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GRAPE POMACE POLYPHENOLS IMPROVE OPHTHALMIC OUTCOMES IN PATIENTS WITH AGE-RELATED MACULAR DEGENERATION (AMD) VIA REDUCING TMAO SERUM LEVELS AND OXIDATIVE STRESS: PRELIMINARY RESULTS FROM A PILOT CLINICAL TRIAL

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Abstract

Background: Thromboembolic and/or atherosclerotic processes have been included among the main etiopathogenetic factors in development of different eye diseases, including retinal artery occlusion, which can evolve in macular degeneration. Similarly, oxidative stress (OxS) is strongly implicated in pathogenesis of age-related macular degeneration (AMD). The close link between vascular alteration and OxS is intuitive and its involvement in eye diseases is well-established. In this sense, individuating novel strategies aimed to contrast these two factors is relevant in management of AMD. Aim: Here we demonstrated the efficacy of a chronic treatment with a novel nutraceutical formulation based on Maltodextrinated Grape Pomace Extract (MaGPE) on ophthalmic outcomes, in both diabetic and non-diabetic patients with AMD. Results: After 6-month treatment, central foveal thickness (CFT) significantly reduced and visual acuity significantly increased. Moreover, serum levels of trimethylamine-N-oxide (TMAO), reactive oxygen metabolites (DROMs), and oxidized-LDL (oxLDL), significantly decreased. Conclusions: Correlation analyses demonstrated that after 6 months, TMAO, DROMs and oxLDL serum level variations, correlated positively with CFT and negatively with visual acuity, suggesting antioxidant and vascular protective activities of MaGPE as potential mechanisms of action for its effect in improving ophthalmic outcomes in AMD patients.

Keywords: *age-related macular degeneration; grape pomace polyphenols; nutraceutical; diabetes; ageing*

Introduction

Among the well-known beneficial effects of polyphenols, their positive role on vision is currently emerging as a relevant beneficial effect of these compounds (1). In particular, due to their anti-ageing potential, polyphenols have been demonstrated to be effective in both prevention and management of age-related eye diseases (2). In this sense, age-related macular degeneration (AMD) can be considered as an attractive target.

AMD is an eye disease mainly affecting middle-aged people (over 65 years) which can culminate with legal blindness and sight loss. This is due to a progressive degeneration of macula, a central retina specialized region, responsible for fine and color vision (3). In advanced stages, AMD can be classified into two types: dry and wet (4). AMD diagnosis based on combination of clinical examination and investigations, including photography, angiography, and optical coherence tomography (OCT) (4). The most common pharmacological treatment for AMD is intraocular injection of anti-VEGF agents (i.e. ranibizumab and bevacizumab), which efficacy has been widely demonstrated, although they are differently tolerated by patients (4).

According to the World Health Organization, about 64 million person in the World suffer from symptoms related to AMD or AMD-caused blindness (5). It is a disease with a multifactorial pathogenesis in which several risk factors are implicated, including age (3), genetic factors (6) and environmental/lifestyle factors, such as smoking, obesity, unhealthy diet, prolonged exposure to blue light and UV (7). Both ageing and environmental/lifestyle factors have been recognized as responsible for increased oxidative stress, whose role in AMD pathogenesis is commonly accepted (3,8).

In addition to oxidative stress, it has been reported that specific cardiovascular and inflammatory markers are correlated to AMD (9–11). More specifically, thromboembolic and/or atherosclerotic processes are among the primary causes of acute retinal artery occlusion (RAO) (12–14), which can evolve in the development of macular degeneration. Investigations, indeed, described the association between AMD and atherosclerosis (15) which, in turn, is linked to diabetes, obesity and altered gut microbiota (16–19). Interestingly, altered gut microbiota has also been reported in AMD patients (20,21). This evidence suggests that specific gut microbiota-derived compounds, whose negative cardiovascular implication is established, may also play a role in AMD.

In the last decade, scientific research spotlighted the microbiota-derived metabolite trimethylamine-*N*-oxide (TMAO) as an important prognostic marker for cardiovascular diseases (CVD), beyond traditional risk factors (22). It is an amine oxide, with structural formula $(CH_3)_3NO$ (23,24), deriving from gut microbiota metabolism of choline and L-carnitine (23), producing trimethylamine (TMA). Once produced in the gut, TMA is absorbed and oxidized by flavin-containing monooxygenase-3 in the liver (25–27). Studies on the CVD-promoting effect of TMAO focused on its pro-atherogenic effect, mainly exerted *via* promoting foam cell formation and interfering with cholesterol transport (22,28). However, besides the growing body of literature reporting the relationship between TMAO and development of CVD, a recent study investigated its role in patients with RAO, finding higher TMAO levels in this class of patients compared to controls (29).

Overall, this evidence leads to consider AMD as an oxidative stress-related disease with relative vascular implications, and this justifies the potential protective role of polyphenols, whose antioxidant and vascular-protection activities are historically-known (30). Interestingly, it has been reported that orally administered polyphenols can cross the blood-retina barrier (3). In this sense, several randomized clinical trials investigated the role of different nutritional compounds (such as polyphenols) on AMD, including the Carotenoids in Age-Related Eye Disease Study (CAREDS), the Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires (ALIENOR) study, the Taurine, Omega-3 Fatty Acids, Zinc, Antioxidant, Lutein

(TOZAL) study (31). These data highlight the interest of both researchers and clinicians in individuating nutraceutical approaches for the management of this degenerative and debilitating disease.

The first aim of the present study was to investigate the effect of a 6-month treatment with a novel nutraceutical formulation based on Maltodextrinated Grape Pomace Extract (MaGPE) on AMD progression, in both diabetic and non-diabetic patients. Then, serum levels of TMAO and other oxidative stress-related biomarkers were monitored, in order to elucidate whether the ability of MaGPE to reduce these markers might be considered as a potential mechanism of action for its protective role against macular degeneration.

