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# Transferrin receptor levels and polymorphism of its gene in age-related macular degeneration

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**The aim of the present study was to investigate the association of age related macular degeneration (AMD) risk with some aspects of iron homeostasis: iron concentration in serum, level of soluble transferrin receptor (sTfR), and transferrin receptor (TFRC) genetic variability. Four hundred and ninety one AMD patients and 171 controls were enrolled in the study. Restriction fragment length polymorphism PCR was employed to genotype polymorphisms of the *TFRC* gene, and colorimetric assays were used to determine the level of iron and sTfR. Multiple logistic regression was applied for all genotype/allele-related analyses and the ANOVA test for iron and sTfR serum level comparison. We found that the genotypes and alleles of the c.–253G>A polymorphism of the *TFRC* gene were associated with AMD risk and this association was modulated by smoking status, AMD family history, living environment (rural/urban), body mass index and age. The levels of sTfR was higher in AMD patients than controls, whereas concentrations of iron did not differ in these two groups. No association was found between AMD occurrence and the p.Gly142Ser polymorphism of the *TRFC* gene. The results obtained suggest that transferrin receptor and variability of its gene may influence AMD risk.**

**Key words**: AMD, gene polymorphism, iron, oxidative stress, TFRC, transferrin receptor
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## INTRODUCTION

Age-related macular degeneration (AMD) is a disease affecting the central part of the retina and is responsible for most of blind registrations among elderly in Western countries (Feret *et al.,* 2007; Hogg & Chakravarthy, 2006). AMD in its advanced stage is classified as a dry (atrophic) or wet (exudative, neovascular) form and leads to photoreceptor and retinal epithelium layer loss (Feret *et al.,* 2007; Hogg & Chakravarthy, 2006; McConnell & Silvestri, 2005). The dry form is characterized by discrete depigmentation area in the center of the macula and is considerably more common, whereas wet form of AMD is manifested by choroidal neovascularization with leakages and bleeding and hemorrhagic detachment of retinal epithelium or sensory retina. The wet form of the disease, although less frequent, is responsible for a majority of total blindness incidents (Coleman *et al.,* 2008). AMD has multifactorial nature linking the influence of genetic and environmental factors (Ambati *et al.,* 2003). Among well proved risk factors for AMD are advanced age (Tomany *et al.,* 2004), sex (women are frequently reported to be at higher risk of AMD than men) (Buch *et al.,* 2005) and Caucasian race (Frank *et al.,* 2000). Hypertension (Klein *et al.,* 2003), obesity (Clemons *et al.,* 2005), cataract and cataract surgery (Klein *et al.,* 1998), high-fat diet (SanGiovanni *et al.,* 2007) and chronic sunlight exposure (Vojnicovic *et al.*, 2007) have been also reported to increase AMD risk. Tobacco smoking is considered as a primary environmental risk factor for AMD (Seddon *et al.,* 2006a; Wang *et al.,* 2008). Numerous family and twin studies suggest that AMD patients' relatives are at higher risk of the disease than general population (Hammond *et al.,* 2002; Shahid *et al.,* 2012).

Oxidative stress and inflammation may be key elements of AMD pathology (Ding *et al.,* 2009; Kaarniranta *et al.,* 2011). It was suggested that iron might be involved in oxidative stress-dependent AMD pathogenesis (Blasiak *et al.,* 2011; Wong *et al.,* 2007). Iron is an essential element in human organism and both its deficiency and excess may be deleterious (Hentze *et al.,* 2004). Majority of the iron pool is required for oxygen transport. It is also a constituent of cytochromes and enzymes (Nadadur *et al.,* 2008; Theil & Goss, 2009). However, iron ions are highly reactive and may participate in the Fenton reaction producing hydroxyl radicals, which may damage proteins, lipids and DNA (Jomova & Valko, 2011; Jia *et al.,* 2008). Elevated level of iron in organism is often caused by perturbation in iron homeostasis associated with genetic defects. A wide spectrum of phenotype-associated point mutations was identified in genes of key iron homeostasis regulators. Hereditary hemochromatosis is a genetically heterogeneous condition of acute iron overload in organism and results from mutation occurring in genes encoding proteins essential for iron homeostasis (Kohgo *et al.,* 2008).

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**Abbreviations**: AMD, age-related macular degeneration; sTfR, soluble transferrin receptor; OCT, optical coherence tomography; FA, fluorescein angiography; ICG, indocyanin green angiography; RPE, retinal pigment epithelium; AREDS, Age-Related Eye Disease Study; SNP, single nucleotide polymorphism; ROS, reactive oxygen species, NTBI, non-transferrin bound iron; LIP, labile iron pool; T2DM, type 2 diabetes mellitus.

