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Lack of association between the c.544G>A polymorphism of the heme oxygenase-2 gene and age-related macular degeneration

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

Background: Age-related macular degeneration (AMD) is a primary cause of blindness among the elderly in developed countries. The nature of AMD is complex and includes both environmental and hereditary factors. Oxidative stress is thought to be essential in AMD pathogenesis. Iron is suggested to be implicated in the pathogenesis of AMD through the catalysis of the production of reactive oxygen species, which can damage the retina. Heme oxygenase-2 is capable of degradation of heme producing free iron ions, thus, diversity in heme oxygenase-2 gene may contribute to AMD. In the present work we analyzed the association between the c.544G>A polymorphism of the heme oxygenase-2 gene (*HMOX2*) (rs1051308) and AMD.

Material/Methods: This study enrolled 276 AMD patients and 105 sex- and age-matched controls. Genotyping of the polymorphism was performed with restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) on DNA isolated from peripheral blood.

Results: We did not find any association between the genotypes of the c.544G>A polymorphism and the occurrence of AMD. This lack of association was independent of potential AMD risk factors: tobacco smoking, sex and age. Moreover, we did not find any association between AMD and smoking in our study population.

Conclusions: The results suggest that the c.544G>A polymorphism of the heme oxygenase-2 gene is not associated with AMD in this Polish subpopulation.

key words: heme oxygenase-2 • *HMOX2* gene • age-related macular degeneration (AMD) • genetic polymorphism • iron metabolism

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BACKGROUND

Age-related macular degeneration (AMD) is the leading cause of vision loss among the elderly in developed countries. The disease affects the macula and leads to a progressive degeneration of retinal epithelium (RPE) cells and photoreceptors [1]. Among individuals aged 75 and older the occurrence of AMD is estimated to exceed 30% and is growing [2]. Early stages of AMD are characterized by drusen deposition in Bruch's membrane, and the disease can develop to dry (atrophic) or wet (exudative) form. The dry form is more common and results in the depigmentation and geographic atrophy of the central retina, while the wet form is characterized by choroid neovascularization with frequent leakages from vessels. The wet form of AMD has faster progression and is responsible for most of blindness cases among AMD patients [3].

The etiology of AMD is not fully understood, but it is known that many genetic and environmental factors are involved in the development of the disease. To date, a number of single nucleotide polymorphisms (SNPs) have been correlated with AMD, including polymorphisms of the complement factor H gene and *ERCC6* flanking region [4], *LOC387715/ARMS2* [5], *VEGFA* and *VEGFR-2* genes [6]. Tobacco smoking seems to be the strongest life style/environmental factor in AMD pathogenesis identified to date [7]. Others are female sex [8], hypertension and obesity [9, 10], and elevated inflammatory and cardiovascular markers in the blood [11–13]. Oxidative stress is considered to play an essential role in AMD pathogenesis. Reactive oxygen species (ROS) can cause severe damage to retinal tissue and antioxidant treatment can protect the retina from ROS-mediated damage [14,15].

Heme is an essential molecule in the human body, displaying a number of functions. It carries oxygen as a component of hemoglobin and it is a part of many other hemoproteins, including cytochromes, catalases, peroxidases and cyclooxygenases [16]. Heme also has an ability to regulate the expression of many genes, including those involved in cell differentiation and proliferation [17]. However, excess heme may be toxic to many organs, especially the kidneys, liver, cardiovascular system and brain [18,19]. Heme toxicity is displayed through its prooxidative action. It may catalyze the formation of reactive oxygen species (ROS) and promote hydrogen peroxide generation, leading to oxidative tissue damage [20]. All heme catabolism products – biliverdin, carbon monoxide and divalent iron ions – are closely bound to diverse metabolic pathways in the body. The effects of deregulation of these pathways can be associated with some pathologies [21,22]. Thus, heme and iron ions can be harmful for different cell types, including retinal cells [23]. The role of iron toxicity as a potential factor in AMD was thoroughly studied by Dunaief et al [24–26].

The products of heme catabolism can exert dangerous, neutral or protective effects, depending on its abundance and tissue type, because various cells can be differentially sensitive to these substances. Therefore, it is possible that some divergence in the heme catabolism pathway may be important for AMD pathogenesis.