Methods

MaGPE Supplement Formulation

The acronym MaGPE refers to a maltodextrinated grape pomace polyphenolic extract, used in this study and object of a patent (n°102020000006493) regarding its efficacy on central foveal thickness (CFT) in AMD patients. More specifically, the chemical composition of MaGPE is equivalent to that of a food supplement consisting of a polyphenol extract obtained from *Aglianico* cultivar grape, collected during the autumn 2016 harvest. Firstly, the Department of Pharmacy, University of Naples Federico II (Naples, Italy), provided the supplement formulation, then the large-scale production was accomplished by MBMed Company (Turin, Italy). For the polyphenol extract production, grapes were extracted with water (50 ◦C), and the solution was filtrated and concentrated and underwent a spray-drying process with maltodextrins as support (40-70%) to obtain a fine microencapsulated powder. As previously reported, the High-Performance Liquid Chromatography-diodearray detector (HPLC-DAD, Jasco Inc., Easton, MD, USA) polyphenol profile of MaGPE revealed the presence of: Gallic acid 1463.4±65.5 µg/g; Syringic acid 539.2±6.02 µg/g, Caffeic acid 20.7±0.76 µg/g, p-coumaric acid 27.9±0.66 µg/g, Ferulic acid 10.5±0.70 µg/g, Resveratrol 13.6±0.64 µg/g, Catechin 4087.0±64.5 µg/g, Epicatechin 886.0±7.82 µg/g, Quercetin 40.22±7.11 µg/g, Rutin 28.4±0.70 µg/g, Procyanidin B1 dimer 62.8±0.59 µg/g, Procyanidin B2 dimer 426.5±5.92 µg/g, Procyanidin B3 dimer 22.05±6.61 µg/g, Procyanidin B4 dimer 56.6±0.88 µg/g, Procyanidin C2 trimer 44.6±0.66 µg/g (32).

Ophthalmic Outcomes Evaluation

CFT was monitored using an Optical Coherence Tomographer (Model Cirrus Zeiss HD-5000, Carl Zeiss, Wetzlar, Germany) to assess retinal structure and measure CFT after pupillary dilation. Twelve 6-mm radial scans spaced 15° were recorded. CFT was measured as the distance between the high-reflectance vitreoretinal interface and the retinal pigment epithelium/choriocapillaris complex based on the vertical and horizontal B-scans of the Stratus or based on the central B-scan of the Cirrus.

Visual acuity was assessed monocularly with the right eye tested first unless otherwise clinically indicated using a distance Snellen chart to determine the power that yielded best-corrected visual acuity.

TMAO Quantification

A High-Performance Liquid Chromatography-mass spectrometry (HPLC/MS) method was performed for quantification of TMAO serum levels as described by Annunziata et al. (33) and reported in previous studies (34–37). Briefly, serum proteins were precipitated adding 160 µL of methanol to 80 µL of serum, vortex-mixing for 2 min and centrifuging at 12,000 rpm for 10 min (4 ◦C); the supernatants were used for the HPLC-MS analysis. The HPLC/MS system used and method conditions are reported in the Supplementary Materials.

Circulating Oxidative Stress-Related Biomarkers Analysis

Serum levels of reactive oxygen metabolites (D-ROMs) and oxidized low-density lipoproteins (oxLDL) as oxidative stress-related biomarkers were monitored. Both D-ROMs and oxLDL analyses were carried out on an automated analyzer (Free Carpe Diem, Diacron International, Grosseto, Italy) using relative commercial kits (Diacron International) according to the manufacturer's instructions, as previously reported (33,38).

Study Design, Setting and Population

This was a single-center, parallel-arm, open-label trial. The study was conducted on patients with AMD recruited by the Cardarelli Hospital (Naples, Italy) in January 2019. Exclusion criteria were: serious hepatic disease (cirrhosis, hepatitis), serious renal disorders (serum creatinine >2.8 mg/dL), heart disease, family history of chronic diseases, drug therapy or supplement intake containing grape polyphenols, heavy physical exercise (>10 h/week), pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding, birch pollen allergy, and donation of blood less than 3 months before the study. Subjects were assigned to the diabetics-group or non-diabetics-group on the basis of the previous diagnosis of diabetes mellitus. Each subject was free supplied with MaGPE supplement and instructed to consume one capsule twice daily (each capsule was acid-resistant and contained 400mg MaGPE). Participants were also instructed to maintain their usual dietary and lifestyle habits throughout the study duration. Clinical visits consisted of evaluation of general medical parameters, ophthalmic outcomes, anthropometric measures and blood sampling, and were conducted at baseline (t0) and after 6-month treatment with MaGPE (t6). In order to blind the data analysis, researchers performing blood analysis and physicians performing clinical visits did not know which group each patient was allocated in; additionally, a different researcher performed the statistical data analysis. Moreover, after 6-month treatment with MaGPE, a second randomized, placebo-controlled, double-blind trial was conducted. In particular, all AMD subjects were randomized into two groups: active (1 capsule of 400mg MaGPE twice daily) and placebo (1 capsule of 400mg maltodextrins twice daily). Treatments were followed for 3 months, and serum levels of TMAO, DROMs and oxLDL were monitored before and after treatment. In both the two trials, blood samples were collected in the morning on 12h-fasting subjects. The study was conducted in accordance with the 1964 Helsinki Declaration (revised in 2000) and approved by the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy) with protocol 106 no. 123512 of 18/06/2018. Additional information concerning the study protocol, including study procedures and statistical analyses, are detailed in the Supplementary Materials.

Results

Enrolment

A total of 37 patients with AMD (23 men and 14 women) with a mean age of 67.59±6.00 years and an average BMI of 31.18±6.83 kg/m^2 were assigned to the study. AMD patients with cataract were also included in the study; however, none needed cataract surgery during the entire study duration. AMD patients were stratified in diabetics and non-diabetics (n=19 and n=18, respectively). Demographic, anthropometric and clinical characteristics are reported in Table 1. As shown, no significant differences were evident for demographic, oxidative stress-related biomarkers and diagnosed diseases, except for diabetes mellitus. No subjects prematurely terminated study participation.

Ma.G.P.E. Treatment Ameliorates Ophthalmic Outcomes in AMD Patients

The effect of 6-month MaGPE treatment on ophthalmic outcomes was evaluated. In particular, changes in CFT and visual acuity were monitored on right and left eye. Significant reductions in CFT and improvements in visual acuity were experienced in 63.1% and 52.6%, respectively, of diabetics AMD patients, while in non-diabetics were observed in 55.5% and 54.4%, respectively. As shown in Fig.1, at the end of the treatment period with MaGPE, globally CFT significantly reduced in both diabetics (right eye: from 442.889±54.091 µm to 334.364±39.721 µm, p=0.01; left eye: from 474.818±66.473 µm to 307.111±18.165 µm, p=0.006) and non-diabetics (right eye: from 370.889±49.087 µm to 287.222±39.496 µm, p=0.002; left eye: from 321.667±47.238 µm to 301.333±50.416 µm, p=0.06). Similarly, visual acuity significantly improved in both diabetics (right eye: from 0.325±0.082 to 0.463±0.094, p=0.004; left eye: from 0.192±0.052 to 0.317±0.085, p=0.02) and non-diabetics (right eye: from 0.120±0.020 to 0.186±0.014; left eye: from 0.165±0.067 to 0.375±0.077, p=0.001).

