life of our aging and diabetic population.

Keywords: vitamin C; lens; cataract; oxidative stress; vitreous humor; vitrectomy

1. Introduction

With an aging and diabetic population, the number of individuals with major eye diseases is increasing, and vision loss in the elderly is projected to be a major public health problem. Cataract or the clouding of the lens is the leading cause of blindness and is responsible for 51% of global blindness [1]. Age is a major risk factor for cataracts [2,3], with the disease progressing gradually, appearing first in the fourth or fifth decade, but not affecting vision until typically the sixth decade. Diabetes is another risk factor, with diabetic patients 2–5 times more at risk for developing cataracts and at an earlier age [4]. The only available treatment for cataract is surgery. This involves replacement of the cataractous lens with an artificial plastic lens which effectively restores sight. However, insufficient surgical facilities in poor and developing countries, and long waiting lists in developed countries, means that alternatives to cataract surgery are required. It has been calculated that delaying the onset of cataract by 10 years would halve its incidence, and therefore reduce the need for, and cost associated with, cataract surgery [5]. Because of the proven association between lens cataract and oxidative damage, antioxidant supplementation has been promoted as a treatment strategy to slow the progression of cataract [6–8]. However, antioxidant supplementation has proven to be largely ineffective as an anti-cataract therapy.

Vitamin C (also known as L-ascorbate or L-ascorbic acid) is present in the lens and surrounding ocular humors, which bathe the lens at a concentration 50-fold higher than that found in plasma [9,10].

It acts as a physiological "sunscreen" to protect the lens from UV (ultraviolet light) induced oxidative damage, and to regenerate vitamin E and glutathione to further increase antioxidant capacity. With advancing age, vitamin C levels in the lens decrease and a decrease in vitamin C in the lens is associated with increasing cataract severity [11]. Consumption of additional dietary vitamin C can increase the concentration of vitamin C in the lens [12], and there is evidence that the incidence of cataract may be higher in persons who have a low plasma concentration of vitamin C [12]. This indicates that vitamin C supplementation may help to replenish and restore vitamin C levels as we age to protect against cataract.

The purpose of this review is to consolidate animal and more recent epidemiological studies to determine future areas of research that could provide more clarity about the role of vitamin C in the lens. By combining our current understanding of lens structure and physiology and the delivery and uptake of antioxidants and nutrients to the different region of the lens [13,14], this review provides new areas of research which can be used to re-evaluate and re-design nutrition based studies. The latter should help provide a more clear and consistent view on whether vitamin C supplementation is beneficial to the lens and whether it affords protective against specific types of cataract. Of particular interest is the potential of vitamin C supplementation to prevent cataract following vitrectomy surgery. Vitrectomy-patients have a high chance of developing cataracts within two years post-vitrectomy [15], providing a unique window with which to test nutritional strategies without many of the variables encountered when studying populations over long periods of time. Hence, an enhanced knowledge on vitamin C pathways in the eye will be key to the design of targeted nutritional strategies aimed at reducing the onset of cataract to avoid the looming cataract epidemic.

2. The Cataract Epidemic

Cataracts are the leading cause of blindness accounting for 51% of global blindness [1]. Given our globally aging population, the social and economic costs of cataract are quite staggering and the demand for cataract surgery far exceeds limited public health resources. In 2010, there were 10.8 million cataract blind people [16], with this number expected to increase to 40 million in 2025 as the population grows and ages, with greater life expectancies [17]. In many countries, cataract surgery remains one of the most commonly performed procedures, with ~8 million cataract operations performed each year worldwide with an additional ~10 million people added to a backlogged system because of the lack of appropriate cataract surgery services in the areas of need [18]. Although the majority of cataracts are due to the aging process [3,19], children can be born with the condition as a result of an inherited genetic condition, or a cataract may develop as a result of a medical condition such as diabetes, other eye diseases, injuries [20], or past eye surgery such as vitrectomy [21].

3. Aetiology of the Different Types of Cataract

Cataracts can form in different parts of the lens with three main type of cataracts classified according to the location in which the cataract first forms; cortical cataract which manifests as an opacity in the peripheral edges of the lens, and is highest amongst diabetic patients (Figure 1A) [22,23], nuclear cataract where the cataract first occurs in the nucleus, or centre of the lens, and is typically associated with aging (Figure 1B) [24], and posterior subcapsular cataract, which forms in the back of the lens, and is often associated with the use of certain medications, including corticosteroids and diabetes medications (Figure 1C) [25,26]. In addition, patients can present with opacity in more than one area of the lens which can cause overlap in the classification of cataracts.

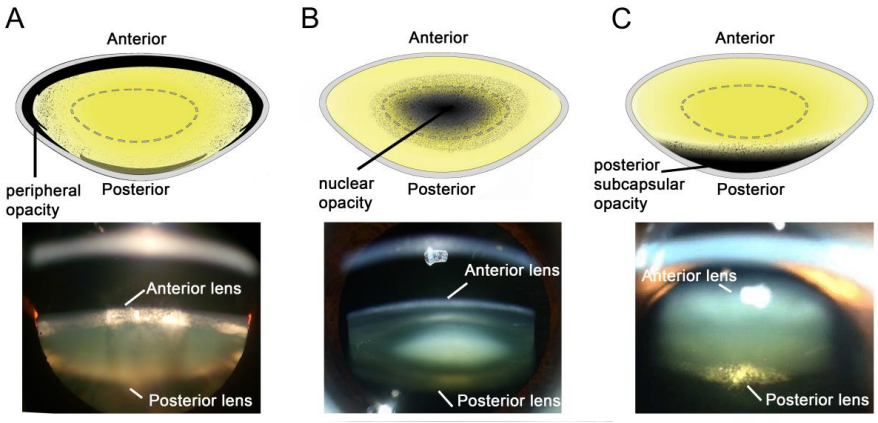

Figure 1. Location of cataract subtypes. Schematic diagrams and scheimpflug slit-lamp photographic images showing the three main types of cataract: (**A**) cortical, (**B**) nuclear, and (**C**) posterior subcapsular (PSC). Source: (**A**) From Uspal NG, Schapiro ES (February 2011). Cataracts as the initial manifestation of type 1 diabetes mellitus. Pediatric Emergency Care. 27 (2): 132–4. Attribution-ShareAlike 4.0 International (CC BY-SA 4.0). (**B**) Ophthalmic Atlas Images by EyeRounds.org, The University of Iowa licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 3.0 Unported License. (**C**) From Chaudhary M, Shah DN, Chaudhary, RP. Scleritis and Takayasu's disease. Nepal J Ophthalmol 2017; Vol 9 (18): 170–174. Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0).

3.1. Diabetic Cortical Cataracts

Diabetes leads to various complications including cataracts (Figure 2A) and with an increasing global prevalence of diabetes, the incidence of cataract formation is rising. Diabetic patients are more likely to get cataracts at an earlier age [27] with cataracts progressing faster in diabetics compared to non-diabetics [28]. The pathogenesis of diabetic cataract is attributed to the accumulation of the impermeable osmolyte, sorbitol, produced from excess glucose by the enzyme aldose reductase (AR), initiating osmotic stress [29,30]. This results in fluid accumulation, lens fibre cell swelling, and tissue liquefaction [29,30]. More recent evidence suggests that hyperglycaemia results in increased polyol activity which generates osmotic and oxidative stress in the diabetic lens [31]. This offers an explanation for the slow development of cataracts that is typically seen in the majority of adult diabetic patients [32]. While initially hyperglycaemia results in osmotic stress, the lens is able to regulate its volume through osmoregulatory mechanism that can accommodate small changes in osmotic pressure [33]. Over time however, the ability of the lens to actively regulate its volume becomes impaired [32] due to oxidative damage to the pathways that regulate fibre cell volume resulting in the localised zone of tissue liquefaction observed in diabetic cortical cataract. However, it should be noted that the association between aldose reductase, osmotic stress, oxidative stress, while very strong in the rat because of the high aldose reductase levels, is not supported by the various clinical trials with aldose reductase inhibitors. This is because while rat lenses have high levels of aldose reductase activity and low levels of sorbitol dehydrogenase activity [34,35], human lenses exhibit low aldose reductase activity and high sorbitol dehydrogenase activity [34]. As a result, using appropriate animal models of diabetic cataract that are translatable to human lenses will be important in identifying additional pathways that contribute to cataract formation [36].

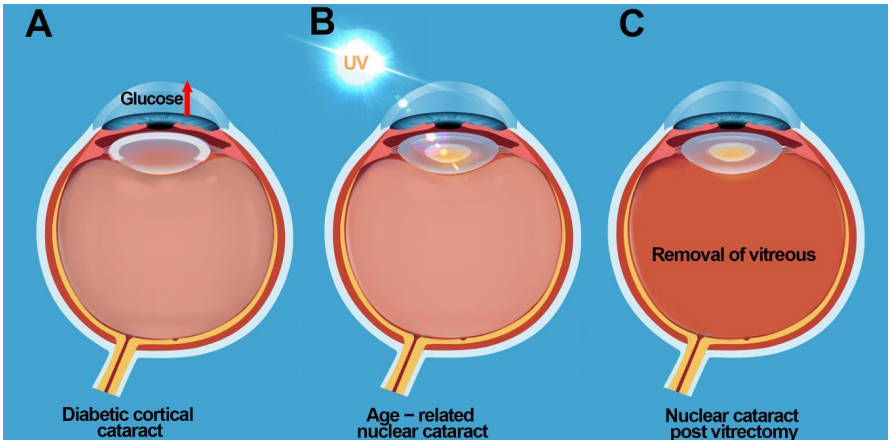

Figure 2. Schematic of the eye showing the development of (**A**) diabetic cortical cataract due to elevated levels of glucose, (**B**) age-related nuclear cataract due to UV exposure, and (**C**) nuclear cataract post vitrectomy due to depletion of vitamin C and elevated PO_2 levels.

3.2. Nuclear Cataracts

The most common form of cataract is age related nuclear cataract (Figure 2B) and is responsible for 50% to 90% of cataracts in developing countries [1,3]. The pathogenesis of age-related nuclear cataract is largely attributed to the chronic exposure of the lens to molecular oxygen, resulting in oxidative damage to proteins in the lens nucleus, protein aggregation, light scattering, and ultimately loss of lens transparency [37–42]. Under normal physiological conditions, the lens exists in a relatively low oxygen environment, with a partial pressure of oxygen <10 mm Hg around the lens [9,21,43]. Low oxygen environment together with high concentrations of vitamin C in the aqueous and vitreous humors [10,44] and high levels of glutathione (GSH) [45] and vitamin C [10] in the lens, ensures protection of the lens against oxidative stress. While vitamin C levels are known to decrease with age in the lens [11], it is unknown in which region of the lens vitamin C depletion initially occurs. However, with increasing age, GSH is known to decrease specifically in the lens nucleus [46], rendering proteins in this region susceptible to oxidative damage. Because of the proven association between lens cataract and oxidative damage, antioxidant supplementation has been promoted as a treatment strategy to slow down the progression of this type of cataract [6–8].

Nuclear cataracts also occur as a secondary consequence of previous ocular surgery such as vitrectomy (Figure 2C). Vitrectomy is a procedure in which the vitreous humor at the back of the eye is removed. Vitrectomy procedures are often done to allow surgeons access to the back of the eye, during operations for retinal conditions, or to drain vitreous fluid filled with blood (common in a person with diabetes), floaters, or clumps of tissue that would obscure vision. While vitrectomy may help to repair damaged or scarred retina or clear the vitreous of debris, studies report that vitrectomy causes rapid progression of nuclear cataracts resulting in the need for cataract surgery in 60–95% of patients within two years [47–51]. As a result, patients who have had to endure the anxiety and stress associated with vitrectomy, are now faced with the prospect of additional surgeries for treatment of cataract.

The molecular mechanisms of cataract formation post vitrectomy was elucidated by David Beebe and colleagues who showed that this was linked to depletion of vitamin C in the vitreous and loss of the tightly managed oxygen gradient [9,52–54]. Under physiological conditions, oxygen enters the eye by diffusion from the retinal vasculature and through the cornea. The lens consumes oxygen to maintain its hypoxic state, while simultaneously, the vitreous humor consumes oxygen through vitamin C. In the vitreous chamber between the retina and the lens there is a decreasing gradient of oxygen,

with the partial pressure of oxygen (PO_2) ranging from 22 mmHg close to the retina and ~9 mmHg close to the lens [55]. However, vitrectomy disrupts this oxygen gradient, and without the constraints of a gel-like vitreous humour, oxygen is able to freely mix though the vitreous chamber resulting in consumption of vitamin C and elevation of oxygen tension levels to ~14 mmHg near the lens [9,55,56]. These abnormally high levels of oxygen persist over many months after the initial surgery [21] and over time lead to elevated oxidative stress in the lens and the formation of nuclear cataracts.

In all three types of cataract described above, it is clear that oxidative stress plays a major role in cataract formation. Vitamin C plays a critical role in consuming oxygen and maintaining low levels of oxygen within the eye, suggesting that replenishing vitamin C in the lens and vitreous is a viable strategy for minimizing oxidative stress and reducing the risk of cataract formation. In the next section, we will describe the roles and biochemical properties of vitamin C in the lens before reviewing a selection of animal studies and human intervention studies investigating the ability of vitamin C supplementation to reduce the risk of cataract.

4. Roles of Vitamin C in the Eye

In humans, high concentrations of vitamin C exist in the aqueous and vitreous humor exceeding plasma concentrations by as much as 20- to 70-fold [9]. Interestingly, vitamin C levels in the ocular humors are quite different between nocturnal and diurnal animals with vitamin C levels much higher in the ocular humors of humans compared to rats [57]. This has led to the suggestion that vitamin C may play a protective role in those animals who are most exposed to light [57]. In humans, the high concentrations of vitamin C in the aqueous humor, together with its ability to absorb UV light, have led to its referral as a physiological "sunscreen" [58], preventing the penetration of UV light and photo-induced oxidative damage to tissues. Vitamin C is effective in scavenging or quenching the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and reactive nitrogen oxide [59], with several studies reporting that vitamin C in the aqueous humor acts to protect the cornea, lens, and other ocular tissues against oxidative damage [60–63]. Vitamin C also protects the reducing powers of other antioxidants such as α-tocopherol (vitamin E) by rescuing α-tocopheryl radicals in membranes [64]. In the lens, vitamin C has been shown to play a role in prevention of membrane lipid peroxidation [65] and in protection against light induced oxidative damage to the Na^+K^+-ATPase pump [63].

5. Biochemical Properties of Vitamin C

Vitamin C's antioxidant properties are due to its ability to donate electrons to free radicals from both the second and third carbon and quench their reactivity [66]. Most animals are able to synthesize vitamin C endogenously. The exceptions are humans, guinea pigs, some fish, birds, and insects [67]. In humans, the conversion of l-gulono-γ-lactone into vitamin C, which is catalyzed by the enzyme gulonolactone oxidase is not functional, due to the accumulation of several mutations that has turned the gene into a non-functional pseudogene [68], meaning that humans must rely on dietary intake of vitamin C. In the process of detoxifying reactive oxygen species, vitamin C becomes oxidized to dehydroascorbate (DHA). However, DHA can be reduced back to vitamin C to regenerate vitamin C pools either via glutathione-dependent enzymes or nonenzymatically using low molecular weight antioxidants such as glutathione or cysteine (Figure 3). In the presence of continued oxidative stress, DHA undergoes irreversible degradation to diketogulonic acid which is implicated in the modification and crosslinking of lens proteins [69] (Figure 3). Under pathological conditions or at high doses, vitamin C in the presence of redox-active ions such as iron or copper, can act as a pro-oxidant contributing to the formation of hydroxy radicals via the Fenton reaction (Figure 3) that can lead to significant oxidative damage [70]. This means that vitamin C can switch from being an antioxidant under physiological conditions to a pro-oxidant under pathological conditions.

