Blood levels of antioxidants during age-related macular degeneration treatment by rheohaemapheresis

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Aims. Rheohaemapheresis treatment influences rheological markers and most likely improves metabolism in affected retinal areas, resulting not only in absorption of soft drusen but also reduction or complete disappearance of drusenoid retinal pigment epithelium detachments. However, the character of the treatment process has raised suspicion that there is a decrease not only in cholesterol but also in antioxidants, such as vitamin E and vitamin A.
Methods. Twenty-three patients with the progressive dry form of age-related macular degeneration were each treated with 8 procedures of rheohaemapheresis. We measured levels of vitamin E (α-tocopherol), the vitamin E/cholesterol ratio in serum and lipoproteins (VLDL, LDL, HDL). Vitamin E in erythrocyte membrane and serum vitamin A (retinol) were also measured. These parameters were determined before and after rheohaemapheresis. Erythrocyte superoxide dismutase, erythrocyte glutathione peroxidase and serum malondialdehyde were analysed as markers of antioxidant activity and lipid peroxidation, respectively.
Results. In serum, the VLDL and LDL fraction ratios of vitamin E/cholesterol increased significantly. Additionally, the HDL fraction ratio showed an increase but this was not statistically significant. The patients showed no clinical signs of vitamin E deficiency, and their serum concentrations of vitamin E did not differ from normal values. The results show that rheohaemapheresis in addition to causing a significant reduction in atherogenic LDL cholesterol, may have favourable additive anti-atherogenic effects due to a relative increase in the content of vitamin E in the lipoprotein fractions.

Key words: rheohaemapheresis, age related macular degeneration, vitamin E, vitamin E/cholesterol ratio, antioxidants

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INTRODUCTION

Age-related macular degeneration (AMD) is a highly complex disease, with demographic, environmental and genetic risk factors[1]. It affects the central region of the retina and choroid, which can result in loss of central vision[2]. AMD is a bilateral disorder; choroidal neovascular membranes develop in more than one fourth (26%) of fellow eyes that are initially free of exudative AMD over a 5-year period[3]. AMD is also the leading cause of vision loss in the developed world, such as in Europe, the USA and Australia, among people older than 55/ 65 years old, accounting for up to 50% of all cases. Additionally, its prevalence is likely to increase as a consequence of increasing longevity[2-6]. The prevalence in adults is approximately 3% (ref.[2]). It is estimated that approximately 30% of adults older than the age of 75 years old have some signs of AMD and that approximately 10% of these patients demonstrate advanced or late stages of the disease[7]. A comparison of statistical data from the Czech Republic with those from other countries provided difficulties because in the Czech Republic, the limit for legal blindness is 1/60, while in other countries, it is 6/60 (ref.[5]).

Clinically, AMD appears in two forms - a non-exudative dry form and an exudative wet form - which in an individual patient can also represent stages of the disease. The non-exudative form involves a variety of presentations, including hard drusen, soft drusen, and geographic (areolar) atrophy of the retinal pigment epithelium (RPE). This non-exudative form of AMD accounts for 80-90% of AMD cases[5,8].

Greater intake of fish, nuts, and dark green, leafy vegetables has been associated with lower risk for AMD. An increase of intake of vegetable fat, mono- and polyunsaturated fatty acids, and linoleic acid was associated with increased risk for AMD (ref.[7]). The Age-Related Eye Disease Study (AREDS), a multi-centre, randomised, controlled clinical trial, demonstrated that oral supplementation with a combination of vitamin C, vitamin E, β-carotene, zinc oxide and cupric oxide in patients with intermediate or advanced AMD in one eye resulted in a 25% relative risk reduction of developing advanced AMD

in the other eye[5,8]. A comparison with placebo demonstrated a statistically significant odds reduction for the development of advanced AMD with antioxidants plus zinc[9]. The US Veterans Administration's LAST (Lutein Antioxidant Supplementation Trial) study found that lutein supplementation, alone or in combination, significantly augmented macular pigment, improved near visual acuity and contrast sensitivity and demonstrated a lack of disease progression over the one-year study period. Gale et al. found that the serum concentration of zeaxanthin was significantly lower in individuals with AMD, compared to those without the disease. Serum concentrations of lutein and of lutein and zeaxanthin combined were also lower but not significantly[10-12].