In Fig.2 are reported OCT images from the same study participant before and after treatment with MaGPE.

Effect of MaGPE in Reducing Circulating Levels of Oxidative Stress-Related Biomarkers

As oxidative stress-related biomarkers, serum levels of TMAO, D-ROMs and oxLDL were monitored in both diabetics and non-diabetics before and after treatment period with MaGPE. All monitored markers significantly reduced at the end of the treatment period in both diabetics (TMAO: from 3.937±0.800 µM to 2.019±0.568 µM, p=0.014; D-ROMs: from 520.30±19.14 UCARR to 251.90±16.93 UCARR, p<0.001; oxLDL: from 778.20±82.97 µEq/L to 415.50±66.18 µEq/L, p<0.001) and non-diabetics (TMAO: from 4.097±0.642 µM to 1.716±0.532 µM, p<0.001; D-ROMs: from 503.10±72.65 UCARR to 258.10±27.61 UCARR, p=0.016; oxLDL: from 747.40±97.80 µEq/L to 344.70±59.37 µEq/L, p=0.002) (as shown in Fig.3).

To test the efficacy of MaGPE to reduce serum levels of oxidative stress-related biomarkers comapared to placebo, all AMD partients were randomised into two treatment groups: active (1 capsule of 400mg MaGPE twice daily) and placebo (1 capsule of 400mg maltodextrins twice daily). Treatments were followed for 3 months, and serum levels of TMAO, DROMs and oxLDL were monitored before and after treatment. As shown in Table 2, MaGPE significantly reduced serum levels of TMAO, DROMs and oxLDL (-16.57%, -17.92%and -27.71%, respectivelly) compared to placebo (+30.95%, +46.68% and +19.90%, respectively).

Correlation Analysis Between Variations of Oxidative Stress-Related Biomarkers and Ophthalmic Outcomes After 6-month Treatment With MaGPE

A Pearson correlation analysis has been performed between TMAO, DROMs and oxLDL serum levels and measured values of CFT and visual acuity. Results reported in Table 3 indicate that oxidative-stress related biomarkers positively correlate with CFT and negatively with visual acuity, suggesting that increased oxidative status and atherosclerotic process have a role in macular degeneration and, consequently, in visual acuity. The absence of statistic significance (except for correlation between DROMs levels and right eye CFT) may be due to the small sample size.

In order to elucidate any eventual involvement of the antioxidant activity of MaGPE (in terms of TMAO, D-ROMs- and oxLDL-reducing effects) in improvement of ophthalmic outcomes, a correlation analysis has been performed. In particular, a Pearson correlation analysis has been performed between (i) variations (defined as the variation between values measured at the end of the treatment period (t6) and at baseline (t0), and indicated as "Δ% (t0-t6)") of TMAO, D-ROMs and oxLDL and (ii) ophthalmic outcomes (CFT and visual acuity). For these last two parameters, values referring to right eye and left eye were correlated. As shown in Table 4, in AMD patients (i) significant positive correlation has been found between variations of CFT and TMAO, D-ROMs and oxLDL, and (ii) significant negative correlation has been found between variations of visual acuity and D-ROMs, while non-significant negative correlation was observed for TMAO and oxLDL.

Discussion

In the present study, the protective effect of MaGPE supplement on AMD is reported. In particular, it was demonstrated that a 6-month treatment with MaGPE significantly reduced CFT and increased visual acuity in both diabetic and non-diabetic patients with macular degeneration. According to the assessment of oxidative stress-related biomarkers, these beneficial effects are due to the ability of MaGPE to reduce serum levels of TMAO, D-ROMs, and oxLDL. In this scenario, thus, antioxidant activity is the key in the management of AMD.

Oxidative stress has been included among the main risk factors for AMD development. This is due to various reasons: (i) retina is among the tissues with higher metabolism rate and oxygen demand, thus, it is highly vulnerable to oxidative stress and consequent oxidative damage (39); (ii) phothoreceptor membranes are rich in polyunsaturated fatty acids, which are a source of reactive oxygen species (ROS) and (iii) prolonged exposure to light/blue light/UV causes photo-

oxidation, resulting in increased production of ROS and lipid peroxidation products (3). In addition to this, it should be taken into account that ageing, *per se*, features high oxidative stress, due to both reduced endogenous antioxidant defenses and increased production of free radicals (40–43). The involvement of oxidative stress in the development and progression of AMD, thus, is rooted in both physiological conditions and consequences of environmental factors. Clinically, increased oxidative stress at eye level can cause a progressive degeneration of various inner parts of the retina, such as macula, resulting in disease development. This can lead to a progressive clinical evolution, also due to limited self-repair and regeneration ability of retina (3). In this sense, interventions aimed to reduce oxidative stress, can be considered as effective strategies for the management of AMD.

Polyphenols are the largest class of plant-derived bioactive compounds exerting protective functions against various negative stimuli, including UV radiations, pathogen aggression and oxidative stress. Although more than 8,000 compounds are included in the polyphenol family, all of them share the same basic chemical structure, consisting of one or more phenolic rings with hydroxyl groups (30). Polyphenols have been historically investigated and appreciated for their antioxidant potential, mainly exerted at two major levels: (i) direct ROS-scavenging and endogenous antioxidant defenses modulation and (ii) inhibition of ROS-producing enzymes and metal-dependent free radical production (44). The beneficial role of these bioactive compounds on eye diseases has been investigated in studies reporting both antioxidant supplementation and polyphenol-rich diets, as effective in slowing progression of intermediate-to-advanced forms of AMD (45,46) or reducing AMD-related risk factors, including oxidative stress, inflammation, and atherosclerosis (47,48).