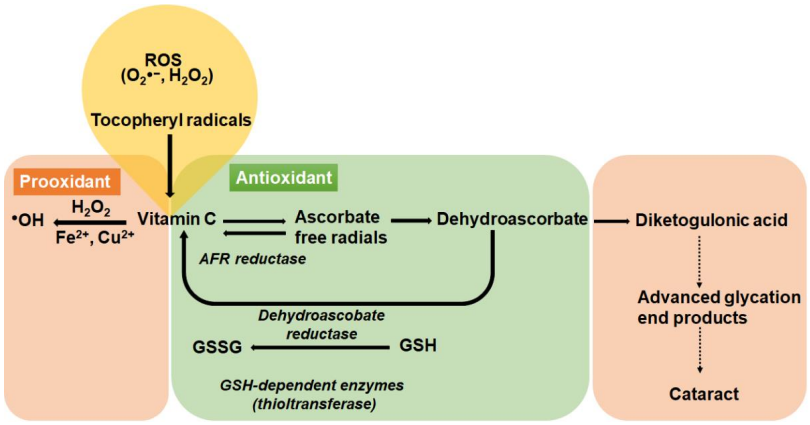

Figure 3. The redox pair vitamin C/DHA. The antioxidant vitamin C is oxidised first to the ascorbate radical and then to DHA which are both reversible reactions due to the enzymes AFR reductase and DHA reductase which relies on GSH as a co-factor. With continued oxidative stress, DHA undergoes an irreversible rearrangement to diketogulonic acid which is linked to accelerated protein cross linking and cataract formation. Vitamin C can also act as a pro-oxidant by reducing metal ions that generate free radicals through the Fenton reaction.

6. Transport of Vitamin C into the Ocular Humors

Early in development, the embryonic human lens is nourished by an external blood supply known as the tunica vasculosa lentis, which is transient, and regresses during the course of development, so that by the fetal period the lens is avascular [71]. While this loss of vasculature is essential for ensuring that light is not absorbed by haem pigments [72], it means that the lens is reliant on the aqueous humor for its nutrients and antioxidants [73].

The aqueous humor is continuously formed from plasma (~2.5 µL/min in humans) and is secreted by the ciliary epithelium. This double layer of epithelium is composed of a pigmented epithelium (PE), which interfaces with the highly vascularized stromal tissue that contains fenestrated capillaries, and a non-pigmented epithelium (NPE) which interfaces with the aqueous humor [73]. The PE and NPE are joined at their apical membranes by gap junctions which forms a functional subunit for aqueous humor section. Aqueous humor formation is driven by chloride (Cl^-) secretion mediated by the PE-NPE pair and involves stromal Cl^- entry into PE cells, diffusion through gap junctions and NPE cell secretion of Cl^- into the anterior chamber of the eye [73].

In the human ciliary epithelium, vitamin C uptake from the stroma is mediated by the Na^+ dependent vitamin C transporter, SVCT2 [74] which was shown to be expressed in the PE [13]. From the PE layer, vitamin C is proposed to diffuse via gap junctions to the NPE (Figure 4A). However, it is unknown how vitamin C in the NPE is transported into the anterior chamber, suggesting unidentified active transporters must be involved. DHA can also be secreted by the NPE cells given that the facilitative glucose transporter GLUT1, the major transporter of DHA (and glucose), is expressed in the NPE layer [13]. However, it has been suggested that the concentration gradient for DHA would most likely suggest that the function of GLUT1 in NPE cells is for the local recycling of DHA back to vitamin C in which DHA is taken up from the anterior chamber into the ciliary epithelium where it is regenerated to vitamin C and then secreted back into the aqueous. In other animals, it should be noted that vitamin C transporter expression is different [13]. In the mouse eye, there is an absence of SVCT2 in the ciliary epithelium, but high expression of SVCT2 in the retina suggesting that the retina is the more likely source of vitamin C in the vitreous for nocturnal animals [13]. This may explain the lower levels of vitamin C in the aqueous humor in mice compared with diurnal species such as humans.

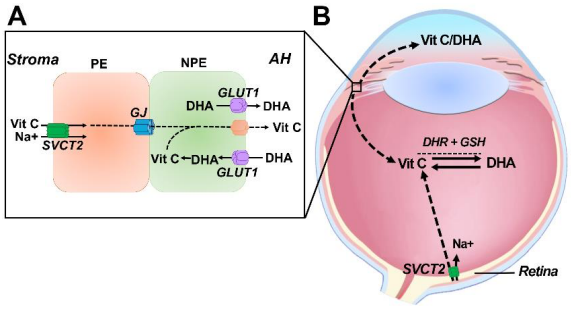

Figure 4. Molecular mechanisms involved in the secretion of vitamin C and DHA by the ciliary epithelium into the ocular humors. (**A**). Located in the apical surface of the PE cell which interfaces with the stroma microvasculature is SVCT2 which transports vitamin C resulting in the high accumulation of vitamin C in the PE. Vitamin C then moves via passive diffusion though a gap junction medicated pathway into the NPE where it then is transported out of the NPE by an unidentified mechanism into the aqueous humor (AH). Depending on the concentration gradient for DHA, GLUT1 which is located in the apical surface of the NPE cells, can either be used to transport DHA from the NPE into the AH, or DHA from the AH can be taken up into the NPE and recycled back to vitamin C. (**B**) Vitamin C is secreted into the aqueous humor and the vitreous humor. Transport of vitamin C via SVCT2 expressed in retinal pigment epithelial cells and active regeneration of vitamin C from DHA are complementary mechanisms most likely used to sustain high levels of vitamin C in the vitreous. PE-pigmented epithelium; NPE-non pigmented epithelium.

In humans, vitamin C levels are even higher in the vitreous humor compared to the aqueous humor. While the ciliary body is likely to be a source of vitamin C in the vitreous, other mechanisms must be required to maintain and sustain high levels of vitamin C in the vitreous. The strong expression of SVCT2 in the human retinal pigmented epithelium and other layers of the retina [13] may indicate that in addition to the ciliary epithelium the retina may serve as a source for vitamin C in the vitreous gel (Figure 4B). Recently, human donor lenses have been shown to export GSH from its posterior surface suggesting that this source of GSH could be used to recycle DHA back to vitamin C [75]

7. Delivery and Uptake of Vitamin C and DHA into the Lens

Uptake of vitamin C in the lens occurs by transport of both vitamin C and DHA (Figure 5). In human epithelial cells, vitamin C uptake was Na^+-dependent with molecular analysis revealing SVCT2 to be the likely transporter involved [76]. SVCT2 gene expression is upregulated in response to oxidants suggesting that vitamin C uptake can increase under oxidative stress conditions [76]. The water channel AQP0 may also be involved in the permeation of vitamin C into lens cortical fiber cells [77] since its expression was increased in diabetic rats and upon vitamin C treatment [78].

Although DHA in the aqueous humor constitutes only about 10% of total vitamin C content, it is DHA which appears to be preferentially transported into the lens and then recycled back to vitamin C [79]. This accumulation is mediated by facilitative glucose transporters of which GLUT1 has been shown to expressed in the epithelium and fiber cells of human donor lenses [80]. While the lens is presumably able to source vitamin C and/or DHA from vitreous humor (Figure 5), it is unknown whether SVCT2 and GLUT1 are expressed at the posterior surface of the lens in order to facilitate this.

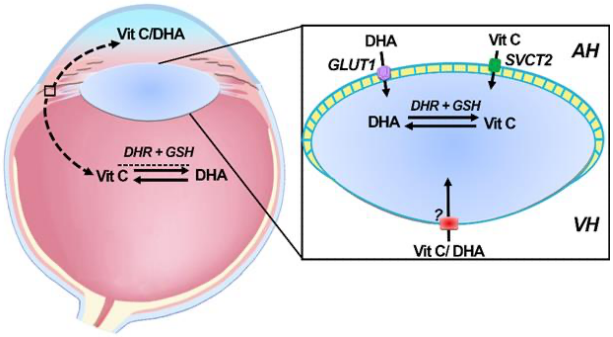

Figure 5. Vitamin C uptake pathways in the lens. In human lenses, SVCT2 localized to the lens epithelium is used to accumulate vitamin C from the aqueous humor (AH), while GLUT1 expressed in the epithelium and cortical fiber cells can function to uptake DHA from the aqueous humor. Whether vitamin C and/or DHA in the vitreous humor (VH) can be taken up from the posterior side of the lens is unknown.

8. Evidence of the Effects of Vitamin C on Cataract Prevention

Given the protective effects of vitamin C in the lens and the link between a decrease in vitamin C with increasing age and with increasing cataract severity [11], it is not surprising that numerous studies exist investigating the relationship between vitamin C and the risk of cataract. While there a number of excellent in depth reviews on vitamin supplementation, diet, and cataract in human populations [7,8,81–85], reviews summarizing the findings from animal studies are lacking. However, these studies are important as they should be used to help inform and guide the design of human therapeutic studies.

8.1. Animal Studies

The evidence of vitamin C as an anticataract agent in animal studies has remained elusive and difficult to prove. In reviewing the literature, there did not appear to be a consistent approach towards studying the efficacy of vitamin C on the lens. For example, there were differences in species selection and the use of nocturnal versus diurnal animals (rodents versus guinea pigs), the method of cataract induction (UV exposure, selenite, buthionine sulfoximine), the type of cataract induced (nuclear vs. cortical cataract), and different set of parameters that were used to assess the ability of vitamin C to protect against cataract (see Table 1). In this section, we provide a selective summary of animal studies in order to demonstrate the type of studies that have been conducted on the lens, and to reflect on how we can develop a more consistent approach that utilizes a standard set of parameters to test the efficacy of vitamin C in appropriate animal models that best mimic the cataract process observed in humans.

Table 1. Summary of animal studies and the effects of dietary vitamin C supplementation or depletion of vitamin C on the lens.

	Species	Method of Cataract Induction	Type of Cataract	Vitamin C Elevation or Depletion	Parameters Measured	Outcome	Ref
In vitro studies	Rats Wistar/NIN inbred strain (3 months old)	Irradiation of lenses at 300 nm for 24 h	No lens opacification	Lenses irradiated in media containing 2 mM ascorbic acid or 2 μM α-tocopherol acetate or 10 μm β-carotene	-Enzyme activity of glycolysis pathways (hexokinase, glucose-6-phosphate dehydrogenase, aldose reductase) -Na, K- ATPase activity -Lipid peroxidation	Addition of ascorbic acid or α-tocopherol or β-carotene to the media, reduced lipid peroxidation and increased activities of enzyme involved in the glycolysis hexomonophosphate pathway	[86]
	Rabbit lens epithelial cells	Buthionine sulfoximine	Not reported. Lenses exhibited a depletion of ~75% GSH	Cells were cultured in 25–50 μM vitamin C or 5–40 μM vitamin E at the same time as BSO treatment for 24 h and then exposed to H_2O_2 for 1 h	-Cell viability: MTS assay, LDH assay -GSH/GSSG levels	Supplementation of vitamin C and vitamin E protects GSH-depleted lens epithelial cells by reducing levels of GSSG	[87]
	Mice CD-1 (25g)	Lenses were cultured in xanthine, xanthine oxidase, and uricase	Not stated	Lenses were cultured in 2 mM ascorbate and ROS-inducing reagents along with ^{86}RbCl	-Membrane transport activity -ATP levels -GSH levels	ROS agents decreased membrane transport activity, ATP and GSH. Ascorbate minimized these effects significantly	[88]
	Calf lenses	NA	NA	Water-insoluble proteins from aged normal human lenses, early stage brunescent cataract lenses and calf lens proteins were reacted with or without 20 mM ascorbate in air for 4 weeks	-Protein modifications (glycation reactions)	AGEs present in aged and cataractous human lenses eluted at the same retention times as those from ascorbic acid glycated calf lens proteins, suggesting that the yellow chromophores in brunescent lenses represent AGEs due to ascorbic acid glycation	[37]
				Water-insoluble proteins from aged normal human lenses, early stage brunescent cataract lenses and calf lens proteins were reacted with or without 20 mM ascorbate in air for 4 weeks	-Amino acid modifications -Protein modifications (glycation reactions)	LC-MS revealed that the majority of the major modified amino acids present in early stage brunescent cataract lens proteins were as a result of ascorbic acid modification	[89]
				Incubation of calf lens extracts with either 10 mM ascorbic acid, 20 mM sorbitol, or 20 mM glucose for 8 weeks	-Protein precipitation and browning -Cross linking of proteins -Protein modifications (glycation)	Only ascorbic acid induced the formation of high molecular weight aggregates with extensive browning	[90]
	Bovine lens crystallin proteins	NA	NA	Bovine lens crystallin proteins incubated with [^{14}C] ascorbic acid for 1 month and the fluorescence spectrum compared to human cataractous lenses	-Browning -Binding of Ascorbic Acid Oxidation Products to Proteins. -Comparison of fluorescence Spectra	Formation of brown condensation products correlated with increased protein radioactivity. Fluorescence spectrum of condensation products was similar to spectrum of human cataractous lenses	[91]
				Bovine lens β-crystallin incubated with increasing concentrations of sugars and sugar derivatives for a period of 2 weeks in the dark at 37 °C	-Protein precipitation and browning -Cross linking of proteins	Protein precipitation and browning reaction was observed with both vitamin C and DHA. No reaction was seen with several other sugars suggesting that vitamin C is a significant glycating agent	[92]

