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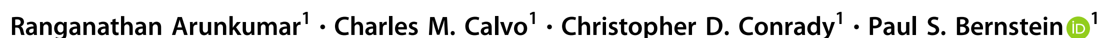
REVIEW ARTICLE

What do we know about the macular pigment in AMD: the past, the present, and the future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Abstract

Carotenoids are lipophilic isoprenoid pigments with a common $C_{40}H_{56}$ core chemical structure that are naturally synthesized by many plants, algae, bacteria, and fungi. Humans and animals cannot synthesize carotenoids de novo and must obtain them solely through dietary sources. Among the more than 750 carotenoids in nature, only lutein, zeaxanthin, *meso*-zeaxanthin, and their oxidative metabolites selectively accumulate in the foveal region of the retina where they are collectively referred to as the macular pigment (MP) of the macula lutea. MP serves an ocular protective role through its ability to filter phototoxic blue light radiation and also via its antioxidant activity. These properties have led to the hypothesis that carotenoids may protect against the development of age-related macular degeneration (AMD), the most common cause of blindness in the aged population >60 years old. Epidemiological studies have supported this by showing that patients with lower concentrations of serum carotenoids and macular pigment optical density (MPOD) measurements are at a higher risk of developing AMD. Conversely, nutritional supplementation and diets rich in lutein and zeaxanthin readily impact MP concentrations and reduce the risk of progression to advanced AMD, and the AREDS2 supplement formulation containing 10 mg of lutein and 2 mg of zeaxanthin is the standard-of-care recommendation for individuals at risk for visual loss from advanced AMD. This article reviews the rich history of research on the MP dating back to the 1700s and outlines their potential for further therapeutic improvements for AMD in the future.

Macular pigment background, history, and relationship to AMD

Structure and chemistry of the macular pigment (MP)

The unique structure of each carotenoid defines its physical, chemical, and biological properties. The macular carotenoids belong to the xanthophyll family with a molecular formula of $C_{40}H_{56}O_2$. They have a conjugated double-bond backbone and cyclohexene end groups with hydroxyl group attached at the 3 and 3′ positions. The chemical structures of the macular carotenoids are shown in Fig. 1. Their rigid conjugated double-bond structure is responsible for carotenoid color and their ability to quench free radicals and reactive oxygen species. The macular xanthophyll carotenoids are relatively more polar compared to carotenes due to the presence of hydroxyl (O–H) groups which alter their polarity and solubility and also contribute to their antioxidant properties [1]. The hydroxyl functional group permits MP and its isomers to cross the blood-brain and blood-ocular barriers, and the position and chirality of the hydroxyl groups of MP contribute to their differences in absorption, transport, metabolism, tissue uptake, and potential orientation in bilayer membrane relative to each other and relative to other carotenoids [2]. The MP carotenoids also differ from each other by the location of one double-bond. The double-bond at the 4′, 5′ position in lutein is shifted to 5′, 6′ position in zeaxanthin and *meso*-zeaxanthin. Lutein exists in eight stereoisomeric forms as a result of three stereocenters at C-3, C-3′and C-6′, but the lutein component of the MP consists of a single stereoisomer, (3R, 3′R, 6R)-β,ε-carotene-3,3′-diol. Zeaxanthin has three stereoisomers which include RR-

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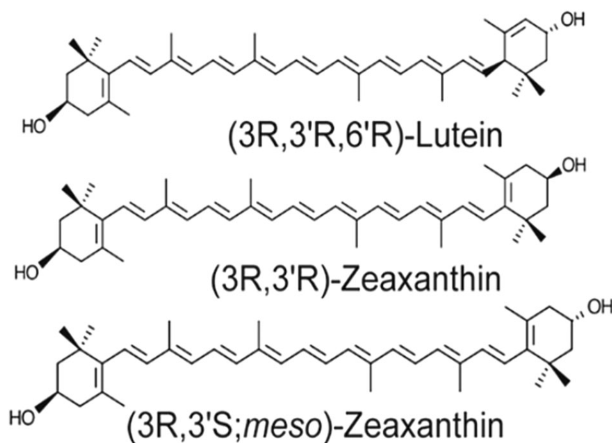

Fig.1 Structure of macular carotenoids. Lutein, zeaxanthin, and *meso*-zeaxanthin are the three carotenoids present in the primate macula. They are structural isomers with the same molecular formula $C_{40}H_{56}O_2$

zeaxanthin [(3R, 3′R) - β, β -carotene-3, 3′diol] (dietary zeaxanthin), SS-zeaxanthin [(3S, 3′S) β, β- carotene-3, 3′-diol] (found only in trace amounts in nature), and *meso*-zeaxanthin [(3R, 3′S)- β, β -carotene-3,3′ diol] (rarely found in nature outside of the primate fovea). The position of the hydroxyl group at the 3′ carbon of lutein is classified as more allelic compared to zeaxanthin and *meso*-zeaxanthin [3].

MP distribution and orientation

Currently, 750 carotenoids have been identified in nature, but only about 40 carotenoids are found in major human foods. About 15 carotenoids have been identified in human blood and tissues, but only lutein, zeaxanthin, and their metabolites are detected in the human retina [4]. MP has a unique distribution in the retina with high concentrations in the fovea and inner plexiform layer of the retina (Fig. 2). The foveal Henle fiber layer has the highest concentration of MP at around 0.1 to 1 mM, and its concentration declines rapidly with increasing eccentricity. The ratio of lutein: zeaxanthin is 1:2.4 in the center (0–0.25 mm) to over 2:1 in the periphery (8.7–12.2 mm) of the human retina. There is a 100-fold drop in the macular pigment concentration in the peripheral retina compared to the fovea, although the MP levels vary widely between individuals. The variation in MP ratio with eccentricity has been linearly correlated with the corresponding rod and cone ratio [5]. Lutein and its isomers adopt perpendicular and parallel orientations to the plane of biological membranes, whereas the zeaxanthins adopt a perpendicular orientation to the plane of the membrane [6]. Transmembrane orientations of lutein and zeaxanthin have been shown to decrease the membrane's susceptibility to lipid oxidation and enhance the rigidity of lipid bilayer and thus act as 'molecular rivets'.