In this study, we investigated the effects of MaGPE, a novel nutraceutical formulation based on grape pomace polyphenolic extract. MaGPE is a supplement rich in polyphenols (32), whose antioxidant potential has been previously described in both animals and humans (33,49). The ability of MaGPE to contrast oxidative stress is herein demonstrated by its efficacy in reducing significantly serum levels of DROMs. These are oxygen metabolites generated as a result of the attack operated by free radicals at the expense of biomolecules. As DROMs are more stable than other free radicals, they are adequately detectable and quantifiable. More specifically, the test used in the present study is based on the concept that, according to the Fenton's reaction, DROMs contained in a serum generate, in presence of iron, alkoxyl (R–O*) and peroxyl (R–OO*) radicals which, in turn, oxidize an alkyl-substituted aromatic amine, producing a pink-colored derivative ([A–NH2*]+), photometrically quantified (50–53). It appears clear, thus, that DROMs are useful biomarkers of oxidative stress, determined on the basis of the following ranges: (i) normal: 250-300 UCARR, (ii) border-line: 300-320 UCARR, (iii) low level of oxidative stress: 321-340 UCARR, (iv) middle level of oxidative stress: 341-400 UCARR, (v) high level of oxidative stress: 401-500 UCARR and (vi) very high level of oxidative stress: >500 UCARR, where 1 UCARR=0.08 mg H_2O_2/dL (50–53). According to this classification, our results showed that 6-month treatment with MaGPE significantly reduced oxidative stress in all study participants, from a very high to normal level. As AMD is an oxidative stress-related disease, it is plausible that the antioxidant potential of MaGPE plays a central role in improving ophthalmic outcomes, suggesting that this effect could be a possible mechanism of action. This is confirmed by the correlation analysis showing that t0-t6 variations of DROMs correlated positively with CFT (p<0.0001 for both eyes) and negatively with visual acuity (p=0.034 and p=0.032, left and right eye, respectively)(Table 3).

In addition to the oxidative stress, previous studies described the relationship existing between eye diseases, including AMD, and CVD, in particular, atherosclerosis (12–14). More specifically, clinical evidence reported that thromboembolic and/or atherosclerotic processes are involved in the pathogenesis of specific eye diseases (i.e. RAO) (12–14), which can evolve in macular degeneration. Similarly, AMD has been associated with atherosclerosis (15) and alterations of gut microbiota (20,21), indicating that specific gut microbiota-derived compounds, whose CVD-

promoting effect is established, may also play a role in AMD. Among these, TMAO is emerging as a biomarker of increased cardiovascular risk (22), in particular, as a pro-atherogenic agent. Interestingly, a recent investigation reported that TMAO levels were higher in patients with RAO compared to controls (29), corroborating the evidence on the eye diseases/atherosclerotic process relationship and providing novel insight on AMD pathogenesis. Our results from the correlation analysis (Table 2) showed, at baseline, that TMAO and oxLDL serum levels correlated negatively with CFT and positively with visual acuity. On that basis, these data are in line with previous evidence and confirm the existence of an atherosclerotic process in AMD patients.

The role of TMAO in AMD pathogenesis is particularly intriguing, and deserves to be further investigated. TMAO is a highly oxidative and reactive molecule, thus, closely linked to oxidative stress and cardiovascular risk (54). The relationship between oxidative stress and CVD, including atherosclerosis, is supported by various evidence (55–57). In atherosclerotic patients, increased ROS levels induce molecular changes that, in turn, cause plaque formation; these changes include oxidization of LDLs which accumulate in the sub-endothelium, inducing adhesion molecule production. It results in recruitment of monocytes and T-cells, pro-inflammatory cytokines, and ROS release, culminating in apoptosis and foam cell formation (58). Interestingly, TMAO is responsible for enhancement of macrophage CD36 receptor expression induced by oxLDL (25,59,60). With regard to the relationship between atherosclerotic processes and eye diseases, it is plausible that these mechanisms may also occur in retinal blood vessel, causing partial occlusions, as previously described in RAO patients (29). In this sense, thus, the involvement of TMAO and oxLDL in AMD progression is intuitive.

Various studies described the ability of polyphenols to contrast atherosclerosis progression, acting *via* different mechanisms, including reduction of LDL oxidizability (61,62). More specifically, several types of polyphenols can exert radical trapping effects, acting as hydrogen donors to α-tocopherol radicals, which results in LDL oxidation prevention (63). Also, *in vitro* studies, aiming to clarify the mechanisms at the base of the well-known French paradox, reported that red wine polyphenols can inhibit copper-catalyzed LDL oxidation (64). In line with these studies, we previously demonstrated the oxLDL-reducing effect of the same grape polyphenol extract used in this study in overweight/obese subjects (33). As in the present study, oxLDL serum levels were evaluated using the LP-CHOLOX test (Diacron, Grosseto, Italy), aimed to measure the levels of lipid peroxidation-derived hydroperoxides, mainly represented by oxidized cholesterol. According to the manufacturer's instructions, oxLDL levels are defined: normal (with values ≤599 µEq/L), slightly high (with values ranging from 600 to 799 µEq/L), moderately high (with values ranging from 800 to 999 µEq/L) and very high (with values ≥1000 µEq/L) (65,66). In agreement with this classification, our results showed that a 6-month treatment with MaGPE significantly reduced serum levels of oxLDL in all study participants from a slightly high to normal level.

Evidence, reporting the TMAO-reducing effect of polyphenols, are limited. However, we previously demonstrated the ability of the same grape polyphenol extract used in this study to reduce serum levels of this gut microbiota-derived metabolite in humans (33,34). Similarly, in the present study, TMAO serum levels significantly decreased in AMD patients after 6–month treatment with MaGPE. Main putative mechanisms responsible for the TMAO-reducing effect of MaGPE are still unclear. Nevertheless, two possible hypothesis can be formulated. On the one hand, polyphenols may contribute to gut microbiota remodeling, in particular, contrasting the growth of TMA-producing bacterial strains (i.e. *Clostridia and Bacteroides*) (67,68) in favor to non-TMA-producing ones (i.e. *Lactobacillus and Bifidobacterium*) (69). On the other hand, MaGPE polyphenols may reduce the levels of TMAO through their intrinsic antioxidant activity. TMAO, indeed, can act as an electron acceptor (70), while it is well-accepted that polyphenols exert their antioxidant activity *via* donating electrons to free radicals molecules. This may support the hypothesis that TMAO and

polyphenols (or their metabolites), at serum levels, might be involved in the same redox reactions, where TMAO, acting as an electron acceptors, is reduced to TMA. This is a proposed mechanism providing novel insights for the TMAO-reducing effect of MaGPE, but it needs to be further investigated.