Table 1. *Cont.*

	Species	Method of Cataract Induction	Type of Cataract	Vitamin C Elevation or Depletion	Parameters Measured	Outcome	Ref
In vivo studies	Guinea pigs (between 280 and 320 g)	UV-B (0.25–0.75 J/cm^2) 10 min exposure time	Not mentioned	Vitamin C depletion via guinea pigs fed an ascorbate-deficient diet	-DNA damage (DNA single strand breaks)	Lenses from ascorbate deficient guinea pigs showed 50% more DNA damage than those from normal guinea pigs after UV exposure	[93]
	Rats Harlan Sprague-Dawley (300 g)	UV-B (0.25–0.75 J/cm^2) 10 min exposure time	Not mentioned	IP injections of sodium ascorbate (1 g/kg)	-DNA damage (DNA single strand breaks)	Increase in vitamin C in AH and lenses; 50% decrease in UV-induced DNA strand breaks compared to non-ascorbate injected rats	[93]
	Guinea pigs (56 days old, 500–600 gm each)	NA	NA	High dietary ascorbate (50 mg/day) vs. low dietary ascorbate (2 mg/day) for 21 weeks. Lens homogenates exposed to UV light.	-Protein damage (high-molecular-weight aggregates and enhanced loss of exopeptidase activity)	Markers of light-induced protein damage were reduced in the HDA animals compared to LDA animals	[94]
	Rat Sprague-Dawley (p8-p21)	IP admin of sodium selenite at postnatal day 10	Nuclear	Daily IP dose of sodium ascorbate (0.3 mmol) at postnatal day 8 until postnatal day 25	-ATP -GSH -MDA -Soluble protein -Lens transparency	Ascorbate was able to restore ATP and GSH levels and reduced MDA levels that were altered in sodium selenite lenses. Significantly reduced cataracts in animals administered with ascorbate	[95]
	Senescence marker protein-30 knockout (KO) mice	UVR-B (200 mW/cm^2) for 100 s twice a week for 3 weeks	Anterior subcapsular cataract	Fed a vitamin C sufficient diet (1.5 g/L) or vitamin C deficient diet (0.0375 g/L) and then exposed to UV-B	-Lens morphology -Protein content -Lens transparency	Less extensive opacities	[96]
	Rats Wistar (18–20 months)	Streptozotocin	Cortical	STZ diabetic rats were fed a Vitamin C (1 g ascorbate/kg feed) and vitamin E (600 mg dl-α-tocopherol acetate/kg feed) supplemented diet	-Lipid peroxidation -GSH -GSH-Px activity	Lowered lipid peroxidation levels in the lens Increased GSH-Px activity No mention of effects on lens opacities	[97]
	Rats Wistar (age not specified)	Streptozotocin	Cortical	STZ diabetic rats were fed vitamin C at 0%, 0.3%, and 1.0% (w/w) to rodent chow	-Membrane integrity -ATP -Lens transparency	Treatment of diabetic group with vit C at 0.3% and 1% lead to decrease in leakage of γ-crystallins into the aqueous and vitreous humor. A reduction in cataract was detected for the 1% dietary vitamin C group	[98]
	Rats Wistar (12 weeks)	Streptozotocin	Cortical	IP administered with vitamin E (20 mg over 24 h), selenium (0.3 mg over 24 h), vitamin E (20 mg) and selenium combination (0.3 mg over 24 h), or vitamin C (30 mg over 24 h). On the fourth day after injection, IP injections of STZ were administered.	-MDA -GSH -GPx activity	Vitamins C and E and selenium can protect the lens against oxidative damage, but the effect of vitamin C appears to be much greater than that of vitamin E and selenium. No mention of lens opacities	[99]
	Transgenic mouse in which SVCT2 is overexpressed	NA	At 12 months of age, transgenic lenses were a yellow colour similar to that observed in older human lenses	Transgenic lenses contained 10-fold greater vitamin C and 25-fold more DHA than WT lenses	-Protein modifications	Transgenic lenses contained increased levels of vitamin C derived advanced ascorbylation end products which are also known to be present in the aging human lens	[100]
	Guinea pigs (6–9 weeks)	UVR-B (80 kJ/m^2)	Superficial anterior cataract	Drinking water supplemented with or without 5.5 mm l-ascorbate for 4 weeks. After supplementation, animals were exposed in vivo to 80 kJ/m^2 UVR-B.	-Lens transparency via forward light scattering measurements	Cataract develops in lenses exposed to UVR-B both in animals given drinking water that is supplemented with ascorbate and those whose drinking	[101]

8.1.1. The Antioxidant Role of Vitamin C in the Lens

In vitro studies have demonstrated that vitamin C protects rodent lenses from oxidative damage induced by UV-B exposure [86], hydrogen peroxide [87], and other ROS-inducing agents [88]. In vivo studies have also reported a protective effect of vitamin C on the lens. Diurnal guinea pigs which have naturally higher vitamin C levels in the aqueous humor, and like humans rely on a diet supplemented with vitamin C, were shown to be more protected against UV-B induced DNA damage to the lens epithelium compared to vitamin C deficient guinea pigs [93]. Guinea pigs placed on high dietary vitamin C (50 mg/day) contained over three times more vitamin C in the lenses than guinea pigs fed low dietary vitamin C (2 mg/day) [94]. In addition, lenses from high dietary vitamin C fed animals contained less high-molecular-weight aggregates following UV exposure compared to low dietary vitamin C fed animals [94]. Knockout mice which cannot synthesize vitamin C due to genetic disruption of the gluconolactonase gene were fed a vitamin C sufficient diet (1.5 g/L) and then exposed to UV-B. This resulted in less extensive opacities compared to knockout mice fed a vitamin C deficient diet (0.0375 g/L) (Ishikawa et al., 2012) [96]. In a selenite model of cataract, treatment of rat pups with vitamin C exerted a marked protective effect against the development of nuclear cataracts compared to those pups that did not receive vitamin C [95]. Biochemical analysis of lenses revealed that selenite plus vitamin C treatment helped to maintain ATP and GSH levels, and resulted in reduced malondialdehyde (MDA) levels, a marker of lipid peroxidation. Streptozotocin (STZ)-induced diabetic rat models have also shown that dietary vitamin C supplementation is beneficial to the lens. For example, dietary vitamin C supplementation was shown to relieve oxidative stress in STZ-induced diabetic aged rats by minimizing peroxidation levels and enhancing glutathione peroxidase activity in the lens [97]. In another study, dietary vitamin C supplementation of STZ-induced diabetic rats resulted in a reduction in cataracts and a decrease of γ-crystallin leakage into the ocular humors [98]. Finally, intraperitoneally administered vitamin C, vitamin E or selenium, or a combination of Vitamin E and selenium in STZ induced diabetic rats revealed that while vitamin C, vitamin E, and selenium can all protect the lens against oxidative damage, the effect of vitamin C appeared to be much greater than that of vitamin E and selenium [99].

8.1.2. The Pro-Oxidant Role of Vitamin C in the Lens

While the above studies demonstrate a protective effect on vitamin C on the lens, other studies suggest a role of vitamin C in stimulating the progression of cataracts. A recent study have revealed that vitamin C in the lens is a source of oxoaldehyde stress that can be beneficial by promoting chaperone activity, or detrimental by removing protein charges [102]. Vitamin C is also know to act as a pro-oxidant due to the metal catalysed reaction of vitamin C which produces ascorbate free radicals, DHA and H_2O_2 which are toxic to the lens and if not reduced by a mechanism such as the GSH redox cycle, can result in the formation of highly reactive carbonyls [103]. This results in rapid glycation of lens proteins [104] and the formation of protein crosslinks capable of scattering visible light [69]. In vitro cross linking of lens crystallin proteins occurs rapidly in the presence of vitamin C (20 mM) and air due to the oxidation products of vitamin C [90,92,105]. It has been suggested that vitamin C can make a larger contribution to cross-linking than glucose and that as a result vitamin C is a significant glycating agent [106]. In vivo studies have shown that guinea pigs supplemented with 5.5 mM vitamin C for four weeks in their drinking water and then exposed to ultraviolet-B (UV-B) radiation were not protected against UV-B induced cataract [101]. Overexpression of SVCT2 in mouse lenses, which typically have low levels of vitamin C and SVCT2 transporter activity, resulted in elevated levels of vitamin C and its associated oxidation products in the lens [100]. In addition, transgenic lenses exhibited a yellow colour and accelerated modification of crystallin proteins by the Maillard reaction [100]. These results are consistent with changes reported for human lenses during normal aging and cataract formation [107,108] suggesting that vitamin C oxidation plays a role in human lens aging and cataract.

Taken together, the available evidence suggests that while maintenance of vitamin C levels are required to prevent oxidative damage to the lens, excessive administration of vitamin C appears to be linked to cataract formation. However, it is difficult to assess from these animal studies the benefits versus risk value of higher than normal intake levels. However, since these animal studies were conducted, our knowledge on lens physiology has significantly grown. It is now accepted that the lens depends on an internal microcirculation system to deliver nutrients and antioxidants to the deeper regions of the lens [7,14]. This opens up new areas of research into vitamin C delivery and uptake into the different regions of the lens and investigations into whether this delivery can be enhanced to provide protection under conditions of oxidative stress. Looking ahead, it will also be important to consider the choice of animal model given that diurnal and nocturnal species exhibit marked differences in their baseline vitamin C levels, the expression of vitamin C uptake transporters, and the ability to synthesize vitamin C. In general it appears that in rodent models of cataract there are benefits of vitamin C supplementation in the prevention or delaying of opacities. However, like most other interventions in other rodent disease models, rodents respond well because the stress is acute and high drug levels can be easily achieved. Furthermore, all rodent strains are inbred, and thus the number of pathogenic pathways is limited. In addition, the selection of the cataract model is equally important as it needs to mimics changes typically associated with age related or diabetic cataract in humans The morphological, physiological and biochemical changes associated with age related and diabetic cataracts are different [36,109], and so the parameters used to assess the efficacy of vitamin C will also be different. Finally, a standard set of parameters or biomarkers should be used amongst researchers to provide more consistent measurement of the relationship between vitamin C and cataract progression that can be used to aid the translation of animal work into human studies

8.2. Evidence of the Effects of Supplemental or Dietary Vitamin C on the Prevention of Cataracts in Humans

Given the role of oxidative stress in cataractogenesis, it is not surprising that the role of antioxidant intake and cataract in human populations has been extensively studied [6,8,110]. While some studies generally support the association an increased intake of vitamin C and other antioxidant nutrients with a decreased risk of cataract [111,112], longer term clinical trials do not tend to support this conclusion, indicating that vitamin C had little or no benefit for treatment durations up to 6.5 years. The Linxian cataract study conducted in a nutritionally deficient population in China (2, 3249 participants aged 45 to 74 years) involved the random assignment of participants to a daily supplement of 14 vitamins and 12 minerals at 2 to 3 times the U.S. recommended dietary allowance. Compared to placebo, a vitamin C/mineral combination had no effect on reducing the prevalence of cataracts for treatment durations of up to seven years [113]. The Age-Related Eye Disease Study (AREDS) in which participants (4629 participants aged 55 to 80 years) were randomly assigned to receive daily oral tablets containing antioxidants (vitamin C, 500 mg; vitamin E, 400 IU; and beta carotene, 15 mg) or no antioxidants found that a high-dose formulation of vitamin C, vitamin E, and beta carotene in well-nourished older adult cohort had no effect on the risk of development or progression of any cataract type [114]. The Roche European American Cataract Trial (REACT) conducted in the U.K. and U.S. in which participants (445 participants over the age of 40 years) received a daily oral antioxidant mixture (beta-carotene 18 mg; vitamin C, 750 mg; and vitamin E, 600 mg) found modest benefits in the U.S. cohort but no significant benefit in reducing the risk of cataract progression in the U.K. cohort [115]. In a more recent search of studies within the last 10 years (outlined in Table 2), Christen et al. reported on the first randomized trial to test the individual effect of vitamin C supplementation on the prevention of cataract. In this study, daily supplementation of 500 mg of vitamin C in healthy US male physicians 50 years or older revealed no effect on risk of cataract or cataract extraction after eight years of treatment [116]. Concerning were findings from the Swedish Mammography Cohort of women aged 49–83 years, which showed that vitamin C supplementation for longer than 10 years was associated with a 25% increase in the risk of cataract extraction. Among women aged 60 years and older, supplementation with vitamin C was associated with a 38% increased risk of cataract extraction [117]. In a follow up

study, the risk of age related cataract was investigated in Swedish men and revealed that the use of multiple supplements in combination with vitamin C was not associated with cataract risk, but that the use of high dose vitamin C may increase the risk of cataract [118]. The use of dietary supplements in the form of multivitamins or a specific vitamin is widespread ranging from 22% to 53% in studies conducted from USA, Canada, Korea, UK, Sweden, Germany, and France [119–125]. While these supplements are taken for a range of health reasons, based on findings from the above clinical trials, the long-term value of vitamin C supplementation in decreasing the risk of cataract progression is questionable, and at high doses may in fact exacerbate cataract progression.

Table 2. Human studies investigating the effect of vitamin C supplements on the development of cataracts.

Study, Type	Nutrients	Population	Disease Outcome	Results	Year, Author
Age-related cataract in a randomized trial of vitamins E and C in men. Eight years of treatment and follow-up RCT	Vitamin E 400 IU or placebo on alternate days and vitamin C 500 mg of or placebo daily	Participants: 11,545 United States male ≥ 50 years	Incidence of age-related cataract	No significant beneficial or harmful effect on the risk of cataract. HR 1.02; 95% confidence interval, 0.91–1.14	[116]
The Swedish mammography cohort study follow up. 8.2 years of follow-up Population-based, prospective cohort of women.	Vitamin C (approximately 1 g) Vitamin c within a multivitamin supplement (approximately 60 mg)	Participants: 24,593 Sweden female 49–83 years	Incidence of age-related cataracts	The use of vitamin C supplements may be associated with a higher risk of age-related cataract among women. The multivariable HR for vitamin C supplement vs. nonusers was 1.25 (95% CI: 1.05, 1.50). The HR for the duration of 10 y of use before baseline was 1.46 (95% CI: 0.93, 2.31). The HR for the use of multivitamins containing vitamin C was 1.09 (95% CI: 0.94, 1.25).	[117]
High-dose Supplements of Vitamins C and E, Low-Dose Multivitamins, and the Risk of Age-Related Cataract Follow-up of 8.4 years Cohort	Vitamin C and vitamin E as single supplements was estimated to be 1 g and 100 mg, respectively. Multivitamins were estimated to contain 60 mg of vitamin C and 9 mg of vitamin E	Participants: 31,120 Sweden male 45–79 years	Risk of age-related cataract	Use of high-dose (but not low-dose) single vitamin C supplements increased the risk of age-related cataract. The multivariable-adjusted HR for men using vitamin C supplements only was 1.21 (95% confidence interval (CI): 1.04, 1.41) in a comparison with that of non-supplement users. The HR for long-term vitamin C users (≥10 years before baseline) was 1.36 (95% CI: 1.02, 1.81). The risk of cataract with vitamin C use was stronger among older men (>65 years) (HR = 1.92, 95% CI: 1.41, 2.60) and corticosteroid users (HR = 2.11, 95% CI: 1.48, 3.02)	[118]

Abbreviations: RCT, Randomized Control Trials, HR, Hazard Ratio, OR, Odd Ration, HRT, Hormone Replacement Therapy.

However, the general consensus of studies evaluating a well-balanced diet rich in fruit and vegetables tends to suggest that intake via a healthy diet enriched with Vitamin C may be a more optimal approach towards slowing down the progression of age related cataracts [126,127]. A search of the literature within the last 10 years found studies which support the evidence for a healthy diet enriched in Vitamin C and the reduced risk of cataract (outlined in Table 3). In the India Study of Age-related Eye Disease (INDEYE), which examined the association between vitamin C and age related cataract in the Indian population, plasma vitamin C and dietary vitamin C was inversely associated with cataract with the authors highlighting that this strong association with vitamin C and cataract in a vitamin C-depleted population may in part, explain the high levels of cataract in India [128]. The European Eye Study (EUREYE) study investigated the relationship between cataract, fruit and vegetable intake, and dietary and blood levels of carotenoids plus vitamins C and E in a Spanish population. High daily intakes of fruit and vegetables and vitamins C and E were associated with a significantly decreased prevalence of cataract or cataract surgery with daily dietary vitamin C intakes above 107 mg inversely associated with reduced odds of cataract [129]. In a U.S. based study assessing the association between healthy diet scores and prevalence of nuclear cataract in women, having a high healthy eating index (HEI) score was the strongest modifiable predictor of low prevalence of nuclear cataracts. Women with higher HEI scores had higher vitamin C intakes than those with lower scores with a trend for a protective association of vitamin C intake from foods alone, but not from

a combination of foods and supplements, suggesting that vitamin C–containing foods rather than vitamin C itself may afford protection from nuclear cataract [130]. Theodoropoulou and colleagues conducted a case-control study to assess the association between diet and the risk of cataract in Greece. The results showed a protective association between cataract risk and intake of vitamins C and E and carotene, with an increase of 185 mg of vitamin C intake/day to reduce, at least by half, the risk of cataract overall, as well as nuclear and posterior subcapsular cataract [131].