Biological function of MP against AMD

MP has its peak absorption at 460 nm (Fig. 3) where it can absorb 40–90 % of incident high-energy, short-wavelength visible light depending on its concentration [7]. A primary function of MP is the reduction of blue-light scattering in the central retina, and the deep yellow color and anatomical location of MP are thought to be ideal to protect the foveal region from photo-oxidative damage. While all MP molecules attenuate blue-light exposure, lutein filters blue light more efficiently than zeaxanthin and *meso*-zeaxanthin due to its orientation within the lipid bilayer [6], and primates fed with a xanthophyll-free diet from birth are more prone to blue-light induced damage [8]. Photoreceptor cells are more prone to oxidation than other cells in the retina and rely on the carotenoids for protection due to their greater oxidative stability. Chucair et al. demonstrated that rat retinal neurons treated with macular carotenoids have greater protection from oxidative stress than an untreated group [9]. The identification of lutein and zeaxanthin oxidative metabolites in the retina by Khachik et al. further supports the function of macular pigment in retinal oxidative protection [4].

Dietary sources of lutein and zeaxanthin

MP is not synthesized de novo in animals and must be obtained by dietary ingestion. Several studies have shown that increased dietary consumption of lutein and zeaxanthin results in a lower incidence of AMD [10–12]. Green leafy vegetables (spinach, parsley, kale, lettuce, broccoli, zucchini, etc.), orange-yellow fruits (orange, tangerine, papaya, and mango) are superior sources of macular carotenoids [13, 14]. Among cereals, durum wheat, corn, and their byproducts contain lutein and zeaxanthin [15, 16]. In animals, lutein and its isomers are mainly found in egg yolk, fish skin, carapace (shell) of crustaceans, and in the skin and integuments of birds. Among macular carotenoids, lutein is the most common carotenoid found in food sources. Zeaxanthin is found in smaller quantities, and *meso*-zeaxanthin rarely exists in the human diet. In 1986, Maoka et al. first reported the presence of *meso*-zeaxanthin in shrimp carapace, fish skin, and turtle fat [17]. In the past, due to difficulties in separating lutein from zeaxanthin, the carotenoid concentration in the foods was often termed as the sum of lutein and zeaxanthin (L+Z). As HPLC methods improved, lutein could be reliably separated from other MP carotenoids, but zeaxanthin and *meso*-zeaxanthin still co-eluted. It was not until the development of chiral chromatography that *meso*-zeaxanthin could be successfully separated from dietary zeaxanthin [18, 19]. Lutein and zeaxanthin supplementation is generally recognized as safe (GRAS) for human consumption, and the European Food Safety Authority (EFSA) recently accepted that consumption of 1 mg/kg of bodyweight of lutein with 80% total carotenoids

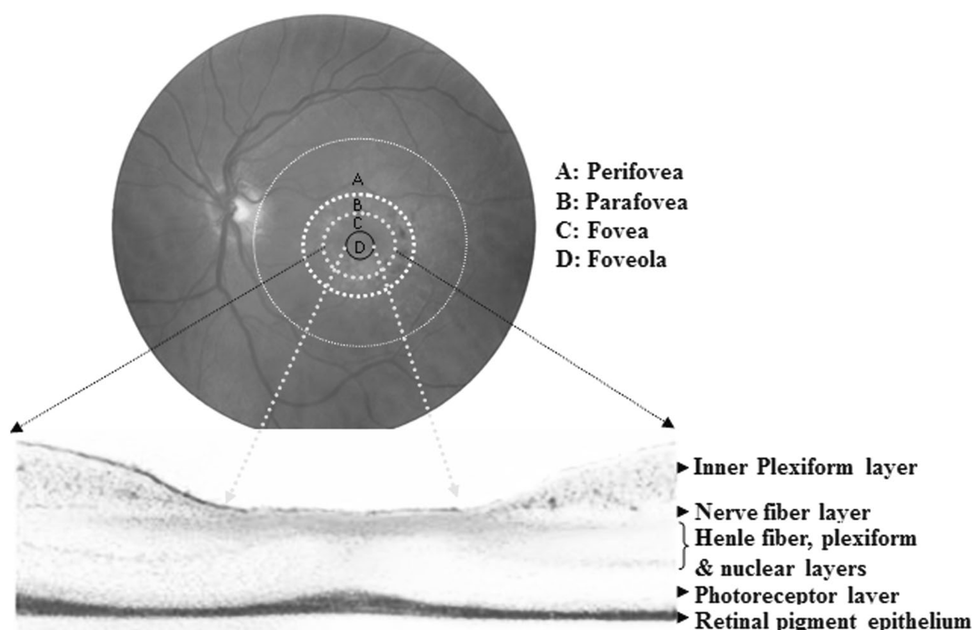

Fig. 2 Topography of the macular pigment, schematically showing the distribution of the yellow macular pigment across the retina: horizontally (top) and vertically (bottom)

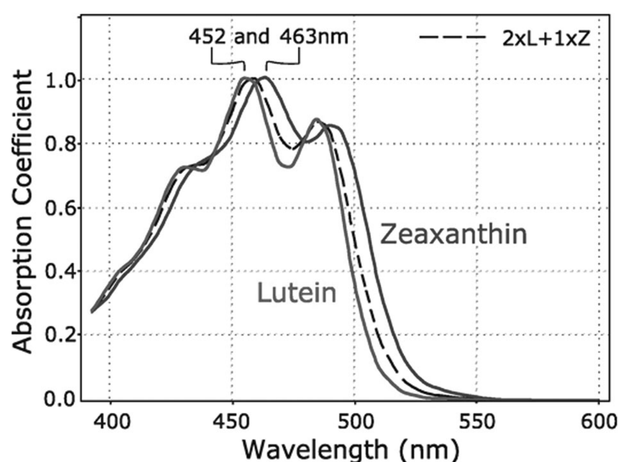

Fig. 3 Absorption spectra of lutein (red) and zeaxanthin (blue) in olive oil. A mixture of lutein plus zeaxanthin (dashed black line) closely approximates the absorption spectrum of the macular pigment in the living human eye

content derived from marigold (*Tagetes erecta*) is safe. The no observed adverse effect level (NOAEL) for lutein and zeaxanthin is 400 mg/kg of bodyweight/day [20], and for *meso*-zeaxanthin it is 300 mg/kg of bodyweight/day [21]