Overall, our results indicate that TMAO- and oxLDL-reducing effect of MaGPE may play a central role in improving ophthalmic outcomes. Results from our correlation analysis (Table 3) corroborate this hypothesis. More specifically, we demonstrated that the decrease in both TMAO and oxLDL serum levels correlated with the decrease in CFT and increase in visual acuity, at the end of the treatment period, suggesting that reducing atherosclerosis progression may be a strategy for the management of AMD.

The major limitations of the present study are described as follows. Firstly, this a pilot study, characterized by a small sample size which is not able to provide an appropriate statistic power. In addition, no data regarding intermediate values during the 6-month treatment (nor 3-month treatment) are available, due to the study design. Nevertheless, to the best of our knowledge, this is the first study demonstrating correlations between oxidative stress reduction and improvement of ophthalmic outcomes in patients with AMD after chronic treatment with grape polyphenol supplement. Evidence herein presented can serve physicians to evaluate the use of nutraceutical approaches for the management of this class of patients.

In summary, in the presentstudy we demonstrated that 6-month treatment with MaGPE significantly reduces CFT and increases visual acuity in both diabetic and non-diabetic patients with AMD. These beneficial effects are related to a marked effect in reducing serum levels of TMAO, as a pro-atherogenic agent, and DROMs and oxLDL, as oxidative stress-related biomarkers. Such promising, these results allow indicating these effects as potential mechanisms of action for the beneficial role played by MaGPE on eye diseases, providing novel insights on the use of nutraceutical for management of oxidative stress- and vascular alteration-related diseases, including ophthalmological disease (as shown in Fig.4).

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Supplementary materials

Materials and methods

TMAO quantification

The HPLC system Jasco Extrema LC-4000 system (Jasco Inc., Ithaca, NY) was coupled to an Advion Expression mass spectrometer (Advion Inc., Ithaca, NY) equipped with an ESI source, operating in positive ion mode. The separation of the analytes was performed using a Luna Hilic (5µ particle size, 150x3mm) and security guard colon both supplied by Phenomenex (Torrance, CA, USA) were used. The column temperature was maintained at 60 °C during analysis. Mobile phase A composition: 0.15% formic acid in water containing a final concentration of 10 mM ammonium acetate; mobile phase B composition: methanol. Mobile phases ratio was 80:20 (A:B), run isocratically at a flow rate of 0.35 mL/min for 6 min, with a 5 µl injection volume.

D-ROMs test (Free Carpe Diem, Diacron International, Grosseto, Italy)

10µl of serum were transferred into 1cm cuvettes containing 1ml of R_2 reagent (acetate buffer, pH4.8). The sample-containing mixture was gently mixed and 20µl of R_1 reagent (a chromogenic mixture consisting of aromatic alkyl-amine, A-NH_2) were added. Cuvettes were mixed by inversion and samples were read at 546nm (5 min, 37°C) on an automated analyzer.

LP-CHOLOX test (Free Carpe Diem, Diacron International, Grosseto, Italy)

10µl of serum were added in a plastic tube containing 1ml of R_1 reagent (indicators mixture) and two drops of R_2 reagent (reduced iron) were transferred. The mixture was mixed by shaking, incubated at 37°C for 2 min and centrifuged at 1,400 g for 2 min. Supernatants were transferred into 1cm cuvettes and read at 505nm (37°C) on an automated analyzer. Blank was prepared following the same procedure, without the addition of sample.

Study population and protocol

Study participants were recruited by the Cardarelli Hospital (Naples, Italy). Subjects were enrolled in January 2019. All subjects underwent a standardised physical examination, assessment of medical history (for up to five years before enrolment), laboratory examination, measurement of blood pressure and heart rate, and evaluation of BMI. Body mass index (BMI) was calculated from body height and body weight. Body fat percentage was measured using a body composition analyzer (TBF-310, Tanita Corp., Tokyo, Japan) and systolic blood pressure, diastolic blood pressure, and heart rate, were measured using a HBP-9020 (OMRON COLIN Corp., Tokyo, Japan). At each clinic visit, subjects had to complete three self-administered questionnaires on quality of life aspects, and their diaries were checked for data completeness and quality of documentation to ensure subject comprehension of the diary items. Subjects aged 18-70 years with diagnosis of AMD were eligible for enrolment.

The subjects received oral and written information concerning the study before they gave their written consent. Protocol, letter of intent of volunteers, and synoptic document about the study were submitted to the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy). The study was approved by the committee (protocol 106 n. 123512 of 18/06/2018), and carried out in accordance with the Helsinki declaration of 1964 (as revised in 2000). The subjects were asked to make records in an intake-checking table for the intervention study and side effects in daily reports.

Subjects were informed not to drink alcohol or perform hard physical activity 48 h prior to blood sampling. Participants maintained their usual dietary and lifestyle patterns throughout the study.

Study procedures

Participants arrived at the research centre in the morning after 12 h of fasting. All blood samples were taken in the morning and immediately after measurement of heart rate and blood pressure. Blood samples were collected from each participant before administration of the reference glucose solutions and the treatment beverages, in 3-mL EDTA-coated tubes (Becton–Dickinson, Plymouth, UK). Plasma was immediately isolated by centrifugation (20 min, 2.200 g, 4 °C). All biochemical analyses including fasting plasma glucose, total cholesterol, fasting plasma TG were performed with a Roche Modular Analytics System in the Central Biochemistry Laboratory of our Institution. Low-Density Lipoprotein (LDL)

cholesterol and HDL cholesterol were determined by a direct method (homogeneous enzymatic assay for the direct quantitative determination of LDL and HDL cholesterol). Plasma insulin concentrations were measured using an enzyme linked immunosorbent (ELISA) assay commercial kit (InterMedical srl, Italy).

Statistics

Methodology

Unless otherwise stated, all of the experimental results were expressed as mean ± SEM. Statistical analysis of data was performed by the Student's t test or Pearson correlation. The statistic heterogeneity was assessed by using Cochran's test ($p<0.1$). The $I2$ statistic was also calculated, and $I2>50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown among the trials. Otherwise, results were obtained from a fixed-effects model. SD values were calculated from standard errors, 95% CIs, p-values, or t if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status, study design, type of intervention, duration, total nutraceutical dose, and Jadad score. Treatment effects were analysed using PROC MIXED with treatment and period as fixed factors, subject as random factor and baseline measurements as covariates, and defined as weighted mean difference and 95% CIs calculated for net changes in fecal and serum parameters, and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levenes test) and normal distribution (determined by residual plot examination and Shapiro–Wilks test) even after log transformation were analysed by a nonparametric test (Friedman). The level of significance (α-value) was 95% in all cases ($P< 0.05$).