Bláha et al. observed, after rheohaemapheresis (RH), not only absorption of soft drusen but also a reduction in or complete disappearance of drusenoid retinal pigment epithelium detachments (RPEDs). RH influenced rheological markers and most likely improved metabolism in the affected retinal areas, leading to the positive results[8]. RH results in a decrease in high-weight molecules, such fibrinogen, immunoglobulins (mainly IgM), LDL cholesterol and other compounds. For this reason, the viscosity of the blood decreases, and the proportions of cytokines and adhesion molecules also change positively. Moreover, the deformability of erythrocytes decreases. Clinical studies on implementing RH in AMD have been guided by evidence-based medicine[13].

However, the character of the treatment process has raised the suspicion that RH also causes a decrease in antioxidants, such as vitamin E and vitamin A.

METHOD

Patient group

From June 2008 to July 2011, twenty-three patients with the non-neovascular form of AMD were randomised. Patients were treated with 8 procedures of RH (9 men, 14 women, mean age 67 years old, range 41-85 years).

Patients admitted to the study had to have received a diagnosis of AMD in both eyes, including dry AMD in one or both eyes confirmed by fluorescein angiography and fundus photography. The exclusion criteria were retinal or choroidal disorders other than AMD, optic nerve disorders, glaucoma, conditions limiting examination of the fundus, and acute bleeding in the study eye. The general exclusion criteria for RH treatment were the usual exclusion criteria for extracorporeal circulation or therapeutic haemapheresis and the absence of peripheral veins suitable for establishing an extracorporeal circuit. Regarding randomisation, patients with the late-stage, high-risk, preangiogenic form of AMD with soft drusen, confluent soft drusen and DPEDs were assigned to RH therapy.

Rheohaemapheresis

The cascade method of plasma filtration was used as our modification of rheotherapy (named rheohaemapheresis by Borberg et al., 2006) (ref.[14-17]) (Fig. 1). After

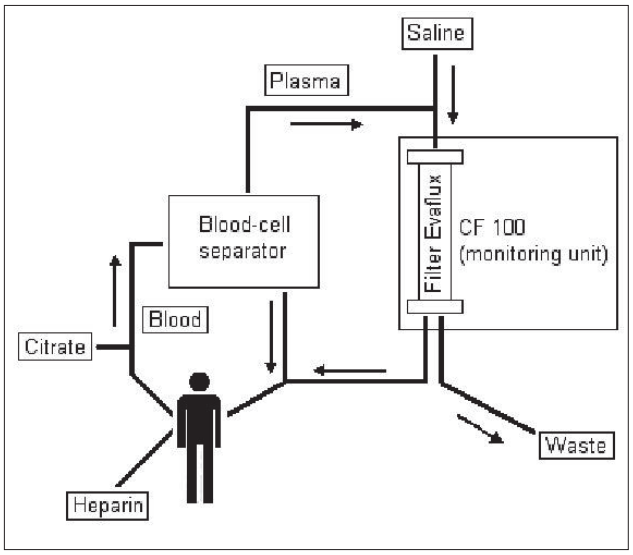

Fig. 1. Scheme of rheohaemapheresis with our modification.

plasma separation (blood-cell separator, Cobe-Spectra, Denver, CO, USA), the separated plasma was pumped through the rheofilter (Evaflux 4A, Kuraray, Japan) to remove high molecular weight rheologic factors. The time schedule of the study was the same as that of the largest published MIRA-1 study[18], i.e., 2 procedures weekly and then a 14-day pause, with the procedure repeated four times. Altogether, 8 procedures were performed within 10 weeks, and 1 to 2 procedures were recently added after one year of follow-up, if needed (if suspicion or symptoms of disease progression were discovered). We treated 1.5 volumes of plasma in one session. Critical values were considered to be decrease in fibrinogen levels to less than 0.7 g/L. In these cases, the volume of filtered plasma was decreased (from 1.5 x body volume to 1 x body volume). The patients were required to have peripheral veins allowing for vascular access to establish the extracorporeal circuit. All of the filters and tubing systems for extracorporeal circulation were for a single use only.