Identification of the macular pigment

More than two centuries ago, the initial observation of yellow pigmentation in the central retina of humans was reported by Buzzi in 1782 [22]. Later, Soemmering discovered the foramine centrali limbo luteo (the central yellow-edged hole) in 1795 [23]. Home and Sommering made a detailed review on the macula lutea in 1798 [24]. They also reported that the yellow spot was not found in dissections of any animals other than humans and primates. This was the first evidence that macular pigment is exclusive to humans and primates. In 1866, the idea that MP may filter blue light and protect against harmful short wavelength radiation was first described by Max Schultze [25]. In 1945, Wald remarked that MP exhibited a characteristic carotenoid spectrum and concluded that the pigment belongs to the xanthophyll family of carotenoids [26]. Forty years later, Bone et al. (1985) first characterized the MP using high-performance liquid chromatography (HPLC) and reported that MP consists of two distinct dietary xanthophyll carotenoids, lutein and zeaxanthin [27], and this was later confirmed by Handelman et al. in 1988 [28]. The third and final component of MP, *meso*-zeaxanthin, was identified in retina by Bone et al. in 1993 [3]. The cross-sectional location of MP within the retina had not been clarified until Snodderly et al. localized the xanthophyll pigments to the Henle fiber layer [29]. Bernstein et al. in 2001 reported that carotenoids are not only located in the macula but are also present in the human lens and in virtually all the tissues of human eyes [30]. The origin of *meso*-zeaxanthin within the macula had not been elucidated until Johnson in 2005 reported that dietary lutein is the precursor for *meso*-zeaxanthin based on feeding studies of carotenoid-depleted monkeys [31]. Johnson's finding was further confirmed by Bhosale et al., where they fed deuterium-labeled lutein and zeaxanthin supplements to quail and determined that dietary lutein is the precursor for *meso*-zeaxanthin and that dietary zeaxanthin is the precursor for 3′oxolutein, β-apo-2′-carotenol, adonirubin, astaxanthin, galloxanthin, and ε,ε-carotene [18]. Recently, Shyam et al. identified RPE65 as the

isomerase enzyme responsible for conversion of dietary lutein to *meso*-zeaxanthin in the retinal pigment epithelium of vertebrates [32].

Bioavailability of MP

Carotenoid bioavailability is one of the vital factors affecting its biological functionality and effectiveness. Bioavailability is termed as the 'fraction of ingested nutrients that is available for normal physiological function or storage.' The fraction of ingested carotenoids that becomes available for physiologic function is highly variable, and it depends on the source and amount of carotenoid consumed and the influence of other dietary factors [33]. Despite their various health benefits, macular carotenoids are poorly bioavailable due to their hydrophobicity. Carotenoid intestinal absorption involves several steps starting with the mechanical and enzymatic disruption of carotenoids from the food matrix, its release into the aqueous solution, followed by its incorporation into lipid droplets, and transfer to mixed micelles. Carotenoids in mixed micelles are absorbed by the intestinal cells via passive or facilitated absorption. Until the discovery of SR-B1 protein and CD36, researchers believed that carotenoid absorption took place by simple diffusion. The carotenoid cleavage enzymes, β-carotene-15,15′-oxygenase (BCO1) which cleaves carotenes symmetrically at the 15-15′ C = C results in formation of retinal from β-carotene, but BCO1 cannot cleave MP carotenoids due to lack of one non-substituted β-ionone ring, a requirement for BCO1's cleavage activity. BCO2 cleaves xanthophyll carotenoids at the 9′, 10′ C = C, resulting in formation of three possible cleavage products: 10′- apo-β-carotenol (C27), β-ionone (C13), and a dialdehyde (C9). BCO2 is relatively inactive in humans, and as a result, lutein and zeaxanthin are stable in humans as opposed to mice where a very active BCO2 cleaves ingested xanthophylls [34]. Next, the uncleaved MP are packed into chylomicrons and transported into the lymphatic system to the liver and then transported to targeted tissues by the general circulation [35].

Each step of carotenoid absorption is influenced by various factors, making it difficult to assess the effects of each factor on overall carotenoid bioavailability [36, 37]. The key factors that affect carotenoid bioavailability are Species of carotenoid, molecular Linkage, Amount of carotenoids consumed, carotenoid Matrix, Effectors of absorption and bioconversion, Nutrient status of the host, Genetic and host-related factors, and mathematical Interactions (collectively known by the acronym SLAMENGI) [36]. There are several studies showing that addition of lipid improves the absorption of macular carotenoids [38, 39]. A complex food matrix also influences carotenoid bioavailability, as carotenoid uptake from oil-based supplements show better bioavailability than from whole vegetable sources [40]. Dietary fiber inhibits macular carotenoid absorption by entrapping them and also inhibiting lipase activity and micelle formation [38]. The presence of other carotenoids such as β-carotene leads to competition for incorporation into micelles and reduction of macular carotenoid absorption [41].