Since iron may play a role in AMD pathogenesis, it is justified to check the effect of the variability in the transferrin receptor gene (*TFRC*) and its product on AMD risk. In the present work we investigated the association between AMD risk and the genotypes of the transferrin receptor gene (*TFRC*) polymorphisms: rs3817672 (p.Gly142Ser) and rs11915082 (c.-253G>A) as well as with serum iron concentration and soluble transferrin receptor (sTfR) level. Transferrin receptor, encoded by the *TFRC* gene, together with transferrin, mediates the delivery of transferrin-bound iron into the cell. It is ubiquitously expressed in diverse cell types, but its expression varies greatly (Ponka & Lok, 1999). Transferrin receptor may be in a membrane-bound form or circulate in the serum. In general, the level of sTfR is an indicator of available iron in organism (Edison *et al.,* 2009) and may also reflect the erythropoietic activity, anemia and other pathological conditions (Muñoz *et al.,* 2009).

## MATERIALS AND METHODS

**Clinical subjects**. The group of 662 individuals – 491 AMD patients and 171 controls from Central Poland were enrolled in this study. Control group comprised persons routinely examined in ophthalmology clinic, in whom AMD and other retinal diseases were excluded. Patients' characteristics are shown in Table 1. Medical history was obtained from all subjects and no one reported any genetic disease. The patients 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 the use of 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 criteria for patients selection were based on the clinical usefulness: dry form corresponded to AREDS category 2, 3 and 4 (geographic athrophy subtype), while wet form to AREDS category 4 (choroidal neovascularisation or neovascular maculopathy subtype) (AREDS Rep. No. 3, 2000). 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). Structured questionnaire was used to gather information from study subjects on their 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 a written informed consent.

**Polymorphisms selection**. We applied several on-line web tools for the selection of polymorphisms (Dayem-Ullah *et al.,* 2012; Yuan *et al.,* 2006). We performed the SNP annotation to find SNPs of probable phenotype influence. Also, we eliminated sites with unknown or too low minor allele frequency in order to obtain genotypes frequencies sufficient to gain enough high power of statistical analysis.

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

| Individuals | Number | Mean age (SD[a)]) | Sex[b)] |
|---|---|---|---|
| All | 662 | 70.4 (9.5) | 441F/221M (33.4%) |
| AMD | 491 | 70.9 (9.1) | 314F/177M (36.1%) |
| Dry AMD | 182 | 71.6 (10.8) | 116F/66M (36.3%) |
| Wet AMD | 309 | 70.6 (8.6) | 198F/111M (35.9%) |
| Controls | 171 | 68.9 (10.2) | 127F/44M (25.7%) |

[a)] S.D., standard deviation; [b)] females (F)/males (M) ratio (males %).

**DNA isolation**. Samples of venous blood were collected to EDTA-containing tubes. DNA was isolated using AxyPrep Blood Genomic DNA Miniprep kit (Axygen Biosciences, San Francisco, CA, USA) and stored frozen (–20ºC) until use.

**Genotype determination**. The genotype determination was performed using polymerase chain reaction – restriction fragments length polymorphism (PCR-RFLP) method. Each reaction tube contained 10 ng of genomic DNA, 1 U Taq Polymerase (Biotools, Madrid, Spain), 1 × reaction buffer, 0.5 mM deoxyribonucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$ and 0.25 μM of each primer (Sigma-Aldrich, St. Louis, MO, USA). The sequences of primers are given in Table 2. PCR was run on a Bio-Rad C1000™ thermocycler (BIO-RAD Laboratories, Hercules, CA, USA). Thermal cycling conditions were: initial denaturation step at 95ºC for 5 min, cycling steps: denaturation at 95ºC for 30 s, annealing at 60ºC for 30 s and elongation at 72ºC for 1 min; final extension at 72ºC for 5 min. Amplified fragments were digested with 1.5 U of allele-specific restriction enzymes (Table 3). Fragments containing p.Gly142Ser and –253G>A polymorphic sites were digested for 4 h with *Hpa*II and *Bsm*AI (Fermentas, Hanover, MD, USA), respectively. After digestion, DNA fragments were separated on an 8% polyacrylamide gel (acrylamide/bis-acrylamide 29:1). Electrophoresis was run at 5 V/cm in Tris-borate-EDTA buffer. pBR322 DNA/*Alu*I ladder was used as a mass marker. After separation, gels were stained with ethidium bromide (0.5 μg/ml) and analyzed by the digital imaging system InGenius Bio Imaging (Syngene, Cambridge, UK). As a quality control, random 10% of samples were re-genotyped and 100% compliance was achieved. Representative gels for genotyping the p.Gly142Ser and –253G>A polymorphisms are shown in Figs. 1 and 2, respectively.