Analytical determination of retinol and α-tocopherol in serum, lipoprotein fractions and erythrocyte membranes

The blood samples were collected before and after the extracorporeal elimination process. The study protocol was approved by the Ethics Committee of University Hospital in Hradec Králové. Informed consent was obtained from all of the participants. The research followed the tenets of the Declaration of Helsinki.

The blood samples were first centrifuged (2000 × g, 10 min, 4 °C), and the serum was separated and analysed immediately or was frozen at -20 °C until analysis.

Lipoprotein fractions were prepared from fresh serum by a gradient ultracentrifugation technique, using saline solution with a determined density with 0.1% EDTA added to avoid oxidation of lipoproteins during ultracentrifugation.

Serum lipoproteins were separated into very low-density lipoprotein (VLDL d < 1.006), low-density lipoprotein (LDL d < 1.063) and high-density lipoprotein (HDL d >

Table 1. Levels of cholesterol and TAG in serum and lipoprotein fractions before and after RH treatment.

Analyte	N	Before Average (±SD) mmol/L	After Average (±SD) mmol/L	Decreased by (%)	Statistical significance
Cholesterol-serum	66	4.25 (±1.35)	1.78 (±0.49)	58.12	0.001
Cholesterol-VLDL	66	0.81 (±0.34)	0.30 (±0.22)	62.96	0.001
Cholesterol-LDL	64	2.28 (±0.71)	0.81 (±0.29)	64.47	0.001
Cholesterol-HDL	66	1.02 (±0.21)	0.64 (±0.15)	37.26	0.001
TAG-serum	65	1.48 (±0.64)	0.74 (±0.44)	50.00	0.001
TAG-VLDL	65	0.93 (±0.49)	0.48 (±0.37)	48.39	0.001
TAG-LDL	64	0.35 (±0.11)	0.17 (±0.08)	51.43	0.001
TAG-HDL	65	0.13 (±0.08)	0.09 (±0.04)	30.77	0.001

Table 2. Levels of vitamin E in serum and lipoprotein fractions before and after RH treatment.

Analyte	N	Before Average (±SD) µmol/L	After Average (±SD) µmol/L	Decreased by (%)	Statistical significance
Vit E-serum	68	22.23 (±6.75)	11.00 (±3.25)	50.52	0.001
Vit E-VLDL	66	6.31 (±3.14)	2.56 (±1.28)	59.43	0.001
Vit E-LDL	66	9.45 (±3.45)	3.83 (±1.34)	59.47	0.001
Vit E-HDL	67	6.45 (±1.81)	3.88 (±1.20)	39.85	0.001

1.063) fractions, using an OPTIMA MAX-XP ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) (ref.[19]).

The method used in this study for the analysis of vitamin A (retinol) and vitamin E (α-tocopherol) in serum and lipoprotein fractions was modified from the method previously published by Urbanek et al.[20].

The level of α-tocopherol in erythrocyte membranes was analysed by the modified HPLC method previously published by Solichova et al.[21].

Analytical determination of cholesterol and triacylglycerols

Serum cholesterol and triacylglycerols (TAG) were determined on a MODULAR analyser (Hoffmann-La Roche, Basel, Switzerland) using commercially available kits, according the manufacturers' instructions. The same methods were used for the evaluation of cholesterol and TAG contents in the lipoprotein fractions.

Analytical determination of enzymes

Erythrocyte glutathione peroxidase (GPx) was determined spectrophotometrically using a commercial kit (Ransel, Randox, United Kingdom), according the manufacturer's instruction, with the elimination of absorbance at a 340 nm wavelength (Cobas Mira, Roche, Switzerland).

Serum malondialdehyde was measured as a red complex with thiobarbituric acid at 485, 532 and 560 nm using a Beckman DU 640 spectrophotometer (Beckman, Palo Alto, USA).