Heme oxygenase is a membrane-bound enzyme catalyzing oxidative degradation of heme. In this reaction biliverdin,

carbon monoxide and divalent iron ions (FeII) are generated [27]. Biliverdin is directly reduced to bilirubin by biliverdin reductase (BVR), and because the activity of BVR is 30–50 times higher than heme oxygenase, the latter appears to be the rate-limiting element in heme catabolism [28]. Three isoforms of heme oxygenase have been identified – 1, 2 and 3 – each being a product of a different gene. Whereas the properties of heme oxygenase-1, encoded by the *HMOX1* gene and heme oxygenase-2 (*HMOX2*) gene are generally understood, the nature of the heme oxygenase 3 (*HMOX3*) gene is unclear [17]. Despite the fact that both heme oxygenases-1 and 2 share the same functions in heme catabolism, their properties are unique. *HMOX2* gene is constitutively expressed in most tissues, including brain, liver, kidneys, vascular system and retina, while *HMOX1* gene expression is inducible and tissue-specific. Various factors, including oxidative stress, hypoxia or cigarette smoke can alter the expression of this enzyme [21]. Genetic variability in the protein related to oxidative stress has been shown to play a role in AMD pathogenesis [29].

In the present work we investigated the role of the c.544G>A polymorphism in the *HMOX2* gene (rs1051308) in AMD. This polymorphism is located in the 3'-untranslated region of the *HMOX2* gene, so it can change the stability of its transcript and affect translation. We chose this polymorphism on the basis of information in the SNP branch of the PubMed database. There is a lack of information about the effect of genetic variability in this gene in AMD, but the present study is justified by the potentially important role of heme oxygenase-2 in AMD pathogenesis.

MATERIAL AND METHODS

Patients

The study was performed on blood samples obtained from 276 AMD patients (average age 72.5 years) and 105 age- and sex-matched controls (average age 68.3 years) seeking medical advice at the Department of Ophthalmology, University of Warsaw, Poland in 2010 due to various ophthalmological disturbances (Table 1). The patients group included 101 individuals with dry form of AMD (average age 72.9 years) and 175 with wet form of the disease (average age 72.3 years). Medical history was obtained from all subjects and none reported current or previous cancer or any genetic disease. The patients and controls underwent ophthalmic examination, including best-corrected visual acuity, intraocular pressure, slit lamp examination, and fundus examination, performed with a slit lamp equipped with either non-contact or contact fundus lenses. Diagnosis of AMD was confirmed by optical coherence tomography (OCT) and, in some cases, by fluorescein angiography (FA) and indocyanin green angiography (ICG). OCT evaluated retinal thickness, the presence of RPE atrophy, drusen, or subretinal fluid and intraretinal edema; angiography assessed the anatomical status of the retinal vessels, the presence of choroidal neovascularization and leakage. The OCT examinations were performed with Stratus OCT model 3000, software version 4.0 (Oberkochen, Germany). The FA and ICG examinations were completed with a Topcon TRC-50I IX fundus camera equipped with the digital Image Net image system, version 2.14 (Topcon, Tokyo, Japan). Subjects with the exclusion of AMD were classified into the control group.

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Table 1. Characteristics of patients with age-related macular degeneration (AMD) and controls.

Individuals	Number	Mean age (SD[1])	Sex[2]
All	381	71.3 (9.4)	2.26
AMD	276	72.5 (8.5)	1.91
Dry AMD	101	72.9 (9.4)	1.97
Wet AMD	175	72.3 (7.9)	1.98
Controls	105	68.3 (10.9)	3.55

[1] SD – standard deviation; [2] females/males ratio.

A structured questionnaire was used to obtain information from study subjects about lifestyle habits and family/personal history of AMD. The genetic analyses did not interfere with diagnostic or therapeutic procedures for the subjects. The Bioethics Committee of the Medical University of Warsaw, Poland approved the study and each patient gave written informed consent.

DNA preparation

DNA was isolated from venous blood samples. DNA was isolated using AxyPrep Blood Genomic DNA Miniprep kit (Axygen Biosciences, San Francisco, CA, USA). DNA was kept frozen at –20°C before use.