**Serum iron assay**. Venous blood samples were collected and centrifuged to avoid hemolysis. Serum samples were portioned and stored frozen (–20ºC). Serum samples were centrifuged at 12000 × *g* for 15 min. Serum iron level was determined using QuantiChrom™ Iron Assay kit (Bioassay Systems, Hayward, CA, USA) according to the manufacturer protocol. The absorbance was read at 450 nm.

**Serum transferrin receptor ELISA**. Serum samples were cen-

**Table 2. Sequences of primers for the PCR-RFLP analysis**

| | Polymorphism | |
|---|---|---|
| | p.Gly142Ser | –253G>A |
| Forward | 5′-TAGGATTTATGATTGGCTACTTGGG-3′ | 5′-CTGCCGCAGTGCAATATCCAAC-3′ |
| Reverse | 5′-TCTACCTTTTCCCCTACCAGT-3′ | 5′-CCGATATCCCGACGCTCTGA-3′ |

**Table 3. Restriction analysis details for PCR-RFLP**

| Polymorphism | Location in the *TFRC* gene | Enzyme | Target sequence | Recognized allele | Genotypes[a)] |
|---|---|---|---|---|---|
| p.Gly142Ser | Coding region | *Hpa*II | 5'...C↓CGG...3'<br>3'...GGC↑C...5' | G | AA 385<br>AG 385/198/187<br>GG 198/187 |
| **c.**–253G>A | 5′-upstream | *Bsm*AI | 5'...GTCTC(N)1↓...3'<br>3'...CAGAG(N)5↑...5' | A | GG 397<br>GA 397/208/189<br>AA 208/189 |

[a)]Restriction products lengths [bp] for each genotype.

**Table 4. Association of AMD with BMI, tobacco smoking, family history of the disease, and living environment.**

| | AMD | |
|---|---|---|
| Factor | OR[a)] (95% CI) | *p*[b)] |
| BMI | 0.98 (0.94–1.02) | 0.329 |
| Smoking (ever) | 0.99 (0.67–1.47) | 0.972 |
| Family AMD | 12.04 (2.90–50.06)↑ | <0.001 |
| Environment[c)] | 0.68 (0.38–1.22) | 0.195 |

[a)]Odds ratio with 95% confidence interval; [b)]Chi-square test; [c)]adjusted to rural; Data in boldface are $p < 0.05$.

trifuged at $12000 \times g$ for 15 min prior to analysis. The serum transferrin receptor level was determined using the Human sTfR ELISA Kit (BioVendor, Brno, Czech Republic), with all steps performed according to the protocol. The plates were read at 450 nm. The final concentration of transferrin receptor was determined from a standard curve.

**Data analysis**. Hardy-Weinberg equilibrium (HWE) was checked using the $\chi^2$ test for each group. The allelic frequencies were calculated by gene counting and genotypes were scored. The significance of the differences between distributions of genotypes and alleles was tested using the $\chi^2$ analysis. Multiple logistic regression analysis was performed to assess the association between the genotypes of the polymorphisms and AMD risk. 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. Sex, family AMD history, inhabitation and smoking (never/ever) were coded binary, while age as a continuous variable. To verify a potential gene-environment interaction, the patients and controls were stratified depending on age, sex, living environment (rural/ urban), smoking status (never/former/current and never/moderate/heavy), BMI (<26/26–30/>30). For all analyses, except stratification, BMI was coded binary — greater than or equal to mean value as 1, lesser as 0). We set the mean value from whole analyzed group to balance the number of individuals between groups. For stratification we set the cut offs: BMI < 26 as a normal weight, 26–30 as overweight and > 30 as obesity. Stratified $\chi^2$ analyses were run to test the association of genotypes and environmental and social factors with AMD risk. To establish a potential polymorphism influence on AMD form progression (dry to wet), the OR analysis was performed between dry and wet groups. The sTfR and serum iron was compared between AMD and control group using Mann-Whitney Rank Sum test. To assess the potential association between genotypes of SNPs and the soluble transferrin receptor level the one-way ANOVA was performed. All statistical analyses were performed using Statistica 9.0 package (Statsoft, Tulsa, OK, USA).

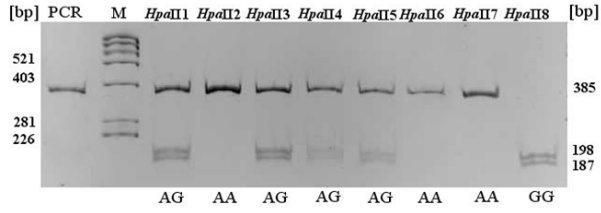


**Figure 1. Analysis of the p.Gly142Ser polymorphism in the *TFRC* gene with RFLP-PCR**
M, DNA mass marker; PCR, undigested PCR product; *Hpa*II1-*Hpa*II8, PCR products digested by *Hpa*I enzyme.