Erythrocyte superoxide dismutase was determined spectrophotometrically with a commercial kit (Ransod, Randox, United Kingdom) according the manufacturer's instructions, as the elimination of absorbance at a 505 nm wavelength (Secomam, Ales, France).

Statistical analysis

NCSS 2007 software (Kaysville, USA) was used for statistical evaluation of the changes in each measured parameter before and after extracorporeal elimination therapy. Evaluation was performed with the nonparametric Mann-Whitney U test and Wilcoxon's signed-tank test. Statistical significance was based on $P \leq 0.05$.

RESULTS

Cholesterol and triacylglycerols

Decreases in cholesterol levels are presented in Table 1. Cholesterol and TAG were measured in serum and different lipoprotein fractions, including VLDL, LDL and HDL fractions. The results are presented in Fig. 2.

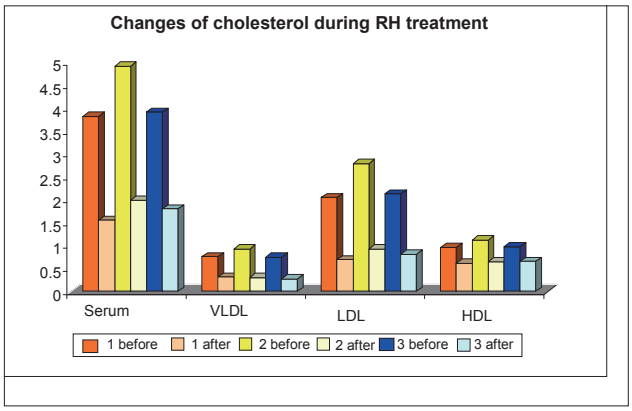

Fig. 2. Changes of cholesterol levels in serum and lipoproteins during RH treatment.

Table 3. Vitamin E/cholesterol ratios in serum and lipoprotein fractions before and after RH treatment.

Vitamin E/ cholesterol ratio	N	Before Average (±SD) 10^{-3}	After Average (±SD) 10^{-3}	Increased by (%)	Statistical significance
Serum	65	5.42 (±1.23)	6.28 (±1.34)	15.87	0.001
VLDL	64	7.88 (±2.29)	9.80 (±5.09)	24.37	0.000309
LDL	57	4.16 (±0.99)	5.11 (±3.64)	22.84	0.000006
HDL	65	6.36 (±1.21)	6.48 (±4.12)	1.89	0.043457

Table 4. Levels of MDA, SOD and GPx before and after RH treatment.

Analyte	N	Before Average (±SD)	After Average (±SD)	Statistical significance
Malondialdehyde (μmol/L)	50	0.48 (±0.41)	0.56 (±0.22)	0.002358
Superoxide dismutase (U/g Hb)	50	1595.78 (±729.02)	1505.74 (±676.66)	0.593154
Glutathione peroxidase (U/g Hb)	50	35.79 (±11.29)	34.46 (±10.20)	0.557875

Vitamin E

Levels of vitamin E before and after the procedure and its decreases in serum and lipoprotein fractions are presented in Table 2.

However, a decrease in the vitamin E/cholesterol ratio was not observed. In serum and in the VLDL and LDL fractions, the vitamin E/cholesterol ratio increased significantly in range, from 1.9% to 24.4%. In the HDL fraction, the ratio showed weak but statistically significant changes. The results are presented in Table 3.

The results of our study confirmed an increase (not statistically significant) in vitamin E levels in the membranes of 2% after rheohaemapheresis (Fig. 3).

Vitamin A

The average levels of vitamin A in serum before and after treatment were 1.56±0.38 μmol/L and 1.22 ± 0.27 μmol/L, respectively. The results constitute a statistically significant decrease (P = 0.001). Nevertheless, the measured values after treatment were at physiological levels.

Other metabolites

During the rheohaemapheresis treatment, there were reductions observed in the levels of erythrocyte superoxide dismutase and erythrocyte glutathione peroxidase, but these changes were not statistically significant. The serum level of malondialdehyde after rheohaemapheresis increased significantly by 17%, as presented in Table 4 and also in Fig. 4.