Table 3. Human studies investigating the effect of diets high in vitamin C on the development of cataracts.

Study, Design	Nutrients Studied	Population	Disease Outcome	Results	Ref
The India Study of Age-related Eye Disease (INDEYE study) a population-based study. Cross-sectional analytic study	Vitamin C and inclusion of other antioxidants (lutein, zeaxanthin, retinol, β-carotene, and α-tocopherol)	Participants:5638 North and South India Male and female ≥60 years	Incidence of cataract in the Indian setting	Vitamin C was inversely associated with cataract (adjusted OR for highest to lowest quartile = 0.61; 95% confidence interval (CI), 0.51–0.74; $p = 1.1 \times 10^{-6}$). Similar results were seen by type of cataract: nuclear cataract (adjusted OR 0.66; CI, 0.54–0.80; $p = 0.0001$), cortical cataract (adjusted OR 0.70; CI, 0.54–0.90; $p = 0.002$), and PSC (adjusted OR 0.58; CI, 0.45–0.74; $p = 0.00003$)	[128]
Healthy Diets and the Subsequent Prevalence of Nuclear Cataract in Women. Participated in the Carotenoids in Age-Related Eye Disease Study—7 years follow up	Vitamin C (40 vs. 207 mg/d); vitamin E (3 vs. 11 mg/d)	Participants: 1808 United States female 50–79 years	Prevalence of nuclear cataract in women.	Adjustment of the OR for nuclear cataract among women with high vs. low HEI-95 scores, for vitamin C intake from foods attenuated the ORs (Multivariate OR (95%CI) = 0.76 (0.50–1.15), suggesting that higher vitamin C intakes partly explained the associations with HEI-95 dietary assessment. There was a significant linear trend for a protective association of vitamin C intake from foods	[132]
The European Eye Study (EUREYE study). Recruited during 1-year period. Multi-center cross-sectional population-based study	Carotenoids, vitamins C (107 mg/d) and E	Participants: 599 Spain Male/female ≥ 65 years	Prevalence of cataract with fruit and vegetable intake	High daily intakes of fruit and vegetables and vitamin c were associated with a significantly decreased prevalence of cataract or cataract surgery (p for trend = 0.008). Increasing quartiles of dietary intakes from 107 mg/d of vitamin C showed a significant decreasing association with prevalence of cataract or cataract extraction (p for trend = 0.047)	[129]
Diet and cataract. Case-control study	Carbohydrates carotene vitamins C and E	Participants: 314 cataract cases and 314 controls Greece Male/Female 45–85 years	Association between diet and risk of cataract in Athens	There was a protective association between cataract risk and intake of vitamin c (OR = 0.50, $p \setminus 0.001$ for cataract overall; OR = 0.55, $p \setminus 0.001$ for nuclear cataract; OR = 0.30, $p \setminus 0.001$ for PSC)	[131]

Abbreviations: EUREYE, The European Eye Study, HR, Hazard Ratio, OR, Odd Ratio.

While it appears that a diet high in fruit and vegetables containing vitamin C may be protective against cataracts, the longitudinal nature of nutritional studies and the number of uncontrolled variables present in populations over long periods of observations may affect the observed rates of cataract progression.

9. Cataract Prevention Post Vitrectomy: Restoring Antioxidant Balance in the Eye?

Cataract formation following vitrectomy is a well-recognized postoperative complication of the procedure with the incidence of cataract development as high as 80% within two years after surgery [47–51]. From a nutritional point of view, studying a cohort of individuals in which cataract develops within a two year time frame provides a much-shorted interval of observation than in studying age related cataract progression in the general population. This would minimize a participants potential exposure to uncontrolled variables and potentially allow the researcher to more definitely evaluate the efficacy of an intervention agent to delay the progression of cataracts.

Studies found through clinicaltrials.gov a database of privately and publicly funded international clinical studies identified two trials linked to testing interventions specifically aimed at delaying the progression of cataracts post vitrectomy. The first was a randomised double blind human clinical trial testing the efficacy administration of two doses of OT-551 eye drops in 164 patients (50 years and above) following vitrectomy (NCT00333060). It is unclear whether OT-551 was an antioxidant compound or whether the trial went ahead, but no outcomes or publications were reported from this trial. The second

trial was also a randomised double blind human clinical trial testing the efficacy of Lenstatin™, an over the counter oral antioxidant nutritional supplement to inhibit cataract post vitrectomy (NCT02131194). The formulation included Riboflavin, L-glutathione, C-phycocyanin, lipoic acid, pryruvate, alpha lipoic acid, quercitin, tumeric, silybin, lutein, zeaxanthin, and astaxanthin. Participants took two Lenstatin™ capsules day versus placebo for six months post vitrectomy with lens densitometry measurements taken at baseline and at six month post-operatively. The study was underpowered in sample size with no significant difference in lens nuclear density between Lenstatin™ and placebo groups [133].

With very few studies reported, future work re-examining the efficacy of vitamin C supplementation via the diet or through nutritional supplements will be of great interest in the future and may represent a cost effective solution in reducing the number of individuals requiring cataract surgery following vitrectomy.

10. Conclusions

In general, clinical trials by and large have failed to show convincing beneficial effects of Vitamin C supplementation on cataract incidence, except in those cases in which patients may have had low vitamin C levels to begin with. Unfortunately, only few studies included plasma vitamin C levels. Certainly there is no basis for supplementing with high doses since Vitamin C in excess of 250 mg/day is excreted in the urine. While it seems clear that a healthy diet rich in fruits and vegetables, coupled with healthy lifestyles can help reduce the risk factors for age related cataract, it is estimated that over 2 billion people do not have regular access to safe, nutritious, and sufficient food, and so further work is still required to find alternatives to delay the cataract epidemic caused by our increasing aging and diabetic population. Avoidance of risk factors such as diabetes, UV sunlight, and steroids should all be considered as part of a strategy to delay the progression of age-related and diabetic cataract. However integrating our knowledge of how the lens delivers and accumulates vitamin C and testing this in well-designed studies will play an important part towards designing effective strategies that reduce the risk of cataract formation.

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Fatty acid status and coronary heart disease mortality risk in older adults before and after a 2-year DHA supplementation: A contribution to healthy ageing

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Abstract: The growing older population comes with a rise in the prevalence of non-communicable diseases (NCDs), in which cardiovascular disease is the leading cause of deaths, that are evidenced to be preventable with an optimized nutrition. The aim of this study was to report and analyze the fatty acid (FA) profile and the coronary heart disease (CHD) mortality risk in older adults before, during and after a 2-year supplementation with docosahexaenoic acid (DHA). This study includes 81 patients randomly allocated in the control or the intervention group, the latter receiving a supplement containing DHA. Plasma samples were analyzed at baseline and after 12 and 24 months to determine the FA status. The omega-3 index (O3I) was additionally calculated to assess the CHD mortality risk. At baseline, the population showed poor n6 and n3 proportions and O3I. After the intervention, participants showed an increased DHA status, a decreased n-6:n-3 ratio and an improved CHD mortality risk by increasing the O3I. DHA supplementation is promising to balance FAs that are related to an optimized health by decreasing NCDs and mortality risk. This line of research deserves further attention given its potential in culminating in actionable, tangible and feasible dietary recommendations to promote healthy ageing.

Keywords: healthy ageing, n-3, n-6, old adults, AMD, cardiovascular health, DHA, NCD, wellbeing, quality of life.

1. Introduction

It is no question that we are living longer. The prevalence of the older adult population has increased noticeably worldwide having 727 million persons aged 65 years or older in 2020. This population is expected to more than double reaching 1.5 billion in 2050 (1). The question is, are we living a longer good life? This population commonly presents degenerative and chronic diseases such as eye and heart disease, diabetes, dementia, musculoskeletal disease and frailty, all part of non-communicable diseases (NCDs) and ageing is linked to increased burden and duration of such (2). Despite of efforts made, NCDs, with cardiovascular disease (CVD) being the number one cause of deaths worldwide (3), still lead to 71% of global deaths (4) and represent a major cause of years lived with disability (YLD) (5). Since NCDs are largely preventable with an active and healthy lifestyle, it is necessary to innovate with practical and sustainable preventive solutions (6).

Most of the NCDs are diet affected making optimal nutrition of utmost importance for treatment and prevention. Therefore, the role that micronutrients play in promoting health should receive substantial consideration because it influences the overall quality of life, which is a high concern associated to healthy ageing (7). For instance, the long chain polyunsaturated fatty acids (LC-PUFAs) play an important role in general well-being in all the stages of life, and the omega-3 fatty acids (FAs), such as the DHA, have been recognized to prevent or aid some pathological conditions associated to the ageing process (e.g., in ocular, cognitive, and cardiovascular health, among others). Nonetheless, controversies about the benefits are also documented (8,9).

The omega-3 FAs have strongly shown beneficial effects on CVD, the highly prevalent NCDs. Among the benefits that these nutrients have shown are lowering blood pressure and heart rate, improving blood vessel function and, at higher doses, lowering triglycerides and easing inflammation, which plays a role in the development of atherosclerosis (10,11). Based on a review from Molfino et al. in 2014, nine out of twelve studies point out that omega-3 status/supplementation positively impacts the cardiovascular function in older adults (12).

Deficiencies in the older adult population are a common issue due to a number of factors, mainly due to the reduced food intake and lack of food variety in the diet (7). So, does the older population have an adequate intake of these nutrients? In regards to the adult population, in 2021 the American Heart Association (AHA) recommends 2 servings (3 oz or 85 g per serving) of fatty fish per week for cardiovascular benefits (13,14). For instance, one 4-oz serving (≈113 g) of salmon per week would provide the recommended daily intake of ≈250 mg of LC-n3 PUFAs (14). Also in 2021, the European Society of Cardiology (ESC) agrees saying fish is recommended 1-2 times per week, in particularly fatty fish, amount that has been associated to lower 16% the risk of coronary artery disease and up to 6%

lower risk of stroke when eaten 2-4 times per week. Since the evidence regarding the use of fish oil supplements in cardio health is still unclear, the ESC does not have a specific recommendation for them (15). On the other hand, in 2022, the National Institutes of Health (NIH) suggests consuming 1.1-1.6 g of omega-3 FAs per day (16) and a study suggested that 3 g per day of omega-3 FAs may lower blood pressure, but it takes up to 4-5 oz (≈113 – 142 g) of salmon to provide those 3 g of omega-3 FAs (17), and supplements usually provide daily 1,000 g of fish oil containing only around 300 mg of omega-3 FAs (EPA and DHA). Although the NIH discusses benefits of fish oil supplementation observed in studies, they do not make a specific recommendation to take them given the ambiguous results (16). In Spain, the recommended intake of fish is at least 2 times per week (125-150 g per serving), being 1-2 portions/week of blue fish. In average, other European countries suggest an intake of 1-4 portions of fish per week, some of them specifying the type of fish (18). As observed, doses differ and might also vary for other populations such as pregnant women and children or people undergoing specific health conditions, making recommended doses even harder for the population to reach.

The FA status from human samples can be analyzed individually and reflect the dietary intake (19). The n-6:n-3 ratio has been widely used to analyze the quality of FA dietary intake, but this metric has contributed to misunderstandings, since ratios are non-specific and insensitive to separate FA levels (20). Nowadays, the Omega-3 Index (O3I), calculated from the sum of the proportions of EPA and DHA in erythrocytes or red blood cells, is being used as a biomarker to assess the risk factor for death from coronary heart disease (CHD). Consequently, the higher the index, the less the risk. The stablished cutoff values have been stablished at <4% for a high CHD mortality risk and >8% for a low risk (21), the latter potentially reducing the risk of fatal CHD by 35%. The O3I was first proposed in 2004 (22) and by 2013 the largest dataset published, 160,000 individuals, on circulating FAs status in humans used this parameter. Later, in 2016, authors proposed equivalent cut-offs (<2.9 and >5.2, respectively) to assess the O3I from plasma samples (23). The O3I has been proposed as a better method to represent long term omega 3- status, just as the biomarker hemoglobin A1c does for glycemic status (24).

As mentioned, CVDs are highly prevalent in the older adult population and DHA is suggested to be good for prevention and treatment. Continuing this line of research and generating evidence-based information can contribute to the development of practical strategies to tackle these highly prevalent NCDs affecting healthy ageing in the world. Therefore, the aim of this study was to report and analyze the FA profile and the CHD mortality risk in older adults before, during and after a 2-year supplementation with DHA.

2. Materials and Methods

2.1. Ethics Statement

This study was carried out in accordance with the ethical standards established by the Declaration of Helsinki (2004), the Good Clinical Practice recommendations of the EEC (document 111/3976/88 July 1990) and the current Spanish legislation governing clinical research in humans (Royal Decree 561/1993 on clinical trials). Additionally, the approval by the Clínica Universidad de Navarra Committee was obtained, and written informed consent was obtained from all the participants before any study procedure.

2.2. Study Population and Design

The design of the study is presented in Figure 1. This study was conducted with 81 adults of the total of 106 participating in the THEA-UNAV study, which is a randomized and observer-blinded trial of a 2-year intervention with a nutritional supplement to measure the influence in AMD in adults aged 50 years or older. The study was carried out in nine sites of Spain and Portugal in a population with a previous diagnosis of AMD. The information regarding the THEA-UNAV study has been published elsewhere (25) and was registered at ClinicalTrials.gov (NCT04756310).

The 81 patients included in the present study were divided randomly into two groups: the control group (n= 43) who received the dietary supplement of Theavit® without DHA; and the intervention group (n= 38), conformed by patients who received the Dietary supplement of Retilut® with DHA included.

2.3. Eligibility criteria

Patients were included if they had complete plasma fatty acid data sets at months 0, 12, or 24 of the study and, naturally, all participants followed the eligibility criteria stablished by the THEA-UNAV study such as: any gender, ≥ 50 years of age, diagnosis of AMD and lesions of wet AMD in at least one eye, comprehension of the conditions and particularities of the study and signature of the informed consent, and others (25).

2.4. Dosage and treatment of patients

Both study groups uninterruptedly took 2 tablets a day of their corresponding treatment for a 24-month period and were not allowed to change the dosage or use any other type of nutritional supplement concomitantly. The control supplement, Theavit® (Laboratorios Mayoli Spindler, Barcelona, Spain) contained: vitamins A, beta-carotene, vitamin C and E, zinc, manganese and selenium. The intervention supplement Retilut® (laboratorios Thea, Barcelona, Spain) contained DHA, lutein, zeaxanthin, resveratrol, hydroxytyrosol, vitamins C and E, zinc and copper. The oil of the microalga *Schizochytrium sp* was used as the source of the DHA. Table 1 shows the nutrient content in one capsule of the supplements.

Table 1. Nutritional information of the supplements used

Nutritional Information	**Intervention Retilut® (2 tablets)**	**Control Theavit® (2 tablets)**
Omega 3		
Docosahexaenoic (c22:6 n-3)	400 mg	
Vitamins		
Vitamin A		800 μg
β-carotene		800 μg
Vitamin C (ascorbic acid)	160 mg	120 mg
Vitamin E (d-α-tocopherol)	24 mg α- TE	20 mg TE
Others		
Resveratrol	30 mg	
Lutein	10 mg	
Zeaxanthin	2.6 mg	
Hydroxytyrosol	3 mg	
Trace elements		
Zinc (Zn)	20 mg	15 mg
Copper (Cu)	2 mg	
Manganese (Mn)		2 mg
Selenium (Se)		50 mcg

2.5. Data and sample collection

General data and dietary intake information were collected in the initial assessment. For FA analysis, blood samples were obtained at the beginning of the study (T0), at 12 months (T12) and at 24 months respectively (T24). Blood samples were collected by venipuncture, in a fasting state, and stored in tubes with EDTA-K3 as an anticoagulant. Plasma was then obtained by centrifugation at 1500g for 15 minutes at 4ºC and stored at -80ºC until analysis.