Genotyping

DNA fragment of *HMOX2* gene containing the c.544G>A polymorphic site was amplified by polymerase chain reaction (PCR) in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Total reaction volume for each sample was 25 µl and contained 10 ng of genomic DNA, 0.75 U Taq polymerase (Biotools, Madrid, Spain), 1 × reaction buffer, 0.5 mM dNTP, 1.5 mM $MgCl_2$ and 0.25 µM of each primer (Sigma-Aldrich, St. Louis, MO, USA). Primers sequences were: forward 5'-AGGTGAGTGGCCTGTAAGTCC-3', reverse 5'-TAGACCCAGAGCAGGAGGTG-3'. Thermal cycling conditions were: initial denaturation step at 95°C for 5 min, 34 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and amplification at 72°C for 1 min; final extension at 72°C for 5 min. Amplified fragments of 393 bp long, containing the polymorphic site were then digested with the restriction endonuclease *Hha*I (Fermentas, Burlington, Canada). The enzyme recognizes the G allele in the c.544G>A site and cleaves DNA generating 2 fragments: 204 and 189 bp long, whereas fragments carrying the A variant remained intact (Figure 1). The digestion of 3 µl of PCR product in total volume of 15 µl was performed with 1 U of *Hha*I enzyme and 1 × digestion buffer for 6 min at 37°C. Then samples were separated on a 8% polyacrylamide gel in TBE buffer at 80 V. Gene Ruller (Fermentas, Burlington, Canada) was utilized as a molecular mass marker.

Data analysis

The allelic frequencies were estimated by gene counting, and genotypes were scored. The significance of the differences between distributions of alleles and genotypes was tested using
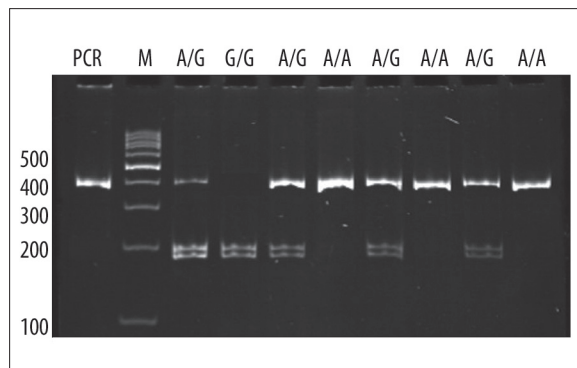

Figure 1. Genotypes of the c.544A>G polymorphism of the *HMOX2* gene (rs1051308) determined by the polymerase chain reaction-restriction fragment length polymorphism with, analyzed by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide and viewed under UV light. Lane PCR represent product M shows GeneRuler™ 100 bp molecular weight marker, all remaining lanes present genotypes indicated in the upper part of the picture.

Table 2. Association of AMD with age, sex, tobacco smoking, AMD in family and living environment.

Factor	OR[1] (95% CI)	p[2]
Age	**1.049 (1.024–1.074)**	**<0.001***
Sex[3]	**0.555 (0.328–0.939)**	**<0.05***
Smoking	0.987 (0.572–1.706)	0.964
Family AMD	**6.854 (2.036–23.077)**	**<0.001***
Environment[4]	0.713 (0.41–1.238)	0.231

[1] Odds ratio with 95% confidence interval; [2] Chi-square test; [3] adjusted to females; [4] adjusted to rural. Data in boldface are statistically significant.

the χ^2 analysis. Unconditional logistic regression analysis was performed to assess the association between the genotypes of the polymorphism and AMD occurrence. The genotype-associated risk was expressed by crude odds ratio with 95% confidence intervals and the *p* value. Odds ratios were then adjusted for possible interfering factors. To verify a potential gene-environment interaction, the patients and controls were stratified depending on age, sex, living environment (rural or urban), smoking status and the occurrence of AMD among first-degree relatives. Multiple unconditioned logistic regression analyses were run to test the association of genotypes and environmental and social factors with AMD occurrence. Statistical analysis was performed using Statistica 9.0 package (Statsoft, Tulsa, OK, USA).

RESULTS

We did not observe any departure from Hardy-Weinberg equilibrium in the distribution of genotypes of the c.544G>A polymorphism in patients and controls (*p*>0.05). Both groups were compared according to age, sex, living environment (rural or urban), AMD in family (first degree relatives) and tobacco smoking (Table 2). AMD was associated with family

Table 3. Distribution of genotypes, frequency of alleles of the c.544G>A polymorphism of the heme oxygenase-2 gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration and controls.