Rodents have been extensively used as animal models for carotenoid research. In one of the earlier reports, High and Day (1951) evaluated the effect of carotenoids on vitamin A storage and growth rate in rats [42]. Several studies have been reported on the bioavailability of xanthophylls in the rodent models [38, 43]. Due to species variations and other factors influencing carotenoid bioavailability, researchers are moving towards the genetically modified knockout mice models to obtain a better model for carotenoid metabolism. The genetically engineered 'macular pigment mouse' model with knockout of BCO2 was developed to study the protective effects of MP in genetic and environmental models of human ocular disease. In contrast to humans, wild-type mice have very active BCO2 xanthophyll cleaving enzymes, resulting in no detectable accumulation of macular carotenoids in their retinas. An unusual insertion of—GKAA— amino acids near the substrate binding tunnel appears to be unique to primate BCO2 and insertion of this sequence into the mouse enzyme leads to its inactivation; germ-line knockout of mouse BCO2 results in MP deposition in the mice retina [34, 44]. Non-human primates are considered even better models to study carotenoid metabolism, but their high cost of care, space requirements, and handling difficulties limit their use. Birds are also used for MP research, but they differ significantly from humans due to a broader diversity of carotenoids, many of which are esterified to long-chain fatty acids and deposited in oil droplets, making them difficult to extract and analyze without artifacts.

Macular carotenoid binding proteins and MP transport to retina

Lipoproteins such as low density lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL) and chylomicrons and chylomicron remnants and their receptors CD36 and SRB1 are involved in macular carotenoid uptake [45]. In vitro studies using intestinal caco-2 cells suggested the possible role of receptor proteins during the process of carotenoid uptake [46]. Scavenger receptor class B member 1 (SR-BI), a cell surface glycoprotein and a non-specific lipid transporter, is involved in the competitive uptake of carotenoids in the gut prior to transport to the liver by chylomicrons. Recently, Lobo et al. reported the presence of ISX (intestine-specific homeobox), a transcription factor that governs carotenoid absorption via SR-B1 and BCO1 mRNA expression, which is regulated by a carotenoid metabolite, retinoic acid [47]. Several studies have revealed that SR-B1 and CD36 can function as

carotenoid transporters into the retina [48, 49]. The role of the third SR-B family member, SR-B2, in carotenoid transport was not clear until Shyam et al. reported that all three human SR-B proteins (SR-B1, SR-B2, and CD36) were capable of binding all three macular carotenoids, and once delivered to the retina, they are stabilized by the presence of high levels of specific binding proteins in the macula [48].

While 15 unique carotenoids have been identified in human blood and tissues, the selective uptake and accumulation of lutein and zeaxanthin in the human retina had puzzled the scientific community until the discovery of their binding proteins. Tubulin, a water-soluble, low specificity carotenoid binding protein was identified by Bernstein et al. in 1997 [50]. Tubulin proteins are found abundantly in the photoreceptor axon layer of the fovea and may be involved in the high concentration and stabilization of MP in the retina, but they exhibit relatively weak and non-specific binding affinity for MP, so the search continued for more specific, higher affinity macular carotenoid binding proteins. Using human macular membrane preparations, glutathione S transferase P1 (GSTP1) was identified as a zeaxanthin-binding protein, and subsequent research revealed that GSTP1 protects the lipid membrane from oxidation [51]. Out of 15 known human steroidogenic acute regulatory domain proteins, steroidogenic acute regulatory domain protein 3 (StARD3) also known as metastatic lymph node 64 protein (MLN64) was identified as a lutein-binding protein [52]. The macular distributions of MP and its binding proteins are shown in Fig. 4. The macular carotenoids share retinoid transporters such as interphotoreceptor retinoid binding protein (IRBP) and retinol binding protein 4 (RBP4) in the process of transport of MP from serum to retina [53]. MP uptake is selective and the possible pathways for MP carotenoid uptake, transport, and accumulation in the human retina are shown in Fig. 5.

HDL, LDL, VLDL and albumin play an important role in transport of these carotenoids to their respective target sites. Carotenes such as β-carotene and lycopene are highly hydrophobic and are transported on LDL, whereas lutein and zeaxanthin are hydrophilic and are transported by HDL [54]. Chickens with a natural genetic defect of HDL (the Wisconsin hypoalpha mutant, WHAM) chicken fed with high levels of lutein in the diet has high levels of lutein in plasma, liver, heart, egg yolk but not in retina, suggesting that HDL is critical for delivery of lutein to the retina [55]. Genetic variations in CD36 and BCO1 can modulate plasma lutein and retinal concentrations [56].

Age-related macular degeneration (AMD)

AMD is an acquired, degenerative disorder of the macula that is the leading cause of irreversible blindness and

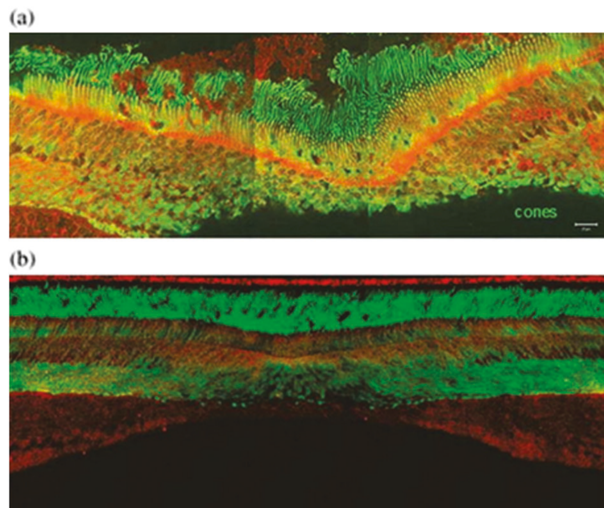

Fig.4 The retinal distribution of macular pigment binding proteins. **a** GSTP1 labeling of foveal cones in the macula of a 3-year-old monkey. This montage shows strongest labeling by antibody against GSTP1 (red) over the myoid and ellipsoid regions of cones identified by monoclonal antibody (7G6, green). **b** A low-magnification view of a near-foveal retina section in which N-62 StAR (red) identifies StARD3, an anti-cone arrestin monoclonal antibody (7G6, green) identifies monkey cones. Images courtesy of Dr. Jeanne M. Frederick