2.6. Determination of total FAs in plasma

The method used to analyze the FA profile in plasma was based on the method developed by our research group and published elsewhere (26). The plasma was initially subjected to a stage of saponification with sodium methylate and anhydrous methanol to obtain FAs in their free form, then FAs methyl esters (or FAMEs) were obtained with the use of boron trifluoride and methanol and, finally, the FAMEs were extracted with hexane and injected

into the gas chromatograph. Quantification was done by normalization, expressing the results in relative amounts (percentages). Saturated Fatty Acids (SFAs), Monounsaturated Fatty Acids (MUFAs), Omega-3 Polyunsaturated Fatty Acids (n-3 PUFAs), Omega-3 Long Chain Polyunsaturated Fatty Acids (n-3 LCPUFAs), Omega-6 Polyunsaturated Fatty Acids (n-6 PUFAs) and Omega-6 Long Chain Polyunsaturated Fatty Acids (n-6 LCPUFAs) sums were created by adding the individual corresponding FAs. Additionally, the ratios n-6:n-3 PUFAs and n-6:n-3 LCPUFAs were calculated for the analysis and the O3I (EPA+DHA proportions) were created to assess the cardiovascular risk.

Other data collection, procedures, and analysis of other variables, such as lutein and zeaxanthin, were previously published (25).

2.7. Statistical analysis

SPSS statistical software package for Windows (version 23.0; SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses. Data were tested for normality using the Kolmogorov-Smirnov test and non-normal data were log-transformed. Characteristics of the participants were described according to ANOVA for quantitative variables and the chi-square test was applied to qualitative variables. We evaluated the O3I and FA evolution and differences at T0, T12 and T24 of each group of study. Since the O3I tool was originally created to assess the FAs from red blood cells, we used the adapted cut-offs proposed by Stark et al. to calculate the O3I from plasma samples. This classification has 4 categories of risk for cardiovascular diseases starting from high risk to low: ≤2.9, >2.9-4.0, >4.0-5.2 and >5.2% (23). The FA analysis was performed by the General Linear Model (GLM) with the Bonferroni post hoc correction and adjusted by fish dietary intake as a covariate given the correlation with DHA plasma status (Table S1). The FA percentages were expressed as means ± standard deviations. The n for each analysis may variate according to data availability. The confidence level was established at 95% for all the tests, thus results obtaining a *p*-value below 0.05 were considered statistically significant and are highlighted in bold.

3. Results

3.1. Characteristics of the population

Basic characteristics of the population are presented in Table 2. As noted, the group who underwent the intervention were older than the control group and most were females. Most of the population were living independently at home with family.

Table 2. Characteristics of the population per study group.

	Control	**Intervention**	
	n= 43	**n= 38**	**P**

Characteristics	Mean (SD)	Mean (SD)	
Age (y)	74.23 (8.41)	78.26 (7.95)	**0.033**
Gender (female, %)	50.0	42.1	0.475
Live Independent (%)			0.642
No, I live in a residence or institution	2.3	0	
Yes, I live at home with family	84.1	86.8	
Yes, I live at home alone	13.6	13.2	

Quantitative data were analyzed with ANOVA and qualitative data with the chi-square test. *p*-values < 0.05 (level of significance) are highlighted in bold.

3.2. Plasma FA evolution per study group

Table 3 presents how the FAs of the intervention group had a higher impact overtime on the FA profile, with strong changes ($p<0.001$) in C22:5n-6, DHA and the ratio n-6 LC: n-3 LC, and also impact in C22:4n-6, n-3 PUFAs, n-3 LC-PUFAs, and the n-6:n-3 ratio ($p<0.05$).

At baseline, FA levels showed no differences between groups, but at the 12 and 24-month follow-up, the DHA, n-3 PUFAs and n-3 LC-PUFAs were higher in the intervention group, while the n-6:n-3 and n-6 LC: n-3 LC ratios were higher for the control group.

As expected, SFAs and MUFAs presented no differences throughout time or between groups.

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Table 3. Fatty acids in plasma per group and time of study.

	T0							T12							T24							*p*	*p*
	Control n=43			Intervention n=38			*p*	Control n=35			Intervention n=34			*p*	Control n=19			Intervention n=13			*p*	FA evolution of control group	FA evolution of intervention group
%	Mean	±	SE	Mean	±	SE		Mean	±	SE	Mean	±	SE		Mean	±	SE	Mean	±	SE			
C12:0	0.11	±	0.01	0.09	±	0.01	0.067	0.09	±	0.01	0.13	±	0.01	0.124	0.09	±	0.02	0.11	±	0.02	0.552	0.267	0.879
C14:0	0.82	±	0.05	0.72	±	0.06	0.445	0.68	±	0.05	0.77	±	0.05	0.216	0.82	±	0.09	0.71	±	0.10	0.537	0.997	0.782
C16:0	21.49	±	0.30	21.01	±	0.35	0.330	20.77	±	0.36	21.24	±	0.40	0.379	21.73	±	0.47	21.62	±	0.56	0.879	0.743	0.779
C16:1n-9	0.48	±	0.02	0.49	±	0.02	0.959	0.45	±	0.02	0.43	±	0.02	0.733	0.45	±	0.02	0.41	±	0.03	0.390	0.634	0.178
C16:1n-7	1.53	±	0.07	1.34	±	0.08	0.115	1.32	±	0.07	1.28	±	0.08	0.948	1.41	±	0.11	1.32	±	0.13	0.362	0.621	0.645
C17:0	0.26	±	0.01	0.26	±	0.01	0.762	0.25	±	0.01	0.27	±	0.01	0.163	0.25	±	0.01	0.26	±	0.01	0.443	0.731	0.986
C17:1	0.16	±	0.00	0.16	±	0.01	0.728	0.16	±	0.01	0.16	±	0.01	0.712	0.17	±	0.01	0.16	±	0.01	0.297	0.921	0.741
C18:0	6.74	±	0.09	6.54	±	0.11	0.163	6.72	±	0.12	6.75	±	0.13	0.858	6.52	±	0.12	6.85	±	0.15	0.124	0.385	0.171
C18:1t	0.18	±	0.01	0.16	±	0.01	0.142	0.18	±	0.01	0.19	±	0.01	0.366	0.17	±	0.01	0.14	±	0.01	0.297	0.668	0.371
C18:1n-9	24.06	±	0.69	25.07	±	0.79	0.462	23.64	±	0.77	24.15	±	0.86	0.669	24.45	±	1.04	24.40	±	1.24	0.782	0.976	0.928
C18:1n-7	1.81	±	0.09	2.00	±	0.10	0.121	1.88	±	0.08	1.96	±	0.09	0.585	2.33	±	0.19	2.55	±	0.22	0.563	**0.005**	0.172
C18:2n-6	28.51	±	0.80	28.95	±	0.92	0.733	30.10	±	0.96	28.78	±	1.07	0.376	27.99	±	1.14	26.43	±	1.36	0.254	0.981	0.276
C18:3n-6	0.45	±	0.02	0.41	±	0.03	0.099	0.43	±	0.03	0.38	±	0.03	0.083	0.45	±	0.03	0.35	±	0.03	**0.010**	0.961	0.552
C18:3n-3	0.31	±	0.02	0.28	±	0.02	0.661	0.29	±	0.02	0.32	±	0.02	0.218	0.29	±	0.01	0.25	±	0.01	0.068	0.969	0.607
C20:0	0.10	±	0.01	0.09	±	0.01	0.294	0.09	±	0.01	0.11	±	0.01	0.528	0.07	±	0.01	0.06	±	0.01	0.066	**0.036**	0.107
C20:1n-9	0.17	±	0.01	0.17	±	0.01	0.967	0.18	±	0.01	0.18	±	0.01	0.928	0.19	±	0.01	0.19	±	0.01	0.916	0.102	0.254
C20:2n-6	0.17	±	0.01	0.16	±	0.01	0.387	0.17	±	0.01	0.17	±	0.01	0.398	0.14	±	0.01	0.14	±	0.01	0.558	0.067	0.184
C20:3n-9	0.10	±	0.00	0.09	±	0.01	0.297	0.10	±	0.00	0.09	±	0.00	0.133	0.08	±	0.00	0.08	±	0.01	0.276	0.083	0.056
C20:3n-6	1.50	±	0.05	1.39	±	0.06	0.175	1.50	±	0.05	1.30	±	0.06	**0.022**	1.59	±	0.08	1.26	±	0.10	**0.021**	0.722	0.179
AA	6.87	±	0.24	6.64	±	0.27	0.519	7.02	±	0.24	6.29	±	0.27	**0.036**	6.91	±	0.34	6.17	±	0.41	0.217	0.999	0.629
EPA	0.71	±	0.07	0.75	±	0.08	0.733	0.70	±	0.09	0.86	±	0.10	0.252	0.77	±	0.11	0.94	±	0.13	0.154	0.880	0.093

C22:4n-6	0.17	±	0.01	0.17	±	0.01	0.574	0.17	±	0.01	0.15	±	0.01	**0.006**	0.18	±	0.01	0.14	±	0.01	**0.006**	0.970	**0.023**
C22:5n-6	0.15	±	0.01	0.14	±	0.01	0.681	0.15	±	0.01	0.31	±	0.01	**<0.001**	0.16	±	0.02	0.26	±	0.03	**0.002**	0.759	**<0.001**
C24:1	0.08	±	0.01	0.09	±	0.01	0.228	0.08	±	0.00	0.09	±	0.01	0.639	0.10	±	0.01	0.09	±	0.02	0.911	0.574	0.996
C22:5n-3	0.43	±	0.02	0.43	±	0.02	0.994	0.40	±	0.02	0.36	±	0.02	0.085	0.53	±	0.03	0.51	±	0.04	0.732	**0.005**	0.076
DHA	2.43	±	0.10	2.34	±	0.12	0.565	2.36	±	0.12	3.12	±	0.14	**<0.001**	2.30	±	0.16	3.22	±	0.19	**0.001**	0.457	**<0.001**
SFAs	29.52	±	0.36	28.70	±	0.41	0.157	28.60	±	0.44	29.27	±	0.49	0.300	29.49	±	0.52	29.61	±	0.63	0.876	0.962	0.533
MUFAs	28.29	±	0.73	29.31	±	0.84	0.451	27.71	±	0.80	28.25	±	0.90	0.634	29.09	±	1.13	29.12	±	1.35	0.837	0.824	0.987
n-6 PUFAs	37.82	±	0.81	37.86	±	0.93	0.925	39.55	±	0.99	37.36	±	1.11	0.138	37.41	±	1.23	34.75	±	1.47	0.125	0.956	0.212
n-3 PUFAs	3.87	±	0.17	3.79	±	0.19	0.566	3.75	±	0.21	4.66	±	0.23	**0.003**	3.89	±	0.25	4.92	±	0.30	**0.014**	0.899	**0.003**
n-6 LC-PUFAs	8.86	±	0.26	8.49	±	0.30	0.364	9.02	±	0.26	8.21	±	0.29	**0.034**	8.97	±	0.37	7.97	±	0.45	0.110	0.991	0.563
n-3 LC-PUFAs	3.56	±	0.17	3.52	±	0.19	0.657	3.47	±	0.21	4.34	±	0.23	**0.005**	3.60	±	0.25	4.67	±	0.29	**0.011**	0.934	**0.002**
n-6 : n-3	10.45	±	0.51	10.97	±	0.59	0.667	11.41	±	0.54	8.69	±	0.60	**0.002**	10.53	±	0.67	7.49	±	0.80	**0.006**	0.970	**0.001**
n-6 LC : n-3 LC	2.63	±	0.12	2.65	±	0.14	0.888	2.81	±	0.12	2.04	±	0.13	**0.002**	2.70	±	0.17	1.81	±	0.21	**0.006**	**<0.001**	**<0.001**

The data were analyzed using the univariate general linear model adding fish dietary intake as a covariate. The presented values are means of the percentages from total FAs. p-values < 0.05 (level of significance) are highlighted in bold.

3.5. The Omega-3 Index and cardiovascular risk.

Table 6 presents the cardiovascular risk in the two study groups, from baseline to the 24-month follow up visit. At baseline, both study groups were at the second highest category of cardiovascular risk (O3I >2.9 - 4.0). Nonetheless, by the 2-year intervention, the supplemented patients presented an O3I of 4.16% reaching the second-best category of low risk for cardiovascular diseases (O3I >4.0 – 5.2), while in the control group remained the same.

Table 6. Omega-3 Index and cardiovascular risk

	Control				Intervention				*p*
Omega-3 Index	**n**	**Mean**	**±**	**SE**	**n**	**Mean**	**±**	**SE**	
At baseline	43	3.14	±	0.16	38	3.09	±	0.18	0.654
At 12 mo follow-up	35	3.07	±	0.20	34	3.98	±	0.22	**0.032**
At 24 mo follow-up	19	3.07	±	0.24	13	4.16	±	0.29	**0.026**
p				**0.709**				**0.009**	

Comparisons determined by the univariate general linear model with fish dietary intake as a covariate. Risk categories for cardiovascular diseases starting from high risk to low: <2.9, >2.9-4.0, >4.0-5.2 and >5.2%.

4. Discussion

This study approaches one of the many ways of how nutrition is linked to healthy ageing. FAs are some of the nutritional components that have extensively shown to be key for optimal health and chronic disease prevention through lifespan (27), and yet, ongoing, and practical efforts are required to tackle the most prevalent diseases in older adults, NCDs, which are not only the main cause of deaths, but also of poor wellbeing and quality of life of our population (28). This research offers 1) a report of the FA status in the older population, 2) shows the CHD mortality risk using the O3I and 3) analyzes the effect on them of a DHA supplementation aiming to contribute to healthy ageing through the development of feasible nutritional strategies.

In regards to the PUFAs, our population evidenced a poor n6:n3 ratio and O3I. As already known, PUFAs are the FAs that have extensively proven to be associated to health outcomes with a recommendation of a balanced n-6:n-3 ratio of 1-2:1 for the prevention of chronic diseases and optimal health (29). Before the supplementation, our population presented a n-6:n-3 ratio in plasma of 10:1, which is considerably higher compared to the general recommendation of 1-2:1. Nonetheless, after the daily supplementation with 400 mg of DHA, our population presented an improved n-6:n-3 ratio (7.49:1) reflecting also a general and positive influence over the rest of FAs in the n-6 and the n-3 series, for instance increasing the anti-inflammatory DHA and nominally reducing the proportion of the pro-inflammatory AA.

Similar to the n-6:n-3 ratio, our population at baseline showed a poor O3I falling into the second highest category of CHD mortality risk (O3I >2.9-4.0%). This was not a surprise, since previous studies have shown that the population has a mean O3I of high or intermediate CHD mortality risk (20,23,30). Also in line with the n-6:n-3 ratio, over the course of the DHA supplementation, the intervention group gradually improved the O3I reaching 4.16% allocating them in a lower risk category (O3I >4.0-5.2%).

In general, at baseline, the older adults of our study did not show n6 and n3 proportions that favor protection against NCDs, however those proportions were improved concerning NCDs prevention with the 400 mg/day DHA supplementation for 12 and 24 months. As confirmed in our study, also dietary intake, such as fish consumption, is a potential and feasible recommendation to improve the n6:n3 ratio and the proportions of the n3 FAs, such as DHA, which compose the O3I and are related to prevent the development or progression of conditions such as cardiovascular and eye disease (20,31).