DISCUSSION

Until now, there have been no effective pharmacological therapies for the dry form of AMD that have been documented by multicentre, randomised, double-blind studies[8,22].

Cholesterol and triacylglycerols

An exactly defined spectrum of high molecular weight, rheologically relevant plasma proteins (i.e., LDL-cholesterol, lipoprotein) is removed by rheohaemapheresis. As described above, this process can lead to sustained microcirculatory recovery and can significantly alter the natural course of this chronic disease. This process should improve the metabolic exchange between the RPE and

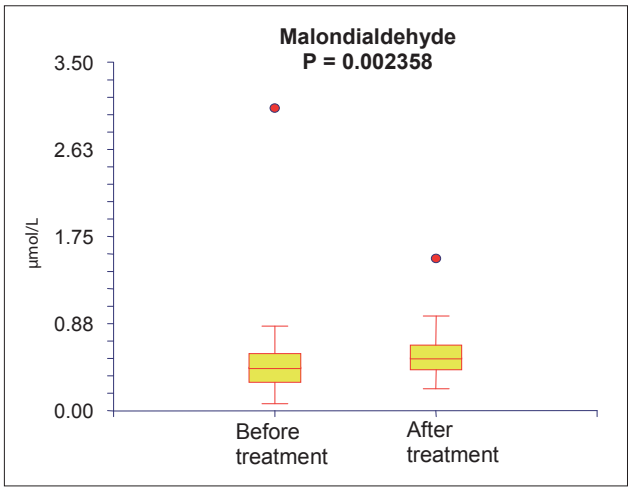

Fig. 3. Levels of MDA in serum before and after RH treatment.

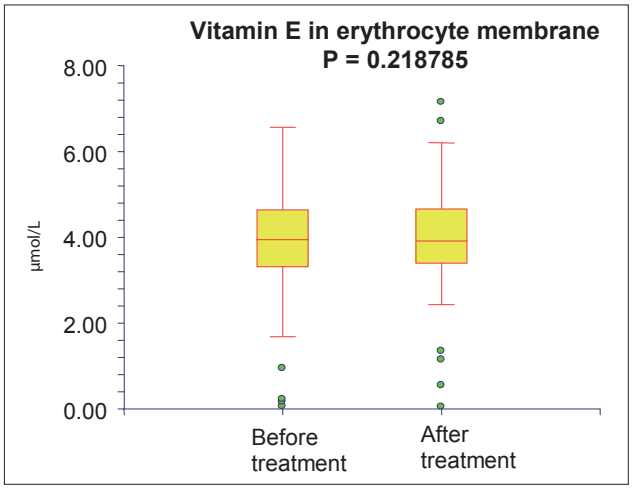

Fig. 4. Levels of vitamin E in erythrocyte membranes before and after RH treatment.

the choriocapillaris and the nutrition of RPED cells and of the neuroepithelium, reducing ischaemia and the production of vascular endothelial growth factor (VEGF). VEGF and pigment epithelium-derived factor (PEDF) are regulated by tissue oxygenation. Expression of VEGF is induced by hypoxia, thus promoting neovascularisation, while PEDF is induced by increase of oxygen, thus inhibiting neovascularisation[23]. However, optimal quantification of the surface structures of erythrocytes (e.g., stability and elongation), granulocytes (e.g., migration and penetration of the vessel wall), lymphocytes e.g., role of regulatory T cells), monocytes (e.g., migration and penetration of the vessel wall) and platelets (e.g., adhesion to endothelial cells) has not yet been achieved[13,24].

In this work, high molecular weight molecules, such cholesterol and TAG, decreased by 31-65%.

Vitamin E

Antioxidant defence systems, such as enzymes or vitamins, protect the host directly and indirectly against the damaging influence of oxidants[25]. Tocopherols react with free radicals, notably peroxyl radicals, and with singlet molecular oxygen, which forms the basis of their function as antioxidants[26].

The term vitamin E covers 8 different forms (α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol) (ref.[27]), and in general, it is the major chain-breaking antioxidant of cellular membranes. α-tocopherol is the most active scavenger of free radicals and the most predominant tocopherol in the human retina and plasma[26].