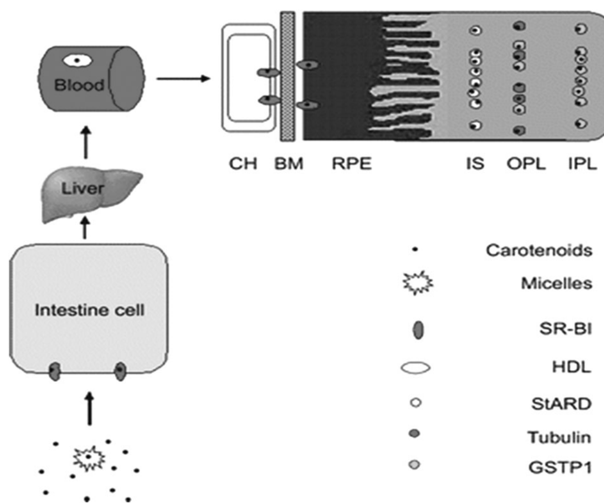

Fig. 5 Possible pathway for MP carotenoid uptake, transport, and accumulation in the human retina. Choroicapillaris (CH); Bruch's membrane (BM); Retinal pigment epithelium (RPE); Inner segments (IS); Outer plexiform layer (OPL); Inner plexiform layer (IPL), (►) Retinal transport pathway

visual disability in people greater than 60 years of age. AMD accounts for 8.7 % of all legal blindness across the globe [57]. By the end of 2020, the patients with AMD in the United States alone will exceed 3 million [58]. AMD can be classified into two forms: non-exudative (dry) and exudative (wet), and fundus images of both the dry and the wet forms compared to a normal eye are shown in Fig. 6.

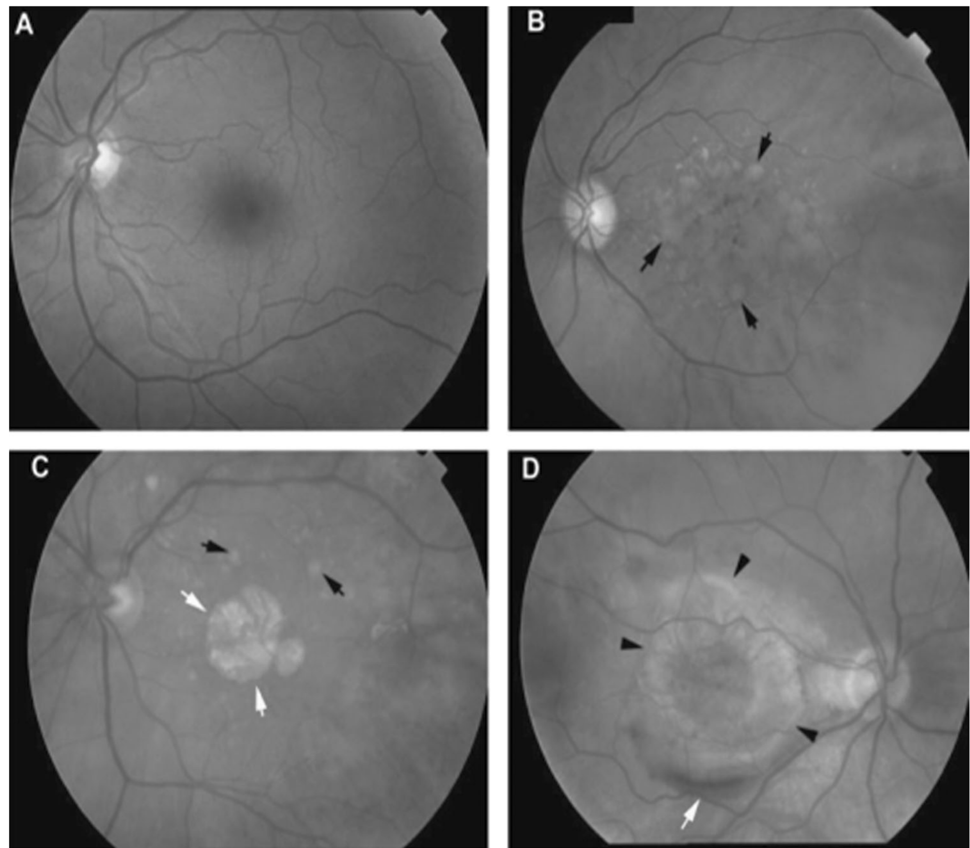

Fig. 6 Fundus images of A, normal macula; B, macula with confluent soft drusen C, macula with dry AMD; D, macula with wet AMD

The dry form is characterized by the accumulation of drusen under the macular retina caused by photo-oxidative damage of lipids and proteins along with depigmentation of the retinal pigment epithelium (RPE). The wet form is characterized by sub-RPE, sub-retinal, or intraretinal neovascularization from blood vessels originating in the choriocapillaris that have broken through Bruch's membrane. This vascular abnormality leads to macular edema, sub-retinal fluid, hemorrhage, and eventual fibrosis, resulting in permanent loss of macular vision. AMD is a complex, multifactorial disease, and its pathogenesis is not yet clearly understood. AMD has a high cost to society and patient quality of life, hence prevention has important public health implications. Hereditary is one of the major risk factors, with risk loci on chromosome 1 in the complement factor H region [59] and on the chromosome 10 in the HTRA1/ARMS2 region [60]. Advanced age is an obvious risk for AMD, with a sharp increase in prevalence in those older than 75 years of age. Other unmodifiable risk factors of AMD are female gender, light complexion, and light-colored irides. Strategies to ameliorate AMD have been based on reduction of modifiable risk factors. Smoking is considered the most important environmental risk factor. Oxidative stress, excessive light exposure, poor nutritional status, hypercholesterolemia, and hypertension are also strongly correlated with AMD.