Genotype/allele	Controls N=105		AMD N=276		OR crude (95% CI)		OR adjusted[1] (95% CI)	
	Number (%)		Number (%)					
AA	55	(0.52)	155	(0.56)	1.16	(0.74–1.83)	1.24	(0.72–2.19)
AG	43	(0.41)	112	(0.41)	0.98	(0.62–1.56)	0.85	(0.48–1.53)
GG	7	(0.07)	9	(0.03)	0.47	(0.17–1.30)	0.70	(0.18–2.69)
A	153	(0.73)	422	(0.76)	2.112	(0.77–5.84)	1.42	(0.37–5.46)
G	57	(0.27)	130	(0.24)	0.86	(0.54–1.35)	0.81	(0.46–1.43)

[1] Odds ratio adjusted for age, sex and family AMD status.

Table 4. Distribution of genotypes, frequency of alleles of the c.544G>A polymorphism of the heme oxygenase-2 gene and odds ratios (OR) with 95% confidence intervals (95% CI) in wet form of age-related macular degeneration and controls.

Genotype/allele	Controls N=105		Wet AMD N=175		OR crude (95% CI)		OR adjusted[1] (95% CI)	
	Number (%)		Number (%)					
AA	55	(0.52)	104	(0.59)	1.33	(0.82–2.17)	1.73	(0.89–3.34)
AG	43	(0.41)	67	(0.38)	0.89	(0.55–1.47)	0.63	(0.32–1.24)
GG	7	(0.07)	4	(0.02)	0.33	(0.09–1.15)	0.46	(0.08–2.76)
A	153	(0.73)	275	(0.79)	3.05	(0.87–10.69)	2.17	(0.36–12.95)
G	57	(0.27)	75	(0.21)	0.75	(0.46–1.22)	0.58	(0.30–1.12)

[1] Odds ratio adjusted for age, sex and family AMD status.

Table 5. Distribution of genotypes, frequency of alleles of the c.544G>A polymorphism of the heme oxygenase-2 gene and odds ratios (OR) with 95% confidence intervals (95% CI) in dry form of age-related macular degeneration and controls.

Genotype/allele	Controls N=105		Dry AMD N=101		OR crude (95% CI)		OR adjusted[1] (95% CI)	
	Number (%)		Number (%)					
AA	55	(0.52)	51	(0.50)	0.93	(0.54–1.60)	0.87	(0.43–1.75)
AG	43	(0.41)	45	(0.45)	1.16	(0.67–2.01)	1.10	(0.54–2.25)
GG	7	(0.07)	5	(0.05)	0.73	(0.22–2.38)	1.21	(0.28–5.30)
A	153	(0.73)	147	(0.73)	1.37	(0.42–4.47)	0.82	(0.19–3.60)
G	57	(0.27)	55	(0.27)	1.08	(0.62–1.86)	1.15	(0.57–2.32)

[1] Odds ratio adjusted for age, sex and family AMD status.

history, sex (women at a higher risk) and age. We found no association between AMD and tobacco smoking and living environment. There was no difference between the distributions of the genotypes of the c.544G>A polymorphism in AMD patients and controls (Table 3). No differences were observed when patients with dry or wet form of the disease were compared with the controls (Tables 4 and 5). Our results suggest the lack of correlation between the c.544G>A polymorphism in the *HMOX2* gene and AMD occurrence, including both forms of the disease. Further stratification in the patients showed no correlation between the c.544G>A polymorphism and tobacco smoking, sex, age and living environment (Tables 6–9).

DISCUSSION

We observed a small age difference between the control and the age group (Table 1); however, this difference is not statistically significant ($p > 0.05$) and it may not be relevant medically at that age in the context of AMD.

We did not observe any association between AMD occurrence and tobacco smoking in our population. Smoking is associated with oxidative stress and this association may be organ-dependent [30]. Smoking is one of the most potent environmental risk factors of AMD, but its actual role in the pathogenesis of AMD may depend on many factors

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Table 6. Distribution of genotypes, frequency of alleles of the c.544G>A polymorphism of the heme oxygenase-2 gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration and controlsamong smokers and non-smokers.