There have been some studies reporting an association between high total vitamin E intake and the risk of late AMD or indistinct soft or reticular drusen and between higher intake of dietary α-carotene and the risk of neovascular AMD (ref.[28]).

There has been much evidence supporting the concept that vitamin E protects against retinal oxidative damage, including that vitamin E deficiency results in retinal degeneration and that darkening, which results in increased vulnerability to retinal light damage, is associated with reduced ascorbate and vitamin E levels in the rat retina[4].

AREDS demonstrated that supplementation with high-dose antioxidant supplements (vitamin C, vitamin E, and β-carotene) and zinc for an average of 6.3 years could reduce the risk of progression to advanced AMD in persons with intermediate AMD or advanced AMD in 1 eye. Nevertheless, AREDS could not determine whether the zinc and antioxidant combination could delay progression of early-stage AMD or could prevent the onset of AMD in persons at usual risk[29]. However, there has been accumulating evidence that taking vitamin E or β-carotene supplements will not prevent or delay the onset of AMD, and some researchers have failed to demonstrate that vitamin E and selenium protect against photochemical damage to the retina[4,26,29].

The optimal combination of antioxidants has yet to be formulated, as has whether antioxidant therapy will be part of a multifaceted approach to the treatment of AMD (ref.[30]). The results of 3 trials, enrolling primarily persons at usual risk, found little evidence that supplementation with vitamin E for 4 to 6 years or with β-carotene for 10 years could materially reduce the risk of AMD; with a treatment duration of 10 years, the current findings extended these earlier findings by showing that very long-term supplementation with vitamin E alone was unlikely to have an important effect on AMD occurrence[29].

Vitamin E does not appear to have a specific carrier protein in the plasma. It is rapidly transferred from chylomicra to plasma lipoproteins, to which it binds non-specifically, whereupon it is taken up by the liver and is incorporated into nascent VLDL (with selectivity in favour of α-tocopherol over the γ-vitamer), which is secreted by the liver. Although the majority of triglyceride-rich VLDL remnants are returned to the liver, others are converted by lipoprotein lipase into LDL. It appears that, during this process, vitamin E also transfers spontaneously to apolipoprotein B-containing lipoproteins, including VLDL, LDL and HDL. Therefore, plasma tocopherols are distributed among these three lipoprotein classes, with the more abundant LDL and HDL classes comprising the major carriers of vitamin E (ref.[31]). Fasting blood levels of vitamin E, after multivariate adjustment, showed a weak negative association with AMD (ref.[31]). Lipid-standardised plasma α-tocopherol had significant inverse relationships with early and late AMD, representing a risk reduction in AMD of 82% for those in the highest quintile versus the lowest quintile, in a study by Delcourt et al.[32].

However, Christen et al. reported that, in a large-scale, randomised trial of male US physicians, alternate-day use of 400 IU of vitamin E and/or daily use of 500 mg of vitamin C for 8 years had no appreciable beneficial or harmful effects on the risk of incident diagnosis of AMD (ref.[33]).

Nevertheless, among the components of LDL investigated in this study, an increased vitamin E-to-cholesterol ratio was the strongest predictor of increased resistance of the lipoprotein to metal ion-dependent oxidation[34], which was why we examined the proportions of vitamin E and cholesterol in serum and in different lipoprotein fractions, and the results showed no decreases in the lipoprotein fractions.

In general, the highest accumulation of vitamin E is in the adipose tissue[35]. In most non-adipose cells, vitamin E is localised almost exclusively in the membranes. Kinetic studies have indicated that such tissues have two pools of the vitamin: a labile, rapidly overturning pool; and a fixed, slowly overturning pool[31].

Very important is the effect on the levels of vitamin E in membranes. As a model of the circumstances in membranes, erythrocyte membranes were selected. Erythrocytes are the major cellular component of the blood, and erythrocyte membrane fluidity can be affected by human diseases associated with oxidative stress. Free radicals are commonly formed in erythrocytes due to oxidation of haemoglobin. Erythrocyte membrane fluidity can thus be viewed as an indirect marker of oxidative stress[36]. For this reason, we measured vitamin E levels in erythrocyte membranes and confirmed no decrease.