AREDS

Through the better understanding of antioxidant macular carotenoids, vitamins, and minerals gained over the previous decades, evaluation of epidemiological data suggested that patients with higher serum concentrations of antioxidants had a significantly lower risk of developing neovascular AMD. The Eye Disease Case–Control Study group compared over 350 patients with advanced AMD with 520 matched, control subjects from similar geographic areas without AMD to identify relationships between carotenoid dietary intake and risk for AMD. The study group determined that an increased dietary intake of carotenoids, particularly spinach and collard greens, was strongly associated with decreased risk for AMD a 42% decrease in risk between the highest and lowest quintile of carotenoid ingestion [12]. There was no significant benefit of vitamin A, C, or E intake; however, there was a trend in risk reduction with high vitamin C intake from food sources. Additional large epidemiologic prospective studies have associated increased lutein and zeaxanthin intake with a decreased long-term risk of advanced AMD [10].

There have been numerous small interventional studies looking at the role of carotenoids and other nutrients in the prevention of AMD that have been reviewed elsewhere [61], but clinicians generally rely on large, randomized, prospective trials to guide clinical practice, so we will

concentrate on the AREDS and AREDS2 studies. The Age-Related Eye Disease Study (AREDS) [62] was commenced by the National Eye Institute in 1990 to study whether supplementation with antioxidants would alter the development and/or progression of AMD. This double-masked, randomized trial enrolled participants at 11 centers with extensive drusen of small, intermediate, or large size, geographic atrophy, or pigment abnormalities in one or both eyes. Additionally, patients with advanced AMD or vision loss from AMD in one eye were enrolled. The aim of the AREDS trial was to evaluate the effect of zinc and other antioxidant vitamins in doses 5 to 15 times greater than the recommended daily allowance in the progression of advanced AMD in older adults. The AREDS formulation was selected through the recommendations of an expert panel of nutritionists, biochemists, and ophthalmologists at National Eye Institute sponsored meetings. β-Carotene (15 mg (25,000 IU/day) was included in the formulation as it was commercially available at the time, unlike lutein and zeaxanthin. β-carotene was well-established in ophthalmic nutritional supplements, and its antioxidant properties were well-documented, although only trace amounts are actually found in the retina. The antioxidant vitamins C (500 mg/day) and E (400 IU/day) were also included. A randomized, placebo-controlled trial of zinc supplementation had reported a significant reduction ($P < 0.05$) in visual acuity loss in the group supplemented with zinc; therefore, zinc oxide (80 mg/day) was included in the mineral arm of the AREDS. Copper oxide (2 mg/day) was selected to prevent a copper-deficiency anemia caused by high serum zinc levels [63].

At the 5-year mark, the probability of progression to advanced AMD was 20% for those assigned to antioxidants plus zinc compared to 28% of study participants assigned to placebo, 23% in the antioxidants group, and 22% in the zinc group [62]. By the time these results were published in 2001, there had been a significant leap forward in the nutritional understanding of AMD since the study had commenced, thus highlighting the need for the AREDS2 clinical trial [63]. Also, there was new evidence during the course of AREDS trial that β-carotene supplementation was associated with an increased risk for lung cancer in smokers [64, 65]. This raised concerns about the inclusion of β-carotene in the AREDS formulation, as almost 50% of the US population at risk for AMD are former or current smokers [63].

Clinical research on macular pigment in the present

AREDS2

The AREDS2 supplement formulation was introduced into the market following the conclusion of the study in 2001. This formulation has since become the standard-of-care for those at risk for visual loss from AMD. AREDS2 was started in 2006 to evaluate the addition of lutein, zeaxanthin, and omega-3 long-chain polyunsaturated fatty acids into the AREDS formula. There was also a lower zinc content (25 mg) subgroup, as there was evidence that no greater amount could be absorbed and that high levels of zinc may result in genitourinary-related hospital admissions [66, 67]. Current and former smokers were enrolled into subgroups without β-carotene. Subgroup analysis of individuals in the entire study cohort that received lutein and zeaxanthin compared with individuals who did not receive lutein and zeaxanthin showed a statistically significant ($P < 0.05$) decrease in progression to advanced AMD [68]. Further analysis comparing groups receiving lutein and zeaxanthin without β-carotene and groups receiving β-carotene without lutein and zeaxanthin showed a significant ($P < 0.05$) reduction in progression to advanced AMD, particularly development of neovascular AMD [68]. However, the AREDS2 formulation was unable to meet the ambitious goal of an additional 25% reduction in progression to advanced AMD above the already effective AREDS formulation, but it was a safer and at least as efficacious option, specifically in former and current smokers [63]. In contrast to other published results [69], AREDS2 did not demonstrate any benefit or harm with omega-3 fatty acid supplementation in AMD.

No evaluation of MP in any fashion was performed in AREDS or AREDS2. In vivo, image-based instrumentation during the time period of these trials was not sufficiently standardized or widespread in the clinical domain [63], and psychophysical methods were considered too time-consuming to add to the already long study visits. Patients enrolled in an ancillary AREDS2 study from the Moran Eye Center, Salt Lake City, Utah, underwent MPOD and MP spatial distribution imaging and total skin carotenoid measurement by resonance Raman spectroscopy prior to the start of the AREDS2 trial [70]. They found that enrolled patients had higher than average baseline MPOD measurements than an age-matched control group, likely due to enrollment of patients taking eye supplements containing lutein and zeaxanthin for many years prior.

Modalities to clinically measure MP

From the findings of large epidemiological studies and AREDS2 that found a reduced risk of AMD progressing to advanced form with higher carotenoid consumption, groups became interested in attempting to quantify macular pigment concentrations to identify patients at risk of developing AMD [10, 62, 68]. Several measurement modalities have been proposed and have been compared elsewhere [71]. The use of high-performance liquid chromatography