Analysis set

The full analysis set population included all randomised subjects, and subjects who did not fail to satisfy a major entry criterion. The per protocol set consisted of all subjects who did not substantially deviate from the protocol. This group included subjects for whom no major protocol violations were detected (for example, poor compliance, errors in treatment assignment).

Statistics

All of the experimental data were expressed as mean ± SEM. Statistical analysis of data was carried out by the Student's t test or Pearson correlation. The level of significance (α-value) was 95% in all cases ($P < 0.05$). The degree of linear relationship between two variables was measured using the Pearson product moment correlation coefficient (R). Correlation coefficients (R) were calculated using Microsoft Office Excel.

Table 1. Baseline characteristics of study participants. No significant differences were evident between the two groups, except for anthropometric characteristics, metabolism blood parameters and diagnosis of diabetes mellitus. *Values are expressed as mean ± SD; statistical significance is calculated by Student's t-test. Abbreviations: BMI, body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; TMAO, trimethylamine N-oxide; D-ROMs, reactive oxygen metabolites; ox-LDL, oxidized LDL cholesterol.

Parameters	Diabetics (*n*=19)	Non-diabetics (*n*=18)	*p*-value
Demographic characteristics			
Gender [male (%)]	73.33	50.00	χ^2 = 2.204, *p* = 0.138
Age (years)*	65.93±5.60	69.67±6.07	0.110
Smokers [yes (%)]	40.00	33.33	χ^2 = 0.302, *p* = 0.582
Physical activity [yes (%)]	13.33	16.67	χ^2 = 0.005, *p* = 0.942
Anthropometric characteristics			
Weight (kg)*	84.70±15.58	67.92±1.31	0.007
Height (m)*	1.68±0.12	1.59±0.09	0.032
BMI (kg/m^2)*	34.36±6.35	27.21±5.33	0.004
WC (cm)*	106.00±9.48	94.33±12.23	0.010
HC (cm)*	108.40±10.73	101.00±11.18	0.093
WHR*	0.98±0.08	0.93±0.08	0.124
Blood parameters			
Metabolism			
Glucose (mg/dl)*	139.60±28.78	93.39±9.87	<0.0001
Insulin (µIU/ml)*	9.44±2.35	7.81±0.91	0.032
Cholesterol (mg/dl)*	199.61±47.93	165.33±29.87	0.041
HDL-c (mg/dl)*	43.74±13.77	56.12±16.83	0.045
LDL-c (mg/dl)*	122.38±45.07	87.53±27.66	0.027
Triglycerides (mg/dl)*	167.47±21.62	108.42±56.26	<0.001
Oxidative stress			
TMAO (µM)*	3.94±3.10	4.10±2.40	0.879
D-ROMs (UCARR)*	520.27±74.12	503.07±2.71	0.815
Ox-LDL (µEq/L)*	778.20±321.34	747.36±365.92	0.811
Diagnosed diseases			
Diabetes mellitus [yes (%)]	100.00	0.00	1.00
Hypertension [yes (%)]	66.67	41.67	χ^2 = 2.165, *p* = 0.141
Hypercholesterolemia [yes (%)]	53.33	41.67	χ^2 = 0.248, *p* = 0.618
Hypertriglyceridemia [yes (%)]	6.67	8.33	χ^2 = 0.424, *p* = 0.515

Table 2. Effects of MaGPE on TMAO, DROMs and oxLDL serum levels on AMD patients after 3-month treatment. Values are expressed as mean±SD of three repetitions. "Initial" and "final" refer to samples collected at months 6 and 9, respectively. Statistic significance was calculated by Student's t-test. *p<0.05, initial vs. final (active); ** p<0.0001, initial vs. final (placebo); ° p<0.05, active vs. placebo (final); † p<0.0001, active vs. placebo (final). Abbreviations: TMAO, trimethylamine N-oxide; D-ROMs, reactive oxygen metabolites; ox-LDL, oxidized LDL cholesterol.

	TMAO (μM)		DROMs (UCARR)		oxLDL (μEq/L)	
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**
Active group	2.10±1.05	1.75±0.93*°	235.47±96.73	193.27±49.43*†	421.47±211.81	304.67±139.96*†
Placebo group	2.98±2.11	3.91±2.25**	298.93±82.58	417.93±107.32**	541.71±263.36	649.50±252.31**

Table 3. Correlation analysis between ophthalmic outcomes and oxidative stress-related biomarkers at baseline in AMD patients. Pearson correlation coefficients between indicated parameters are represented; values in bold type are statistically significant (p< 0.05). Abbreviations: TMAO, trimethylamine N-oxide; D-ROMs, reactive oxygen metabolites; ox-LDL, oxidized LDL cholesterol.

	TMAO levels		DROMs levels		oxLDL levels	
	Coefficient	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**
			Central Foveal Thickness			
Left eye	0.133	0.638	0.242	0.304	0.195	0.454
Right eye	0.391	0.297	**0.607**	**0.036**	0.161	0.636
			Visual acuity			
Left eye	-0.195	0.487	-0.193	0.495	-0.374	0.140
Right eye	-0.012	0.975	-0.350	0.365	-0.039	0.942

Table 4. Correlation analysis between ophthalmic outcomes and oxidative stress-related biomarkers at the end of the treatment period in AMD patients. Pearson correlation coefficients between indicated parameters are represented; values in bold type are statistically significant (p< 0.05). "Δ% (t0-t6)" refers to variations observed between values measured at the end of the treatment period (t6) and at baseline (t0). Abbreviations: TMAO, trimethylamine N-oxide; D-ROMs, reactive oxygen metabolites; ox-LDL, oxidized LDL cholesterol.