Our intervention group also showed an increase in the C22:5n6 as a result of the DHA supplement source, the algae *Schizochytrium sp.*, which contains large amounts of DHA, but it is also rich in this other FA.

DHA has been widely implicated in healthy ageing. Benefits in multiple patient outcomes have been seen by improving function and clinical course, for instance in cognitive health, decline of muscle mass, cancer treatment, surgical patients, and critical illness (32). Overall, n-3 LC-PUFAs reduce the inflammatory response by competing against the n-6 AA that mainly activates eicosanoids of pro-inflammatory series (33). Additionally, FAs are an important structural component of cell membranes which impacts their physicochemical properties, therefore, naturally influencing organ functions (11).

Knowing the current health status of our population can help identify and develop preventive strategies to contribute to better lives. Our study, voting for nutrition as a key player in NCDs prevention and healthy ageing, contributes with information of the current FA status and CHD mortality risk in older adults before, during and after a DHA supplementation. We acknowledge and identify limitations in this study, extending from the sample size to dropouts, treatment compliance and the lack of complete dietary, lifestyle and subjective data of participants. Although risk factors, such as socio-demographic information and diet, allowed us to adjust our statistical models for potential confounders, we cannot rule out residual confounding given the complexity and nature of this data collection.

5. Conclusion

This study endorses the role of nutrition in healthy ageing. The results allow us to confirm 1) the link between FAs and health; 2) the need to balance n6 and n3 proportions; 3) that an increased intake of omega sources improve the FA status; and 4) that DHA supplementation can improve two markers of health, n6-n3 ratio and the O3I, therefore decreasing the risk for NCDs responsible of the majority of global deaths and poor quality of life. This research supports the need of promoting and truly implementing the frequently overlooked preventive healthcare and shows the potential of DHA dietary recommendations in culminating in an actionable, tangible, and feasible small step towards healthy ageing and NCD prevention. To enhance the extent of nutritional research, it is crucial to include complete and clear dietary intake information, other health indicators, lifestyle and subjective data of participants advocating for their wellbeing and quality of life.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Association of fish dietary intake and DHA and n-6:n-3 ratio in plasma at baseline.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Materials

Table S1 shows the positive association of fish dietary intake with DHA and the negative association of n-6:n-3 ratio in plasma at baseline of all included patients.

Table S1. Association of fish dietary intake and DHA and n-6:n-3 ratio in plasma at baseline.

Plasma FAs	**Fish dietary intake n= 81**	
	β	*p*
DHA	0.248	**0.033**
n-6:n-3	-0.257	**0.015**

Associations were determined using the linear regression analysis.

nutrients

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Article

A Randomized Study of Nutritional Supplementation in Patients with Unilateral Wet Age-Related Macular Degeneration

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Abstract: The purpose of this study is evaluate the efficacy and safety of medicinal products containing the original Age-Related Eye Disease group (AREDS) formulation at doses approved in Europe (EU, control group; $n = 59$) with a product that adds DHA, lutein, zeaxanthin, resveratrol and hydroxytyrosol to the formula (intervention group; $n = 50$). This was a multicenter, randomized, observer-blinded trial conducted in patients aged 50 years or older diagnosed with unilateral exudative Age related Macular Degeneration AMD. At month 12, the intervention did not have a significant differential effect on visual acuity compared with the control group, with an estimated treatment difference in Early Treatment Diabetic Retinopathy Study (ETDRS) of −1.63 (95% CI −0.83 to 4.09; $p = 0.192$). The intervention exhibited a significant and, in most cases, relevant effect in terms of a reduction in some inflammatory cytokines and a greater improvement in the fatty acid profile and serum lutein and zeaxantin concentration. In patients with unilateral wet AMD, the addition

of lutein, zeaxanthin, resveratrol, hydroxytyrosol and DHA to the AREDS EU recommended doses in the short-term did not have a differential effect on visual acuity compared to a standard AREDS EU formula but, in addition to improving the fatty acid profile and increasing carotenoid serum levels, may provide a beneficial effect in improving the proinflammatory and proangiogenic profile of patients with AMD.

Keywords: age-related macular degeneration; AREDS; Theavit®; Retilut®; carotenoids; polyunsaturated fatty acids; inflammatory markers; angiogenic factors at month 12

1. Introduction

Age-related macular degeneration (AMD) is a leading cause of loss of vision and is associated with a substantial burden for the individual. Globally, the prevalence of AMD has been estimated to be 8.7% in individuals aged 45 to 85 years [1]. In Europe, despite the observation of a decrease in the prevalence in the last two decades, it is expected that the number of affected subjects will increase, with AMD remaining a significant public health problem [2]. In 2015, AMD was the fourth cause of blindness globally and the third cause of moderate-to-severe visual impairment [1]. Individuals with AMD may exhibit an important deterioration in quality of life depending on visual acuity and other factors, such as the disease stage and comorbidities [3–5].

Advanced forms of AMD include geographic atrophy or dry AMD and choroidal neovascularization or wet AMD. Anti-vascular endothelial growth factor (anti-VEGF) agents are effective in patients with wet AMD in terms of maintaining visual acuity [6] and should therefore be considered a standard of care for these patients [7]. Despite its proven efficacy, due to the difficulties in implementing strict intravitreal treatment patterns in clinical practice, anti-VEGFs may not be associated with the expected outcomes [8]. In addition, dry AMD is a more resistant form of the disease, and no drug has yet been approved for its treatment. Therefore, there is considerable interest in identifying therapeutic options that could delay the occurrence of advanced forms of the disease.

The recognition of the role of oxidative stress in macular degeneration and the fact that the retina is particularly susceptible to it has led to the proposal of several antioxidants for reducing the risk of progression of AMD [9]. In this context, a large, multicenter, randomized trial, namely, the Age-Related Eye Disease Study (AREDS), demonstrated that a supplement containing vitamin C, vitamin E, beta-carotene and zinc compared to placebo reduced the five-year risk of developing AMD by 25% in patients at risk, a modest but significant benefit [10]. Afterward, in light of the potential antioxidant benefit of other micronutrients and potential risks of beta-carotene, the AREDS2 randomized trial was conducted to evaluate, using a factorial design, the efficacy and safety of the addition of lutein plus zeaxanthin and/or omega-3 long-chain polyunsaturated fatty acids (LCPUFAs) to the original formula and to assess the omission of beta-carotene and/or reduction in the dose of zinc from the formula [11]. The primary analysis of the trial did not find a further reduction in the risk of AMD with the addition of lutein plus zeaxanthin and/or omega-3 LCPUFAs. However, a secondary analysis showed that individuals randomized to lutein plus zeaxanthin and the AREDS formula without beta-carotene compared to those who received no lutein plus zeaxanthin and the AREDS formula containing beta-carotene exhibited a significant 18% reduction in the likelihood of progression to advanced AMD and a 22% reduction in the likelihood of developing neovascular AMD; there was no significant effect on the evolution of central geographic atrophy [12]. Further studies have demonstrated the beneficial role of lutein plus zeaxanthin in AMD [13–15]. PUFAs play a role in inflammation and its resolution [16,17] and have a beneficial effect in AMD [18,19], and their role in the maintenance of vision has been endorsed by approval from the European Food Safety Authority [20]. Other substances, such as resveratrol and hydroxytyrosol, have been investigated and have shown antioxidant and/or antiangiogenic properties

in cultured retinal pigment epithelial cells [21,22]. However, there are insufficient data on the role of docosahexaenoic acid [DHA]/eicosapentaenoic acid [EPA] combined with the recommended dietary allowances of vitamins and minerals or the addition of other micronutrients, such as resveratrol, vitamin B or vitamin D, as preventive strategies in AMD [20].

We report herein the results of a randomized, observer-blinded trial aimed at evaluating the efficacy and safety of medicinal products containing the original AREDS formulation at doses approved in Europe with a product that adds to the formula DHA, lutein, zeaxanthin, resveratrol and hydroxytyrosol.

2. Materials and Methods

This was a randomized, observer-blinded trial conducted at nine sites in Spain and Portugal between November 2014 and April 2018. Every patient provided written informed consent before performing any study procedure. The protocol was approved by the ethics committee of the Clínica Universidad de Navarra, and each participant site endorsed that approval. The study was conducted following the principles included in the Declaration of Helsinki. Trial registration: ClinicalTrials.gov identifier NCT04756310.

2.1. Study Subjects

We included patients aged 50 years or older diagnosed with unilateral choroidal neovascularization secondary to AMD or any of its sequelae (i.e., disciform scar, pigment epithelium detachment secondary to subretinal fluid, and/or subretinal hemorrhage (stage V of the modified AREDS classification)) with no exudative involvement in the contralateral eye (study eye) and who provided written informed consent.

Patients were excluded if they met any of the following criteria: had myopia of six diopters; had posterior pole abnormalities that could lead to choroidal neovascularization such as choroidal nevus, angioid streaks, central serous choroidopathy, inherited degenerative retinal diseases, and diabetic retinopathy; had coexisting media opacities that prevent assessment of the fundus; were considered to be at risk of becoming lost to follow-up based on the investigator's judgment; had participated in a therapeutic trial within the last three months; had received any nutritional supplement within one month of the study entry; had suspected or confirmed diagnosis of substance use disorder (illegal drugs); and/or were not able to understand the study procedures.

2.2. Interventions

Patients were randomized in a 1:1 ratio with a block design to receive a supplement containing the components of the AREDS original formulation (i.e., vitamin C, vitamin E, beta-carotene and zinc) manganese and selenium (Theavit®, laboratorios Mayoli Spindler, Barcelona, Spain; the control group) or a supplement containing the AREDS original formulation, except for beta-carotene, plus copper, DHA, lutein, zeaxanthin, resveratrol and hydroxytyrosol (Retilut®, laboratorios Thea, Barcelona, Spain; the intervention group). The doses of the AREDS formulations complied with European requirements for these supplements and are specified in Table S1. The boxes containing the control and intervention products were identical in appearance and were consecutively numbered according to the randomization schedule. Patients were instructed to receive two capsules daily regardless of the assigned group.

2.3. Evaluations and Outcomes

Patients were evaluated at the inclusion visit (baseline) and at 6 and 12 months.

At baseline, we recorded information on medical and ophthalmologic history, a brief nutritional questionnaire was applied, the best-corrected visual acuity (BCVA) was assessed in the study eye in a sitting position using Early Treatment Diabetic Retinopathy Study (ETDRS) testing charts, an anterior segment biomicroscopy was performed, and stereoscopic fundus evaluation and digital fundus photography were performed; in addition, blood samples were obtained for biochemical analysis (see below). During the follow-up, BCVA, biomicroscopy and fundus evaluation were performed every six months, while digital retinography and biochemical analysis were performed at baseline and 12 months. Adverse events were recorded at each study visit.

2.4. Biochemical Analyses

Biochemical analyses included the determination of several inflammation and oxidative stress markers, vascular endothelial growth factor and the fatty acid profile.

2.4.1. Determination and Analysis of Lutein and Zeaxanthin

The extraction of lutein and zeaxanthin from the serum samples (600 µL) was carried out with a mixture of hexane:dichloromethane (5:1) [23], and the extract was reconstituted with a mixture of methanol:methyl-tert-butyl-ether (50:50) and injected (20 µL) into the chromatograph. A duplicate analysis was performed on 20% of the patients' samples, and all of them contained an internal standard (tocopherol acetate).

Lutein and zeaxanthin analysis was performed by high-performance liquid chromatography (HPLC) using a kit consisting of a model 600 pump, a Rheodyne injector, and a diode array detector (PDA) (Waters, Milford, MA, USA). The system included a C30 YMC column (5 µm, 250 × 4.6 mm i.d.) and a precolumn (Aquapore ODS type RP-18). The mobile phase consisted of methanol with triethylamine (0.1%) and methyl-tert-butyl-ether in a linear gradient from 95:5 to 70:30 in 30 min as described in Olmedilla-Alonso et al. [24]. The flow rate was 0.9 mL/min. The response was recorded using the Empower 2 software application (Waters). The identification of the compounds was carried out by comparing the retention times with those of standard compounds and comparing the UV-VIS spectra online. Quantification was performed using a calibration curve. The repeatability of the response to the concentration of these carotenoids was verified by repeated injections of the standards on the same day and on different days.

2.4.2. Multiplex Cytokine Analysis of IL-1b, -6, -8, -9, -10, -12p70, IFN-γ, MCP1 and TNF-α

Cytokine analysis for IL-6, -8, -18, IFN-γ, MCP1 and TNF-α was performed using FirePlex Firefly® Analysis Workbench (Abcam), which is software for multiplex protein expression assays from Abcam Laboratories. A total of 100 µL of each sample (plasma) was assayed. All cytokines are expressed in pg/mL.

2.4.3. MMP-10 Analysis by ELISA

Plasma samples were assayed for MMP-10 levels using the BioAim ELISA kit (BioAim Scientific, Scarborough, ON, Canada) following the manufacturer's instructions. Data are presented as pg/mL.

2.4.4. VEGF Measurement by Western Blot

Western blotting for VEGF determination was performed as previously described [25]. Briefly, 2 µL of plasma samples (diluted 1:10) was mixed with Laemmli buffer (Bio-Rad), boiled for 5 min, separated on 10 to 12% SDS PAGE gels and transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk (*w*/*v*), 0.1% Tween-20 (*w*/*v*) in TBS (1 h, RT), membranes were exposed to rat monoclonal anti-VEGF antibody (1:5000, 512808, BioLegend, San Diego, CA, USA) at RT for 1 h followed by incubation at RT for 1 h with a horseradish peroxidase-conjugated goat anti-rat IgG-peroxidase conjugated antibody

(1:5000, 31470, Pierce Biotechnology, Waltham, MA, USA). Signals were detected with an enhanced chemiluminescence (ECL) kit (ECL Prime Western blotting detection kit, GE Healthcare) and captured with ImageQuant 400 (GE Healthcare, Fairfield, CT, USA). The relative intensities of the immunoreactive bands were analyzed with ImageQuantTL software (GE Healthcare). The loading was verified by Ponceau S red, and the same blot was stripped and reblotted with an anti-β-actin monoclonal antibody (Sigma-Aldrich) to normalize the VEGF levels.

2.4.5. Fatty Acid Profile Analysis

For the fatty acid (FA) profile analysis, we used a method described elsewhere [26]. The plasma was initially subjected to a saponification step with sodium methylate and anhydrous methanol to obtain FAs in their free form. Then, FA methyl esters (or FAMEs) were obtained with the use of boron trifluoride and methanol, and finally, the FAMEs were extracted with hexane and injected into the gas chromatograph. Quantification was performed by normalization, expressing the results in relative amounts. Omega-3 polyunsaturated fatty acid (n-3 PUFA), omega-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA), omega-6 polyunsaturated fatty acid (n-6 PUFA) and omega-6 long-chain polyunsaturated fatty acid (n-6 LCPUFA) sums were created by adding the individual FAs. Additionally, the ratios of n-6:n-3 PUFAs and n-6:n-3 LCPUFAs were calculated for the analysis.

2.5. Statistical Analysis

The primary outcome was the mean change from baseline in the BCVA in the non-affected eye by choroidal neovascularization secondary to AMD (study eye). Secondary outcomes included the mean change from baseline in the evaluated cytokines and lipids and the frequency of adverse reactions, mainly the development of choroidal neovascularization in the study eye.