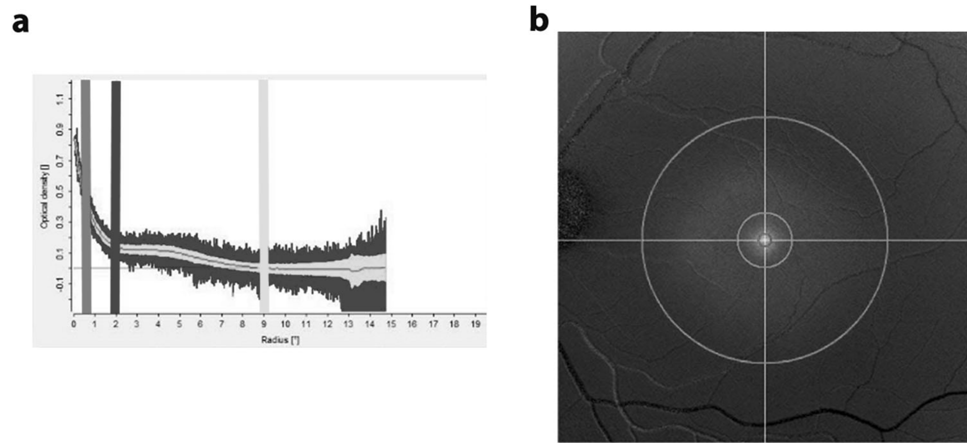

Fig. 7 Macular pigment readout from an unsupplemented normal control obtained by dual wavelength autofluorescence imaging on a Heidelberg Spectralis. **a** Macular pigment tracing at 0.5° (red line), 2° (blue line), and 9° (green line). **b** Autofluorescent image showing the fovea and the degrees (0.5°, red; 2°, blue; 9°, green) from the center of the macula lutea

(HPLC) is the gold standard analytical method to measure MP within the eye, but it is tissue destructive, time-consuming, and provides little-to-no spatial distribution information [18]. Consequently, several less invasive techniques have been designed as biomarkers of MP concentrations, but unfortunately, no technique is perfect. There are two types of methods for measuring MP in living humans: psychophysical and physical. Heterochromatic flicker photometry (HFP) and other related psychophysical methods require test subjects to make iso-iluminance matches between flickering green and blue lights to measure MP at one or a few eccentricities; however, this technique requires significant patient training to produce meaningful results. HFP also provides little information on spatial distribution of MP [61]. Despite the technical expertise required and the relative lack of spatial information gleaned, the technique can be reliably used to detect changes in macular pigment optical density in patients taking oral supplementation [72]. Standardized protocols have been designed to ease the difficulty in both administering and performing the test, and pre-adolescent children can also reliably perform the test [73, 74]. However, due to the difficulty and expertise required in administering the test, researchers have subsequently tried to identify other image-based methods to measure MP objectively.

Imaging-based techniques such as reflectometry, autofluorescence attenuation, and resonance Raman spectroscopy have gained interest, as they are less technically demanding tests to administer and provide high-resolution, quantitative spatial distributions of MP. Raman spectroscopy uses the Raman Effect in which photon scattering by molecules results in a measurable shift in wavelength specific to the chemical structure. Fundus autofluorescence exploits the fluorescent properties of lipofuscin and the predictable attenuation of the fluorescence by MP. Reflectometry, or fundus reflectance, measures reflected light from the retina and choroid and then uses complex mathematical models to derive MP data. There has been concern that these methods maybe affected by visual axis opacities (e.g., cataract) and are quite expensive to manufacture [61]. Fortunately, most of these methods have been shown to correlate with other previously mentioned techniques including HFP [71, 75]. There has been notable measurement discordance between HFP and reflectometry-based techniques [75].

In regards to dual wavelength autofluorescence (Fig. 7), we have found that volumetric measurements appear to be superior to previously used optical densities in regards to their correlative power with other carotenoid measurement techniques [71]. Groups have argued that visual axis opacities such as cataracts may alter measurements; however, we did not find a significant 'cataract effect' on volume measurements [71, 76]. The technique is also highly reproducible even in the face of significant ocular pathology such as exudative AMD and diabetic macular edema [71]. Unfortunately, dual wavelength autofluorescence is not perfect either, as it requires pupillary dilation and expensive equipment to obtain the measurements.

Others have attempted to use systemic measurements of carotenoids as a surrogate for ocular concentrations. These methods include HPLC analysis of serum, dietary assessments, and skin resonance Raman spectroscopy. Unfortunately, HPLC requires time-consuming extractions and analyses, and prior attempts at using dietary assessments have shown that they are both tedious and fraught with recall errors and bias. While skin Raman spectroscopy can be easily performed, it cannot readily differentiate various carotenoids and requires specialized equipment; it correlates well with HPLC total serum, biopsied skin tissue, and volumetric concentrations obtained by dual wavelength autofluorescence [71]. While there are numerous techniques available to quantify MP both invasively and non-invasively, each technique is hindered by either its availability, cost of production, and/or expertise in implementation. As

more individuals become interested in MP and how to measure it, the need for readily available equipment will become even more significant.

Visual performance improvement after carotenoid ingestion

Over the last decade, we have seen the development of psychophysical visual function research and the correlation of MP density with visual performance in AMD. While a majority of research on carotenoids has focused on identifying ways to quantify risks associated with AMD, emerging data from randomized, placebo-controlled, double-blinded studies have demonstrated a correlation between increases in MP optical density with improvement in visual function. This is hypothesized to also be due to the short wavelength filtering effects of MP, resulting in reduced glare, light scatter, and chromatic aberration, thereby enhancing contrast sensitivity [61]. Several randomized, controlled trials have corroborated this, with the most striking responses found in individuals with the lowest baseline MP concentrations [77]. Thus, there appears to be a beneficial role of MP supplementation in both diseased and healthy retinas; however, it is possible to develop a crystalline maculopathy from extreme supplementation [78]. While improvements in best corrected visual acuity (BCVA) are not commonly observed, gains in contrast sensitivity is significant bright light and low light contrast sensitivity, letter contrast sensitivity, glare disability, and mean reading speed showed significant association with MP after controlling for age, sex, and cataract changes [79]. Prior studies have shown that individuals who use computers or smartphones >6 h per day have benefited from carotenoid supplementation in not only increased visual performance measures, but also increased sleep quality, reduced headaches, less eye fatigue, and decreased photophobia [80, 81]. While no significant difference was measured in the National Eye Institute Visual Functioning Questionnaire 25 survey [79, 82], data exist to support contrast sensitivity as a better marker of real-world vision than BCVA [83, 84]. Consequently, macular pigment supplementation may be beneficial even to those without AMD.