	TMAO Δ% (t0-t6)		DROMs Δ% (t0-t6)		oxLDL Δ% (t0-t6)	
	Coefficient	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**
			Central Foveal Thickness Δ% (t0-t6)			
Left eye	**0.764**	**<0.0001**	**0.927**	**<0.0001**	**0.953**	**<0.0001**
Right eye	**0.595**	**0.041**	**0.858**	**<0.0001**	**0.867**	**<0.0001**
			Visual acuity Δ% (t0-t6)			
Left eye	-0.533	0.091	**-0.532**	**0.034**	-0.413	0.126
Right eye	-0.687	0.132	**-0.968**	**0.032**	-0.760	0.136

Figure 1. Effects of 6-month MaGPE treatment on central foeval thickness and visual acuity in both diabetic and non-diabetic patients with age-related macular degeneration. to and t6 refer to baseline and after 6-month treatment, respectively. Data are expressed as mean±SEM. Statistic significance was calculated by Student's t-test. *indicates a significant difference between to and t6 ($p<0.05$).

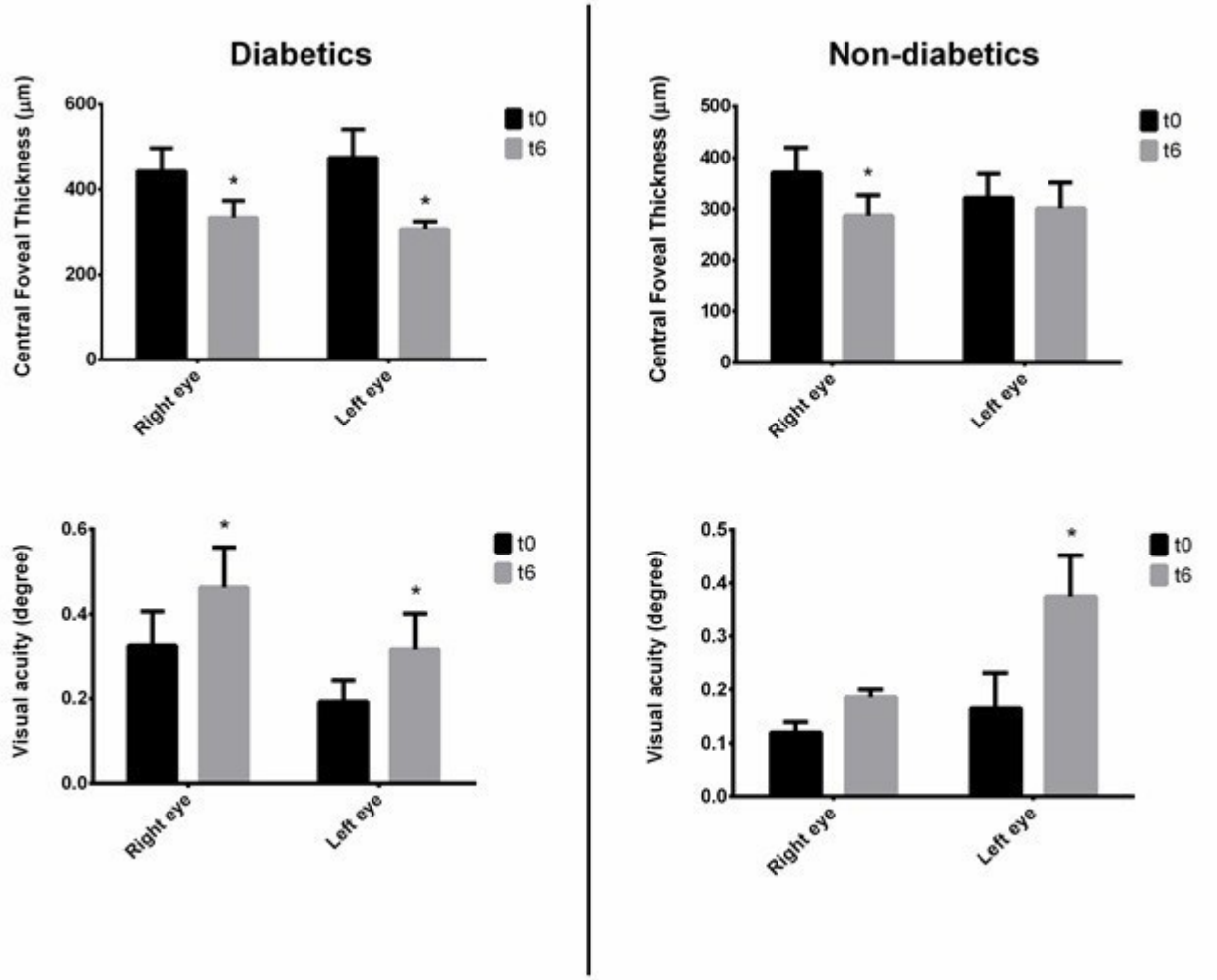

Figure 2. Optical Coherence Tomography (OCT) before and after 6-month treatment of MaGPE (a) OCT-Angiography performed above the plane of the retinal pigment epithelium – the neovascular network is perfectly visualized. There are peripheral anastomoses corresponding to the outer edge of the lesion. (b) The OCT mapping confirms the presence of an abnormal retinal thickening. (c) Horizontal OCT B-scan (passing through the lesion) shows subretinal edema associated with a hyper-reflective subretinal lesion. (d) OCT-Angiography highlights the neovascular network is smaller and the disappearanceof peripheral anastomoses. (e) The OCT mapping shows the reduction of central retinal thickness. (f) Horizontal OCT B-scan (passing through the lesion) shows total absence of perilesional subretinal edema.