To detect a mean difference between treatments in the BCVA of 4.6 letters (standard deviation 8.9), assuming a high correlation between the baseline assessment and the determination to be compared (minimum correlation of 0.8 between both), with a two-sided significance level of 5%, a power of 90%, and an anticipated number of dropouts of 10 to 20%, a sample size of 40 patients per treatment arm was required.

All efficacy outcomes were analyzed in the intent-to-treat population using a visit-wise approach. To compare the mean changes from baseline in the different efficacy outcomes, we used the unpaired Student's *t*-test or the Mann–Whitney U test. All tests were two-sided and considered significant if $p < 0.05$. Effect sizes for the difference in mean changes between the intervention and control groups were calculated using Cohen's d [27]. We considered Cohen's d of <0.20, 0.20 to 0.49, 0.50 to 0.79, and ≥0.80 to reflect trivial, small, moderate, and large effect sizes, respectively [27]. Effect sizes that were at least moderate were interpreted as relevant changes. All analyses were performed with SPSS 20.0.

3. Results

3.1. Patient Disposition and Baseline Characteristics

We randomly assigned 109 patients to treatment. Fifty patients received the intervention, and 59 received the control. Patients had a mean age of 77.1 years (standard deviation [SD]; 7.6) and were evenly distributed with respect to sex. Baseline characteristics were generally well-balanced between the intervention and the control, except for the AMD status, which showed the worst result in patients from the intervention group (Table 1).

Of the 109 randomized patients, 93 completed the trial. The number of participants discontinuing treatment prematurely was 5 (10%) with the intervention treatment and 11 (18.6%) with the control treatment (Figure S1).

Table 2. Changes in carotenoids and polyunsaturated fatty acids at month 12.

	Intervention			Control			Mean Difference (Intervention-Control)	*p*-Value	95% CI		Effect Size
Variable	***N***	**Mean Change**	**SD**	***N***	**Mean Change**	**SD**		**Student's *t*-Test**	**Lower**	**Upper**	**Cohen's d**
CAROTENOIDS (μg/dL)											
Lutein	42	24.41 **	27.93	43	−1.57	6.58	26.0	<0.001	17.08	34.91	1.29
Zeaxanthin	42	2.88 **	3.52	43	−0.09	1.29	2.98	<0.001	1.83	4.13	1.13
POLYUNSATURATED FATTY ACIDS (as % of total fatty acids in plasma)											
DHA	34	0.74	0.59	35	0.04	0.66	0.701	<0.001	0.4	1.004	1.12
Σ n-3 PUFAs	34	0.82	1.1	35	0.05	1.04	0.763	0.004	0.248	1.279	0.72
Σ n-6 PUFAs	34	−0.97	4.4	35	2.47	7.28	−3.444	0.021	−6.347	−0.541	0.57
Σ n-3 LCPUFAs	34	0.79	1.11	35	0.08	1	0.715	0.006	0.207	1.222	0.67
Σ n-6 LCPUFAs	34	−0.35	1.15	35	0.3	2.19	−0.654	0.126	−1.5	0.188	0.37
Ratio of n-6/n-3 PUFAs	34	−2.18	2.59	35	1.05	3.35	−3.227	<0.001	−4.7	−1.785	1.08
Ratio of LCn-6:LCn-3 PUFAs	34	−0.6	0.64	35	0.18	0.89	−0.783	<0.001	−1.155	−0.412	1.00

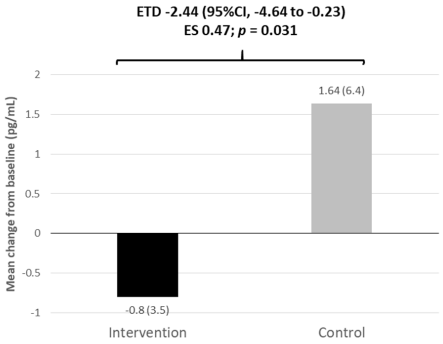
DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acid; N, number of evaluable patients; PUFA, polyunsaturated fatty acid; SD: standard deviation; CI, confidence interval. * $p < 0.05$. ** $p < 0.001$.

3.4. Inflammatory and Oxidative Stress Markers and Vascular Endothelial Growth Factor

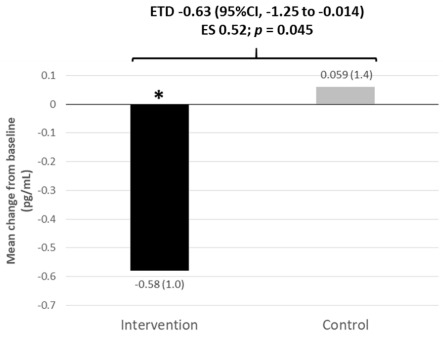
The intervention was associated with significant reductions in the levels of interferon-γ, IL-1β and tumor necrosis factor (TNF)-α, while changes from baseline in the levels of cytokines in the control group were not significant. The intervention treatment reduced the levels of some cytokines, such as interleukin IL-8, IL-1β and TNF-α, to a significantly greater extent than the control group, with the effect size being small for IL-8 and moderate for IL-1β and TNF-α (Figure 1a–k; Table S3). Both treatment groups reduced matrix metalloproteinase (MMP)-10 and VEGF to a similar extent, and the difference between the groups was not statistically significant with a trivial effect size (Figure 1j,k; Table S3).

3.5. Tolerability and Safety

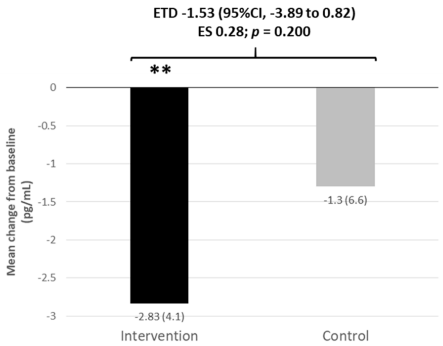
Overall, there were 21 adverse events reported throughout the study–13 in the intervention group and eight in the control group. Of them, 17 were related to the eyes. The most common adverse events related to the eyes were the development/progression of cataracts (five cases in the intervention group and one case in the control group) (Table S2). An adverse event of special interest was the development of exudative AMD in the study eye, which occurred in three patients in the intervention group and two patients in the control group.



(**a**) Interleukin-8.

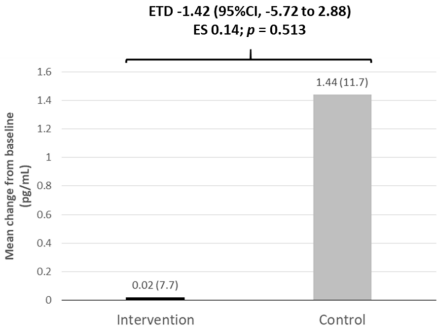


(**b**) Interleukin-1 β.

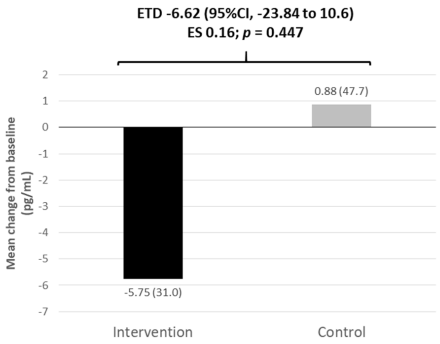


(**c**) Interferon-γ.

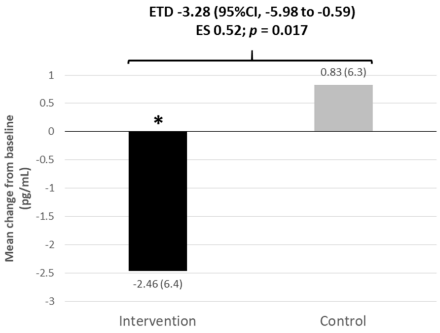
Figure 1. *Cont.*



(**d**) 1g. Interleukin-9.

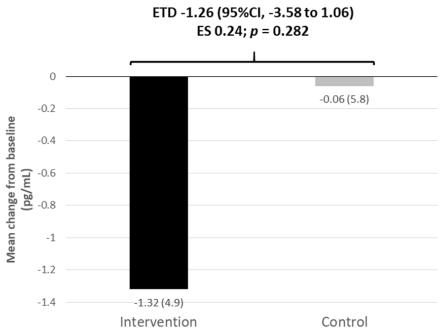


(**e**) Monocyte chemoattractant protein 1.

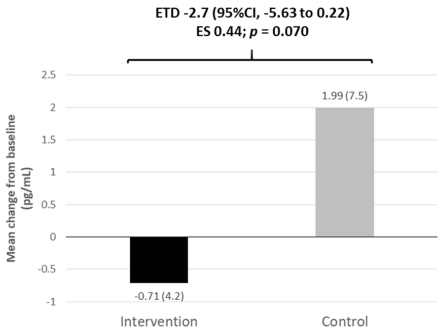


(**f**) Tumor necrosis factor-α.

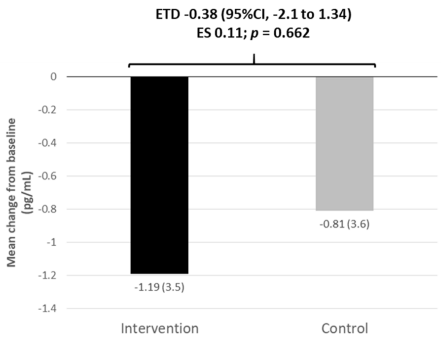
Figure 1. *Cont.*



(**g**) Interleukin-10.

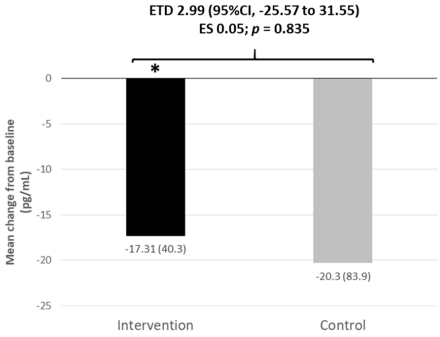


(**h**) Interleukin-6.

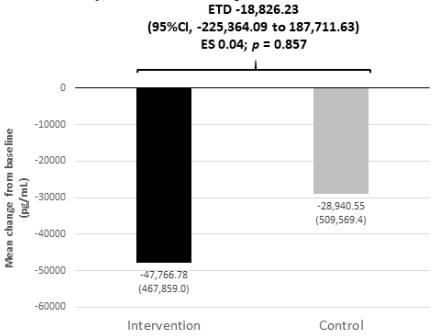


(**i**) Interleukin-12p70.

Figure 1. *Cont.*



(**j**) Matrix metalloproteinase 10.



(**k**) Vascular endothelial growth factor.

Figure 1. (**a**–**k**). Inflammatory markers and angiogenic factors at month 12. CI, confidence interval; ETD, estimated treatment difference; ES, effect size (Cohen's d). Figures between brackets are standard deviations. * $p < 0.01$ vs. baseline; ** $p < 0.001$ vs. baseline.

4. Discussion

Overall, our results indicate that in patients with unilateral wet age-related macular degeneration, short-term treatment with the original AREDS formulation at doses approved in Europe supplemented with DHA, lutein, zeaxanthin, resveratrol and hydroxytyrosol has no significant differential effect on visual acuity compared with the original AREDS formulation at doses approved in Europe. The supplemented formula exhibits a significant and, in most cases, relevant effect in terms of reduction of some inflammatory cytokines and a greater improvement in the fatty acid profile and serum lutein concentration. Both formulations were generally well-tolerated.

Both supplements were associated with minimal changes in visual acuity, with no significant differences between them. Although difficult to compare, these results are consistent with those of the AREDS trial. In the AREDS trial, the median visual acuity score was maintained in the overall population, with a small reduction from a median of 86 to 85 after one or two years among patients with no or few drusen at baseline and no change in the same period among those with large drusen at baseline [28]. In the AREDS2, none of the AREDS formulations were associated with relevant worsening or improving of visual acuity [11].

Compared to the original AREDS formulation at doses approved in Europe, the supplemented formula was associated with a significant and relevant increase in DHA, total n-3 PUFAs and total n-3 LCPUFAs and a significant and relevant decrease in total n-6 PUFAs and the ratios of n-6/n-3 PUFAs and LCPUFAs. These results indicate that the

addition of DHA to the original formula has a positive and relevant impact on the fatty acid profile. However, there is great controversy about the beneficial effect of these fatty acids in AMD. Fish consumption appears to be associated with a significant reduction in the risk of developing AMD according to some meta-analyses [29,30]. However, a systematic review analyzing two placebo-controlled trials of omega 3 fatty acid supplements found that this supplementation in subjects with AMD for periods up to five years is not associated with a reduced risk of the progression of AMD or the development of relevant visual loss [31]. The results of the AREDS2 trial [11] support the results of that systematic review. However, an analysis of the Nutritional AMD Treatment 2 (NAT2) trial comparing the prophylactic effect of oral DHA with placebo showed that patients who maintained high levels of red blood cell membrane EPA/DHA had a reduced likelihood of choroidal neovascularization compared to those who maintained consistently low levels (hazard ratio 0.32, 95% CI 0.10 to 0.99) [18]. Bearing in mind the overall evidence, some authors consider that the beneficial effect of omega-3 fatty acids should be further evaluated using other formulations and/or populations of AMD patients [32].

Chronic inflammation is involved in the pathogenesis of AMD, as shown by the elevations in local and systemic proinflammatory markers in patients with AMD [33–35]. Chronic inflammation has been linked to a transformation of the tissue microenvironment into a senescence-associated secretory phenotype, releasing proinflammatory cytokines such as IL-1β, TNF-α, IL-6 and C-reactive protein [36,37]. In our study, the supplemented formula was also associated with a significantly greater reduction in inflammatory markers compared to the standard formula, more specifically with a significant reduction in IL-8, IL-1β and TNF-α and, albeit not significant, with a reduction in IL-6. All these changes were of moderate effect size with a Cohen's d of approximately 0.50. IL-1β is a potent proinflammatory cytokine whose upregulation induces angiogenesis and neuroinflammation [38] and has been reported to be an inflammatory mediator in the development of wet AMD, since these patients have shown increased plasma and vitreous levels of this cytokine [39,40]; in contrast, in animal models, its inhibition significantly reduced the development of subretinal neovascularization and has been shown to prevent choroidal neovascularization [39,41,42]. IL-6 has been associated with choroidal neovascularization [43,44], and IL-8 also plays a role in inflammation and angiogenesis [45]. High levels of IL-6 and IL-8 have been found in the aqueous humor of patients with AMD compared to patients with cataracts [46], and a recent meta-analysis confirmed that the levels of IL-8 are increased in patients with wet AMD [47]. A study that compared the cytokine profiles in aqueous humor in patients with neovascular AMD found positive correlations between interleukin IL-6 and IL-8 and monocyte chemoattractant protein (MCP) 1, a key chemokine that, in turn, has been associated with wet AMD [48,49]. TNF-α has also been involved in the pathogenesis of AMD [50]. In animal models, TNF-α contributes to laser-induced choroidal neovascularization formation [51], probably by upregulating VEGF production in retinal pigment epithelium cells [52], suggesting that TNF-α could be a therapeutic target for the prevention and treatment of AMD [51]. However, despite the initial interest in anti-TNF-α drugs for the treatment of retinal disease [53], in addition to safety concerns, anecdotal case reports provide controversial results on the use of anti-TNF-α drugs in patients with AMD [54,55]. Interestingly, resveratrol, a component of the supplemented formula, can decrease the secretion of proinflammatory cytokines such as IL-6, IL-8, and TNF-α [56] and, therefore, could have contributed to the observed effects on cytokines in our study in the intervention group.