Genotype/allele	Never-smokers					Ever-smokers				
	Controls N=52 number (%)		Patients N=104 number (%)		OR[1] (95% CI)	Controls N=32 number (%)		Patients N=65 number (%)		OR[1] (95% CI)
AA	30	(0.58)	59	(0.57)	1.02 (0.45–2.12)	16	(0.50)	41	(0.63)	1.41 (0.54–3.71)
AG	20	(0.38)	43	(0.41)	1.10 (0.53–2.31)	14	(0.44)	19	(0.29)	0.64 (0.23–1.74)
GG	2	(0.04)	2	(0.02)	0.31 (0.03–2.94)	2	(0.06)	5	(0.08)	1.28 (0.20–8.04)
A	80	(0.77)	161	(0.77)	3.17 (0.34–29.54)	46	(0.72)	101	(0.78)	0.78 (0.12–4.90)
G	24	(0.23)	47	(0.23)	0.98 (0.47–2.03)	18	(0.28)	29	(0.22)	0.71 (0.27–1.87)

[1] Adjusted for age, sex and family AMD history.

Table 7. Distribution of genotypes, frequency of alleles of the c.544G>A polymorphism of the heme oxygenase-2 gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration and controls among females and males.

Genotype/allele	Females					Males				
	Controls N=82 number (%)		Patients N=180 number (%)		OR[1] (95% CI)	Controls N=23 number (%)		Patients N=91 number (%)		OR[1] (95% CI)
AA	44	(0.54)	98	(0.54)	1.05 (0.54–2.04)	11	(0.48)	56	(0.61)	2.00 (0.64–6.18)
AG	32	(0.39)	77	(0.43)	1.05 (0.54–2.06)	11	(0.48)	31	(0.34)	0.45 (0.14–1.44)
GG	6	(0.07)	5	(0.03)	0.48 (0.09–2.72)	1	(0.04)	4	(0.04)	1.22 (0.11–12.98)
A	120	(0.73)	273	(0.76)	2.06 (0.37–11.57)	33	(0.72)	143	(0.79)	0.82 (0.08–8.70)
G	44	(0.27)	87	(0.24)	0.95 (0.49–1.84)	13	(0.28)	39	(0.21)	0.50 (0.16–1.54)

[1] Adjusted for age and family AMD history.

influencing an individual's susceptibility to this disease, including his/her ability to metabolize xenobiotics included in tobacco smoke. We did not perform any study aimed at assessing the role of factors that may affect relationships between smoking and AMD in our study population. Moreover, the average age in our population exceeded 70 years and many persons enrolled in our study simply did not remember how many cigarettes they smoked and how long they had been smoking.

The c.544G>A polymorphism in the *HMOX2* gene encoding heme oxygenase-2 is located in the c.544 position of the coding sequence, inside 3'-untranslated region (3'-UTR). Changes in this region may affect the stability of the transcript and the process of translation. This is our first study on the significance of the variability in the *HMOX2* gene for AMD and we realize that an association or its lack between only 1 SNP in this gene and AMD do not demonstrate the role of genetic variability in the disease. This is a limitation of our study, but we are in the process of studying other SNPs of this gene. We chose this specific polymorphism because, due to its location, it can directly change the amount and/or function of the heme oxygenase-2 protein.

Heme oxygenase-2, the product of the *HMOX2* gene, is an important enzyme catalyzing heme degradation. Both, free heme (the substrate of the reaction) and iron ions as one of its products are potentially toxic since they can induce ROS generation [20]. Oxidative stress is believed to play a role in AMD pathogenesis [31]. Furthermore, oxidative stress may induce inflammatory processes in the eye, which is believed to contribute to AMD [32]. In contrast to heme and iron, carbon monoxide, which is the second product of heme degradation process, has anti-inflammatory properties [33], can modulate apoptosis [34] and promote angiogenesis [35]. Furthermore, it can reduce oxidative stress by an indirect action, through ROS signalling and upregulation of antioxidant enzymes [36]. The protective properties against oxidative damage are also attributed to the third product of heme catabolism, biliverdin and its derivative, bilirubin. Both have antioxidative properties possibly also in the retina [37]. In addition to their antioxidant roles, they can also reduce inflammation [38]. These data suggest a potentially significant role of both heme and the products of its catabolism in a number of pathological conditions. This includes effects linked with AMD pathogenesis as inflammation, neovascularization or oxidative defence. Therefore, variability in heme oxygenase-2 gene may affect

Table 8. Distribution of genotypes, frequency of alleles of the c.544G>A polymorphism of the heme oxygenase-2 gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration and controls among individuals aged 72 or less and 73 or more.