The future of AMD and MP

Since the discovery of MP, there has been tremendous information and knowledge gathered about MP and its role in ocular health, particularly AMD. Researchers have identified the three distinct macular pigments, MP metabolism, and specific binding proteins of MP in the retina, but there are still many questions to be answered. Carotenoid physiology, metabolism, and molecular aspects of its antioxidant property have not yet been studied in sufficient detail. There are still knowledge gaps with regard to carotenoid absorption, transport, and distribution to the target tissues. The precise function of the metabolite *meso*-zeaxanthin and the interaction of carotenoids with BCO1 and BCO2 are unclear. The individual variations in MP peaks in the fovea and diverse responses to identical supplement interventions have not been solved at molecular levels.

The majority of the MP investigations in AMD over the last two decades were performed without the consideration of genetic predisposition to AMD or alterations in carotenoid metabolism. While many genetic risks factors for AMD have been identified, in 2018 it is still standard-of-care to assess genetic risk by obtaining only a family history. Twenty-four single nucleotide polymorphisms (SNPs) within five genes involved in the metabolism and transport of lutein and zeaxanthin have been identified in individuals with early or intermediate AMD [85], further supporting the protective role of macular carotenoids in the prevention of AMD. A large study of twins demonstrated that there was moderate heritability in the MPOD response to lutein and zeaxanthin supplementation [86]. These genetic variations seen in patients afflicted with AMD may offer an explanation on why some epidemiologic studies did not show a protective effect with lutein and zeaxanthin intake. There is a growing body of evidence supporting the variable response to carotenoid intake in increasing MP density [86, 87]. Variations in the genes complement factor H (CFH) and LOC387715/age-related maculopathy susceptibility 2 (ARMS2) have been associated with varying response to AREDS supplementation [88]. The question begs should we test all our patients with AMD prior to making supplement recommendations? Routine genetic testing for CFH and ARMS2 mutations to guide supplementation has been highly controversial and is likely to remain so. In this genomic era, there will certainly be greater numbers of identified genetic risks factors for AMD and more feasibility in testing for them in the clinical environment. However, further research will be needed to better understand the complex relationship between genetic risks, MP density, dietary intake, serum concentrations of macular carotenoids, and other lifestyle factors.

As the lifespan of individuals worldwide increases, AMD continues to be the number one cause of blindness in the elderly and is the third most common cause of blindness (8.7%) following cataracts and glaucoma [89, 90]. In order to effectively and efficiently screen individuals most at risk for AMD, there is a crucial need for the development of diagnostic equipment that is portable, cost-effective, accurate, and quick. In the future, prospective studies could be designed to elucidate whether skin and/or macular

resonance Raman spectroscopy or heterochromatic flicker photometry (HFP) are sufficient tools to accurately identify persons at risk of developing AMD associated with low MP concentrations or anomalous distributions [91, 92], thus allowing for preventative interventions prior to the onset of visual impairment. Normative databases will need to be created much like optical coherence tomography and retinal nerve fiber layer thickness to better understand an individual's carotenoid status. It is already clear that MP concentrations are readily impacted by oral supplementation through both diet and vitamins [93]. Carotenoid benchmarks could also be defined to aid in dosing concentrations of oral supplementation and monitoring compliance. Long-term studies could then be used to define at what point in an individual's life supplementation should be started, maybe even before the clinical development of AMD.

Dietary supplements containing macular carotenoids are given to AMD patients in the form of capsules, powders or beadlets to ameliorate AMD. In the future, these supplements may be given in the form of nanoformulations such as nanocapsules, and functional foods with lutein supplementation are expected to reach the market soon. Patients often inquire about various herbal supplements and alternative medicines to treat AMD such as anthocyanins [94], goji berries [95], and saffron [96]. However, the safety, pharmacokinetics, and efficacy of these substances are largely unknown, and the potential for adverse interactions with other medications or supplements cannot be overlooked. While it is highly unlikely for more large studies on the scale of AREDS2 to be conducted, there would be benefit to discovering some information about these alternative agents that some of our patients are consuming [63].

While *meso*-zeaxanthin was identified as a macular carotenoid prior to the commencement of the AREDS2 trial, it was not commercially available at that time and was not included in the formulation. *Meso*-zeaxanthin is now available commercially as a supplement, although many questions remain on how it should be utilized. *Meso*-zeaxanthin is not normally a part of the human diet and is thought to be the result of lutein metabolism [97], yet its longitudinal effect on the progression of AMD as a dietary supplement is unknown. Supplementation of *meso*-zeaxanthin along with lutein and zeaxanthin has shown improvements in psychophysical testing compared to placebo, but its impact on the progression of AMD is unclear [77]. Carotenoid-binding proteins have been demonstrated to deposit *meso*-zeaxanthin into the macula, thus supporting the hypothesis that oral supplementation of *meso*-zeaxanthin will augment MP density [48]. It is now available commercially, and logically, it should be included in one treatment arm of any potential AREDS3 trial, if that study were to be conducted one day.

Clinicians and researchers in the future will have the benefit of improved, standardized methods of quantifying MP density and monitoring changes longitudinally. As the research in psychophysical visual function and MP matures, this information could facilitate the evaluation of a patient's vision in a more comprehensive manner than the Snellen chart alone. Correlating MP changes with alterations in psychophysical testing may allow for optimization of dietary supplementation such as the lutein and zeaxanthin ratio or addition of *meso*-zeaxanthin to AREDS2 [98]. These novel techniques in conjunction with the better interpretation of the data will permit a more complete understanding and management of age-related macular degeneration.

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Compliance with ethical standards

Conflict of interest P.S.B. and the University of Utah hold a patent on the use of resonance Raman spectroscopy to measure carotenoid levels in the eye, skin, and other tissues. The remaining authors declare that they have no conflict of interest.

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