The main limitation of our study is the short-term follow-up. A one-year follow-up is appropriate for detecting changes in biochemical parameters but not in visual acuity or in the occurrence of clinical events. Another possible limitation is the use of only systemic cytokines to evaluate the macular inflammatory and stress oxidation levels. The determination of these biomarkers in blood is an indirect determination and it has not been possible to demonstrate that they reflect what is happening at the macular level. However, as occurs with the main genetic factors related to factor H (fH)-related

complement activation, which takes place locally in the retina, our group has shown in previous studies that the concentration of fH variants in plasma varies between controls, AMD patients and aging patients, which can help explain the association of the fH-H402 protein with AMD [57]. These results indicate that the AMD pathology is not an exclusively ocular process.

Although there is no generalized agreement on which are the most important components of micronutrition, supplementation is currently included in the routine management of AMD by many ophthalmologists in Europe [58]. Our study shows that in patients with unilateral wet AMD, the addition of lutein, zeaxanthin, resveratrol, hydroxytyrosol and DHA to the AREDS EU recommended doses in the short term did not have a differential effect on visual acuity compared to a standard AREDS EU formula, but, in addition to improving the fatty acid profile and increasing carotenoid serum levels, it may provide a beneficial effect on improving the proinflammatory and proangiogenic profile of patients with AMD. The impact that these changes could have on the long-term progression of AMD to more advanced stages of the disease requires further investigation. It would be worth evaluating the long-term impact of this supplementation in other AMD subpopulations, such as those with intermediate AMD stages.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13041253/s1, Table S1. Composition of Retilut® (intervention) and Theavit® (control); Table S2. Adverse events; Table S3. Changes in inflammatory markers and angiogenic factors at month 12; Figure S1. Patient disposition.

Author Contributions: Designed the clinical trial or Conceptualization and methodology: A.G.-L.; Laboratory assessments: S.R., M.H., P.F.-R., B.O.-A., M.C.L.-S.; Conducted the clinical trial: A.G.-L., J.N., E.H.-G., M.A.Z., P.C., N.P.-B., S.M.-M., M.J.A., R.S., J.J.E.-B., M.C.A.; Analyzed the data: A.G.-L., S.R., M.H., P.F.-R.; Review & Editing: A.G.-L., S.R., M.H., P.F.-R. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Every patient provided written informed consent before performing any study procedure.

Data Availability Statement: Data is available upon reasonable request from the corresponding authors.

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9.4 CONGRESS COMMUNICATIONS

Maternal Body Mass Index Alters Breast Milk Fatty Acid Composition– The PREOBE Follow up

PT06.2

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BACKGROUND & AIM

Breast milk fatty acid (**FA**) composition plays an important role in **children's growth** and **development**, but there is limited information about it corresponding to **maternal nutritional status**.

The aim of this study was to analyze the differences of **mature breast milk FA composition** in lactating women of different **pre-pregnancy** body mass index (**BMI**).

METHODS

Women (**n=65**) from a population-based pregnancy cohort of the **PREOBE Project** were divided in 3 different groups according to **pre-pregnancy BMI**; **normal-weight** (BMI: 18.5-24.9), **overweight** (BMI: 25-29.9) and **obese** (BMI≥30). Collection of **mature breast milk** was performed **28-32 days postpartum** and samples were used for **FA analysis,** which were expressed as percentages of the total amount of FAs.

RESULTS

Fatty acid levels in breast milk differed according to maternal weight…

Compared to normal-weight mothers, mature milk of:

Overweight mothers showed → **> C22:5n-6** and **n6:n3 ratio**, **< DHA** and **EPA:AA ratio**

Obese mothers showed → **> c23:0, SFAs, C22:2n-6, C22:5n-6**, **< C18:1n-9, MUFAs** and **C18:3n-3**

	NORMAL-WEIGHT		OVERWEIGHT		OBESE	
	Mean	SD	Mean	SD	Mean	SD
SFAs						
C6:0	0.08	0.03	0.10	0.04	0.08	0.07
C8:0	0.23	0.07	0.24	0.09	0.24	0.10
C10:0	1.29	0.37	1.36	0.33	1.47	0.32
C12:0	4.86	1.80	4.86	1.92	5.32	1.65
C14:0	4.79	1.71	4.67	1.66	5.23	1.22
C15:0	0.21	0.06	0.20	0.05	0.22	0.08
C16:0	19.56	2.29	19.53	2.02	21.20	2.55
C17:0	0.29	0.08	0.29	0.06	0.32	0.07
C18:0	5.74	0.41	5.88	0.67	6.03	0.49
C20:0	0.18	0.01	0.17	0.03	0.18	0.04
C22:0	0.06	0.02	0.08	0.03	0.07	0.02
C23:0	**0.05**	**0.02**	0.06	0.03	**0.09**	**0.07***
C24:0	0.05	0.02	0.06	0.03	0.06	0.03
SFA	**25.83**	**2.48**	25.95	2.49	**27.80**	**2.62***
MUFAs						
C14:1	0.10	0.03	0.11	0.04	0.12	0.06
C16:1, n-9	0.43	0.06	0.45	0.07	0.46	0.10
C16:1, n-7	1.83	0.62	1.80	0.49	1.88	0.58
C17:1	0.17	0.03	0.18	0.04	0.17	0.05
C18:1, n-9	**39.69**	**4.23**	38.73	5.53	**36.63**	**0.96***
C18:1, n-7	1.58	0.24	1.63	0.23	1.59	0.32
C20:1, n-9	0.50	0.07	0.46	0.05	0.48	0.06
C22:1, n-9	0.09	0.02	0.09	0.02	0.09	0.01
C24:1	0.06	0.02	0.06	0.04	0.07	0.03
MUFA	**43.73**	**4.17**	42.76	5.70	**40.74**	**1.37***

	NORMAL-WEIGHT		OVERWEIGHT		OBESE	
	Mean	SD	Mean	SD	Mean	SD
Omega 6						
C18:2, n-6 (LA)	13.60	3.21	15.20	3.86	13.90	3.14
C18:3, n-6 (GLA)	0.17	0.06	0.18	0.05	0.17	0.05
C20:3, n-6 (DGLA)	0.47	0.11	0.48	0.06	0.52	0.14
C20:4, n-6 (AA)	0.49	0.05	0.49	0.12	0.47	0.10
C22:2, n-6	**0.04**	**0.01**	0.06	0.03	**0.06**	**0.02***
C22:4, n-6 (AdA)	0.10	0.02	0.12	0.04	0.12	0.03
C22:5, n-6 (DPAn6)	**0.05**	**0.01**	**0.07**	**0.03***	**0.09**	**0.05***
n-6 PUFA	15.24	3.31	16.89	3.93	15.64	3.19
n-6 LC-PUFA	1.48	0.22	1.52	0.24	1.58	0.34
Omega 3						
C18:3, n-3 (ALA)	**0.59**	**0.21**	0.58	0.16	**0.46**	**0.08***
C20:5, n-3 (EPA)	0.07	0.03	0.06	0.02	0.07	0.02
C22:5, n3 (DPAn3)	0.11	0.03	0.11	0.03	0.12	0.04
C22:6, n-3 (DHA)	**0.28**	**0.11**	**0.22**	**0.06***	0.25	0.07
n-3 PUFA	1.05	0.24	0.96	0.21	0.90	0.13
n-3 LC-PUFA	0.45	0.16	0.38	0.11	0.44	0.09
Ratios						
EPA:AA	**0.12**	**0.03**	**0.09**	**0.04***	0.12	0.07
DHA:AA	0.77	0.19	0.76	0.24	0.90	0.34
n6:n3	**15.08**	**3.91**	**18.28**	**5.33***	17.80	4.97
n6:n3 LC-PUFA	3.62	1.18	4.28	1.24	3.73	0.92

Independent-sample T Test was used to evaluate significant differences (*p<0.05) with normal-weight group.

CONCLUSIONS

- ✓ In conclusion, **maternal weight affects FA concentrations in mature breast milk.**
- ✓ Our results suggest that the quality of breast milk is compromised in women with a BMI>25 which could also affect the quality of nutrients supplied to the neonate.
- ✓ Since diet influences breast milk FAs, overweight and obese women could benefit from dietary recommendations to optimize breast milk FA composition.

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Breast milk fatty acids influence infant growth & cognition: The PREOBE Study

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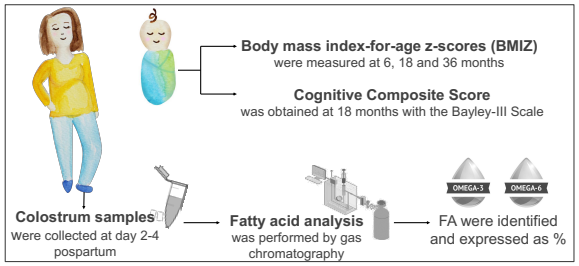
BACKGROUND & METHODS

Breast milk reflects the maternal nutritional status and is a key source of polyunsaturated fatty acids (PUFAs), crucial in growth and development, especially arachidonic (AA) and docosahexaenoic acid (DHA).

…This study aimed to analyze the effect of **colostrum fatty acids** (FAs) **on infant outcomes** to raise awareness about the programming effect of maternal nutrition and promote a healthy diet in women.

For this analysis, **78 mother-child pairs** of the PREOBE cohort were considered. Associations were evaluated using lineal regression analyses and corrected for maternal pre-pregnancy BMI, maternal smoking, weight gain during pregnancy, maternal education, sex of the child and type of infant feeding practice.



RESULTS

INFANT BMIZ

was positively associated with

Colostrum n6:n3 ratio

and negatively associated with

Colostrum AA, DHA, n3 PUFA, n6 LC-PUFA and n3 LC-PUFA

Colostrum FAs	Infant age	Infant BMIZ β	Infant BMIZ *p*
C20:4n6 (AA)	**6 m**	**-0.44**	**0.016**
	18 m	-0.03	0.89
	36 m	0.30	0.30
C22:6n3 (DHA)	**6 m**	**-0.37**	**0.043**
	18 m	0.14	0.42
	36 m	0.42	0.29
n6 PUFA	6 m	0.21	0.32
	18 m	0.16	0.41
	36 m	0.20	0.587
n3 PUFA	**6 m**	**-0.38**	**0.047**
	18 m	0.16	0.38
	36 m	-0.20	0.60
n6 LC-PUFA	**6 m**	**-0.38**	**0.047**
	18 m	-0.05	0.77
	36 m	0.40	0.22
n3 LC-PUFA	**6 m**	**-0.43**	**0.020**
	18 m	0.07	0.70
	36 m	0.28	0.44
n6:n3	**6 m**	**0.42**	**0.031**
	18 m	-0.04	0.82
	36 m	0.30	0.34

N= 37, 38 and 16 according to low-to-high age of assessment.

INFANT COGNITION

was positively associated with

Colostrum LA and n6 PUFA of normal-weight mothers
Colostrum DHA and n3 LC-PUFA of overweight mothers

and negatively associated with

Colostrum n6:n3 ratio of overweight mothers

Colostrum FA	Maternal pre-pregnancy BMI	Infant Cognition β	Infant Cognition *p*
C18:3n3 (ALA)	Normal-weight	0.29	0.581
	Overweight	0.44	0.393
	Obesity	0.55	0.468
C18:2n6 (LA)	**Normal-weight**	**0.84**	**<0.001**
	Overweight	-0.95	0.061
	Obesity	0.12	0.869
C20:4n6 (AA)	Normal-weight	-1.23	0.136
	Overweight	0.32	0.594
	Obesity	0.48	0.416
C22:6n3 (DHA)	Normal-weight	-0.73	0.124
	Overweight	**0.88**	**0.045**
	Obesity	-0.03	0.954
n6 PUFA	**Normal-weight**	**0.81**	**0.002**
	Overweight	-0.97	0.111
	Obesity	0.16	0.803
n3 PUFA	Normal-weight	-0.23	0.687
	Overweight	0.70	0.057
	Obesity	0.13	0.829
n6 LC-PUFA	Normal-weight	0.01	0.984
	Overweight	0.53	0.227
	Obesity	0.28	0.650
n3 LC-PUFA	Normal-weight	-0.71	0.113
	Overweight	**1.01**	**0.004**
	Obesity	0.04	0.949
n6:n3	Normal-weight	0.74	0.067
	Overweight	**-0.97**	**0.002**
	Obesity	0.01	0.985

N= 14 (normal-weight), 11 (overweight) and 12 (obese).

CONCLUSIONS

The **early supply of n6 and n3 impacts infant nutritional status and cognition**, at 6 and 18 months of life, respectively. This study endorses the need for preventive health care. Since breast milk influences the early nutritional status of the child, which is related to health conditions through life span, a healthy diet in women should be encouraged to increase the quality of breast milk and promote healthier future generations.

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Breastfeeding promotes a healthier fatty acid composition in the offspring compared to artificial and mixed feeding: The PREOBE Study

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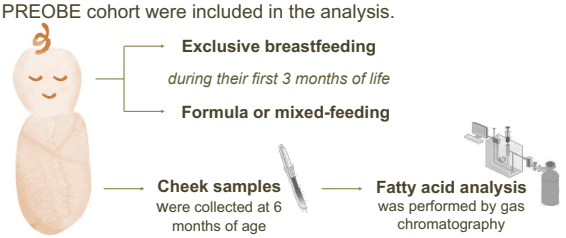
BACKGROUND & AIM

Early life nutrition has a programming effect in the future health of the child and, even when the World Health Organization recommends exclusive **breastfeeding** up to 6 months of age with continue to 2 years of age or beyond, the rate of breastfeeding is alarmingly low. Since polyunsaturated fatty acids (**PUFAs**) and long-chain PUFAs (**LC-PUFAs**) play a crucial role in survival, growth and development of the child…

…the aim of this study was to **analyze the effect of infant feeding practices in their FA levels** and to discuss the implications in future health to promote breastfeeding in the scientific community and citizens.

METHODS

We analyzed the association of infant PUFAs with exclusive breastfeeding, over mix or formula-feeding. 102 children of the PREOBE cohort were included in the analysis.



RESULTS

Association of infant PUFAs with exclusive breastfeeding, over mix or formula-feeding

	ß	p
c18:2n6 (LA)	**0.37**	**0.000**
c20:4n6 (AA)	**0.22**	**0.026**
n6 PUFA	**0.39**	**0.000**
n6 LC-PUFA	**0.23**	**0.019**
c18:3n3 (ALA)	**-0.41**	**0.000**
c22:5n3 (DHA)	**0.29**	**0.003**
n3 PUFA	0.08	0.440
n3 LC-PUFA	**0.22**	**0.032**

Associations were evaluated using lineal regression analyses and corrected for maternal pre-pregnancy BMI and infant gender.

Infant fatty acids **positively** associated with exclusive breastfeeding

LA, AA, PUFAn6, LCPUFAn6, DHA, LCPUFAn3

Infant fatty acids **negatively** associated with exclusive breastfeeding

ALA

(possibly related to an increased DHA conversion)

CONCLUSIONS

Exclusive breastfeeding is associated to a healthier infant fatty acid composition, known to enhance neurodevelopment and growth and protect from conditions, such as cardiovascular diseases and allergies. This information provides further evidence of the positive programming impact of breastfeeding in future health. Dissemination of these results can contribute to raise public awareness and engage society and health care providers to promote breastfeeding beyond other feeding practices.

FUNDING

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