Genotype/ allele	72 and less						73 and more					
	Controls N=58 number (%)		Patients N=121 number (%)		OR[1] (95% CI)		Controls N=47 number (%)		Patients N=155 number (%)		OR[1] (95% CI)	
AA	29	(0.50)	67	(0.55)	1.36 (0.64–2.90)		26	(0.55)	88	(0.57)	1.13 (0.48–2.64)	
AG	25	(0.43)	50	(0.41)	0.78 (0.37–1.67)		18	(0.38)	62	(0.40)	0.88 (0.36–2.12)	
GG	4	(0.07)	4	(0.03)	0.61 (0.09–4.35)		3	(0.06)	5	(0.03)	0.99 (0.16–5.93)	
A	83	(0.72)	184	(0.76)	1.64 (0.23–11.68)		70	(0.74)	238	(0.77)	1.01 (0.17–6.08)	
G	33	(0.28)	58	(0.24)	0.73 (0.34–1.56)		24	(0.26)	72	(0.23)	0.88 (0.38–2.07)	

[1] Adjusted for sex and family AMD history.

Table 9. Distribution of genotypes, frequency of alleles of the c.544G>A polymorphism of the heme oxygenase-2 gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration and controls among city and countryside inhabitants.

Genotype/ allele	City						Countryside					
	Controls N=52 number (%)		Patients N=114 number (%)		OR[1] (95% CI)		Controls N=32 number (%)		Patients N=50 number (%)		OR[1] (95% CI)	
AA	23	(0.44)	64	(0.56)	1.55 (0.76–3.16)		22	(0.69)	30	(0.60)	0.81 (0.27–2.40)	
AG	27	(0.52)	44	(0.39)	0.64 (0.31–1.32)		8	(0.25)	19	(0.38)	1.58 (0.50–5.00)	
GG	2	(0.04)	6	(0.05)	0.98 (0.18–5.39)		2	(0.06)	1	(0.02)	0.33 (0.02–4.60)	
A	73	(0.70)	172	(0.75)	1.02 (0.18–5.63)		52	(0.81)	79	(0.79)	3.03 (0.22–42.18)	
G	31	(0.30)	56	(0.25)	0.65 (0.32–1.32)		12	(0.19)	21	(0.21)	1.24 (0.42–3.68)	

[1] Adjusted for age, sex and family AMD history.

all of these processes. A growing body of evidence supports this thesis. Heme oxygenase-2 plays a protective role in the brain. It is cytoprotective to neural cells by antioxidant action and also by blood flow regulation in the brain [39]. The retinal expression of heme oxygenase-2 was confirmed in a number of animal studies [38,40]. The enzyme may play a role in neural modulation by carbon monoxide generation [40]. It was also shown that heme oxygenase-2 protected cerebral microvascular cells from TNFα-dependent oxidative stress and apoptosis [41]. The deletion of this enzyme caused 3-fold upregulation of vascular endothelial growth factor receptor 1 (VEGFR1) and angiogenic response, elevated inflammation and oxidation status in endothelial cells [42]. The toxic effect of heme in brain tissue was manifested by lipid peroxidation and subsequent cell death and it was abrogated by heme oxygenase-2 [43]. Additionally, it was shown that a suppression of heme oxygenase-2 caused a considerable reduction in the activity of extracellular superoxide dismutase as well as stress- signalling kinases Akt, and Ask1 and neurotrophic factor 3-NT, leading to the apoptosis of vascular cells [44]. Finally, mouse lung lacking heme oxygenase-2 in hyperoxia had an elevated level of hemoproteins and free iron ions, suggesting the role of this protein in protection from oxidative stress by regulation of iron turnover [45]. It is possible that a similar effect exists in the retina since this organ is under high oxygen pressure due to its metabolism [46].

Conclusions

In summary, the c.544G>A polymorphism of *HMOX2* gene may not be associated with AMD, but an important role of the product of this gene in many processes directly or indirectly linked with AMD justifies further studies on the implications of the variability of the *HMOX2* gene in AMD.

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