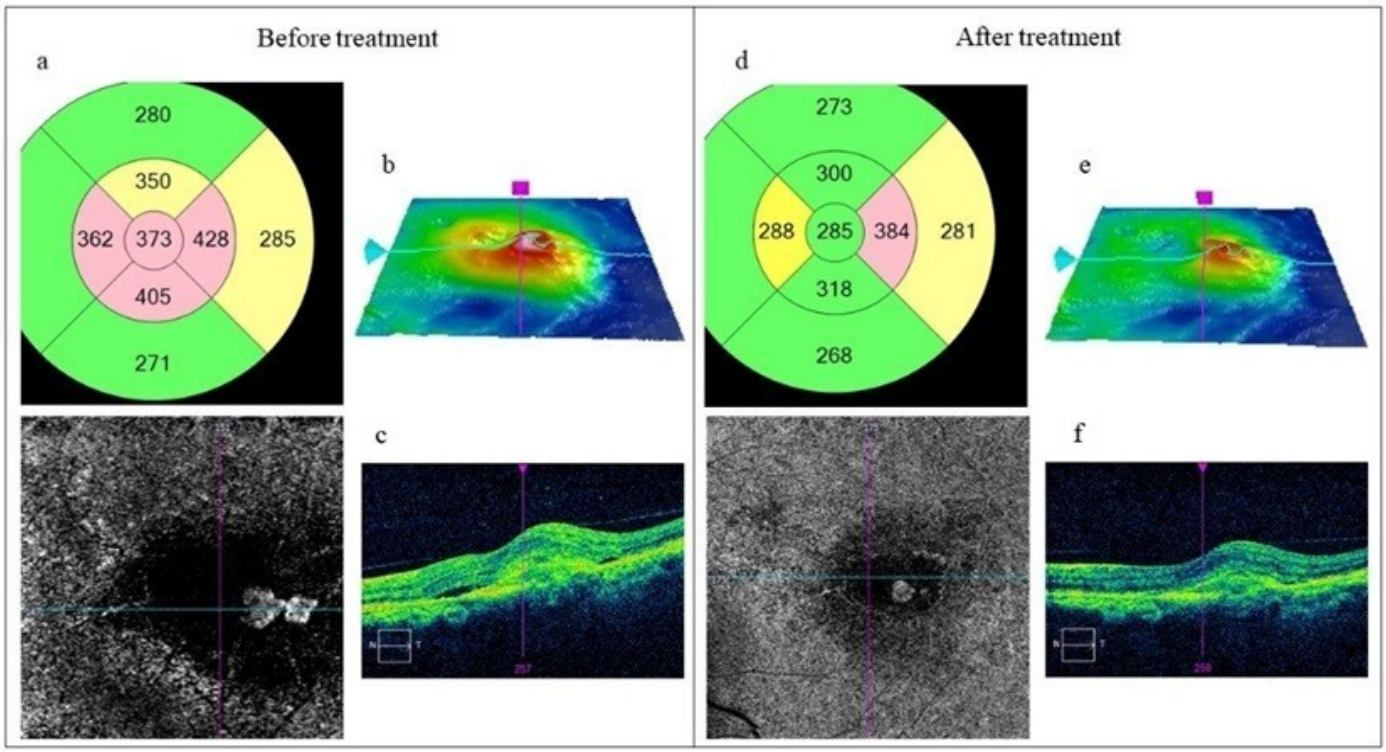

Figure 3. Effects of 6-month MaGPE treatment on serum oxidative stress-related biomarkers in both diabetic and non-diabetic patients with age-related macular degeneration. to and t6 refer to baseline and after 6-month treatment, respectively. Data are expressed as mean±SEM. Statistic significance was calculated by Student's t-test. *indicates a significant difference between to and t6 ($p<0.05$). Abbreviations: TMAO, trimethylamine N-oxide; D-ROMs, reactive oxygen metabolites; ox-LDL, oxidized LDL cholesterol.

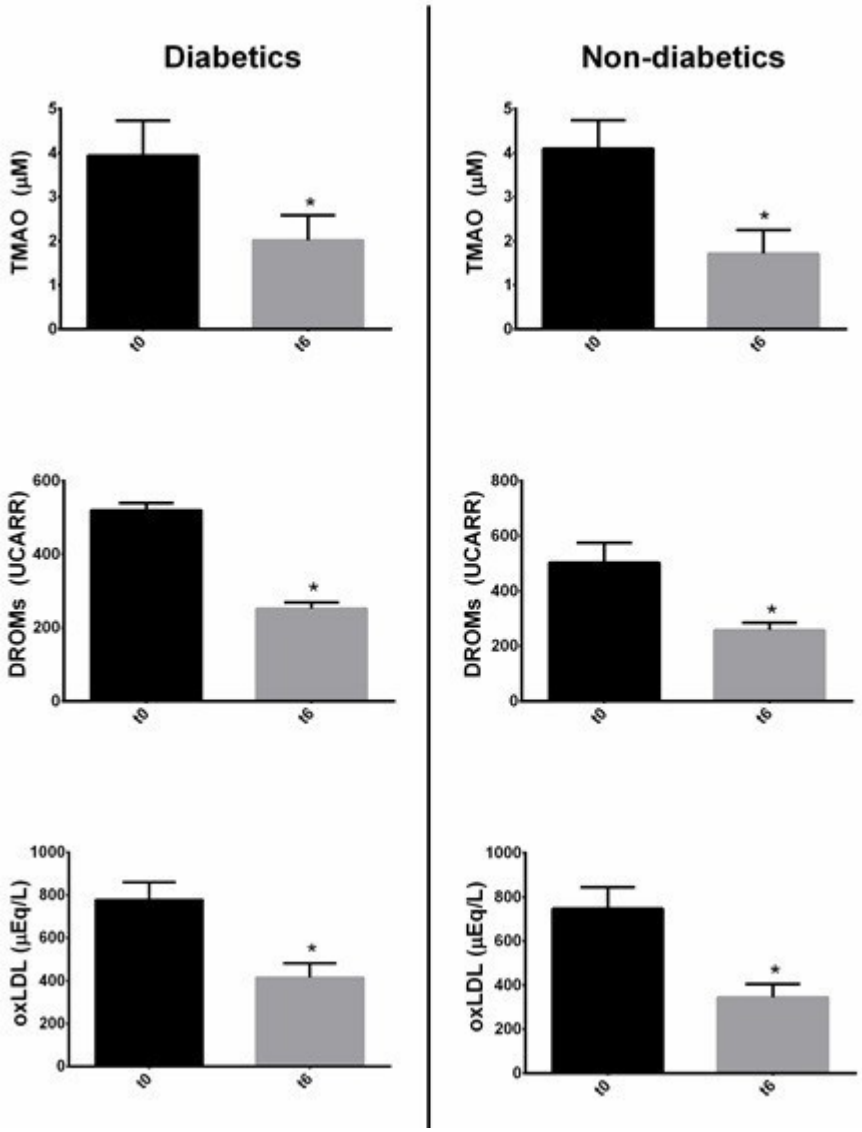

Figure 4. Effect of MaGPE in improving macular degeneration. Thromboembolic and/or atherosclerotic processes, in addition to oxidative stress, are closely implicated in pathogenesis of AMD. Ma.G.P.E., reducing serum levels of TMAO, as pro-atherogenic agent, and DROMs and oxLDL, as oxidative stress-related biomarkers, significantly ameliorates ophthalmic outcomes in patients with AMD, improving the global eye health.