Vitamin A

Vitamin A exists in the following three oxidation states: an alcohol (retinol), an aldehyde (retinal), and an acid (retinoic acid) (ref.[4]). Retinol is essential for vision since it must be available in the retina as a precursor of 11-*cis*-retinal for the regeneration of rhodopsin.

In healthy individuals, plasma retinol is maintained within a narrow range (0.53 – 2.1 μmol/L in adults; typically approximately half of that in new-born infants) despite widely varying intakes of vitamin/provitamin A (ref.[31,37]).

Vitamin A is also involved in the repair of cells that have been oxidatively damaged. Notably, in the retina, vitamin E is believed to protect vitamin A from oxidative degeneration[4].

Nutritional treatment of retinal disease has proved at least partially successful in common retinitis pigmentosa (vitamin A), Bassen-Kornzweig disease (vitamins A, E, and K), and Sorsby fundus dystrophy (vitamin A) (ref.[10]). Nevertheless, regarding plasma retinol and AMD, the POLA study failed to detect a significant association[4].

Other metabolites

Other compounds, such as malondialdehyde (MDA), in the serum and the enzymes erythrocyte superoxide dismutase (SOD) and erythrocyte glutathione peroxidase (GPx) were investigated as markers of antioxidant activity and lipid peroxidation in this study. MDA is a common lipid peroxidation product that accumulates in many pathophysiological processes, including AMD (ref.[38]). Enzymes, superoxide dismutase and glutathione peroxidase demonstrated antioxidant activity[4].

SOD catalyses the quenching of the superoxide anion to produce hydrogen peroxide and oxygen. No association could be detected between systemic SOD activity and AMD in the POLA study[4]. In every way, high levels of SOD were also associated with an increased risk of nuclear cataracts[39].

GPx uses glutathione as an electron donor to reduce organic hydroperoxides. The POLA study analysed the relationship between antioxidant enzymes and age-related macular disease and found out that higher plasma levels of GPx were significantly associated with a nine-fold increase in the prevalence of late AMD, but they were unassociated with early AMD. Delcourt et al. demonstrated a strong association of high levels of plasma GPx with age-related eye diseases. More data are needed at the biochemical and epidemiologic levels for a better understanding of these findings[4,39].

MDA is an abundant oxidation-specific epitope that accumulates in a number of oxidative stress-related diseases. Chou et al. recently demonstrated that 15% of all IgM natural antibodies bound to MDA adducts in mouse plasma[40]. This observation illustrated the importance that the natural selection process of the innate immune system places on MDA. Recently, Weismann et al. identified complement factor H (CFH) as a major MDA-binding protein that could block both the uptake of MDA-modified proteins by macrophages and MDA-induced proinflammatory effects in vivo in mice[38].

As a summary of our study, we should mention that the levels of vitamin E in erythrocyte membranes did not decrease after rheohaemapheresis. These results suggest that tissue concentrations of vitamin E remained unchanged despite a reduction in the lipid carrier in the blood. In serum and lipoprotein fractions, vitamin E decreased. However, more informative data were obtained from the ratio of vitamin E to cholesterol in serum and in various lipoprotein fractions, in which the relative content of vitamin E even improved significantly. The monitored patients did not show any clinical signs of vitamin E deficiency, and their serum concentrations of vitamin E did not differ from normal values.

CONCLUSION

In conclusion, the level of serum vitamin A statistically decreased but not to less than physiological levels. Serum malondialdehyde significantly increased, and erythrocyte superoxide dismutase and erythrocyte glutathione peroxidase were not statistically significantly involved in rheohaemapheresis processes.

Rheohaemapheresis did not result in a negative antioxidant balance. On the contrary, the results show that, rheohaemapheresis might have had favourable additive anti-atherogenic effects due to the relative increase in content of vitamin E in the lipoprotein fractions, in addition to causing a significant reduction in atherogenic LDL.

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Author contributions: JA: manuscript writing; MB: study design; EK, BH, BC, LKK, JP: data analysis; ML, LS: data interpretation; DS: statistical analysis.

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