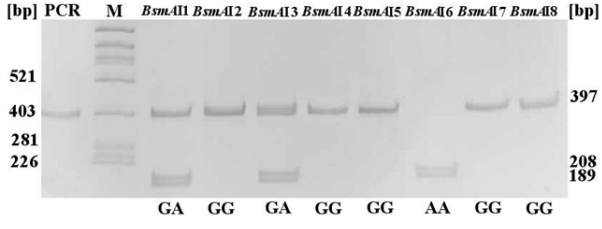


**Figure 2. Analysis of the –253G>A polymorphism in the *TFRC* gene using RFLP-PCR**
M, DNA mass marker; PCR, undigested PCR product; *Bsm*AI1-*Bsm*AI8, PCR products digested by *Bsm*AI enzyme.

## RESULTS

We considered the following environmental and social factors, which may play a role in AMD pathogenesis: age, sex, BMI, family history of AMD among 1st degree relatives, living environment (urban/rural) and tobacco smoking. Those factors were analyzed between both groups (AMD/Control) independently from genotypes (Table 4). No significant difference was found in the distribution of BMI, smoking status and environment of living between AMD patients and controls. However, an association was found for age (OR 1.023; $p < 0.05$ –for +1 year) and family AMD history (OR 8.88; $p < 0.001$). Next, we analyzed the distribution of genotypes and alleles of 2 polymorphisms in the *TFRC* gene: p.Gly142Ser (rs3817672) and –253G>A (rs11915082). Genotypes and alleles frequencies were compared between the control and AMD patients, and between the control and the dry and wet forms of AMD separately. For the p.Gly142Ser polymorphism, genotypes distribution in patients and controls did not departure from the HWE ($p > 0.05$), whereas in the case of the –253G>A polymorphism the genotype distribution was not in the Hardy-Weinberg equilibrium for both groups ($p < 0.05$). We did not found any direct correlation between both polymorphisms and AMD, neither for general AMD occurrence, nor for dry or wet form, respectively (data not shown). We then

**Table 5. Distribution of genotypes, frequency of alleles of the c.–253G>A polymorphism of the TFRC gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration (AMD) patients and controls among males and females.**

| | MALES | | | FEMALES | | | |
|---|---|---|---|---|---|---|---|
| Genotype /allele | Control (N = 38) | AMD (N = 125) | OR[a] (95% CI) | Control (N = 101) | AMD (N = 244) | OR*(95% CI) | $p$[b] |
| -253G>A | N (%) | N (%) | | N (%) | N (%) | | |
| GG | 13 (0.34) | 44 (0.36) | 0.83 (0.26–2.66) | 37 (0.37) | 106 (0.43) | 2.71 (1.25–5.84)↑ | 0.011 |
| GA | 19 (0.50) | 53 (0.42) | 0.69 (0.23–2.09) | 38 (0.37) | 102 (0.42) | 0.83 (0.40–1.71) | 0.619 |
| AA | 6 (0.16) | 28 (0.22) | 2.54 (0.50–12.95) | 26 (0.26) | 36 (0.15) | 0.33 (0.14–0.79)↓ | 0.013 |
| G | 45 (0.59) | 141 (0.56) | 0.71 (0.33–1.56) | 112 (0.55) | 314 (0.64) | 2.11 (1.29–3.45)↑ | 0.003 |
| A | 31 (0.41) | 109 (0.44) | 1.40 (0.64–3.07) | 90 (0.45) | 174 (0.36) | 0.47 (0.29–0.78)↓ | 0.003 |

[a]Odds ratio adjusted for age and living environment; [b]Chi-square test; data in boldface are $p < 0.05$.

**Table 6. Distribution of genotypes, frequency of alleles of the c.–253G>A polymorphism of the *TFRC* gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration (AMD) patients and controls among urban and rural residents.**

| | URBAN | | | RURAL | | | |
|---|---|---|---|---|---|---|---|
| Genotype /allele | Control (N = 43) | AMD (N = 108) | OR[a] (95% CI) | Control (N = 25) | AMD (N = 46) | OR*(95% CI) | $p$[b] |
| -253G>A | N (%) | N (%) | | N (%) | N (%) | | |
| GG | 15 (0.35) | 44 (0.41) | 1.42 (0.65-3.06) | 5 (0.20) | 21 (0.46) | 3.67 (1.13-11.94)↑ | 0.030 |
| GA | 19 (0.44) | 44 (0.41) | 0.82 (0.39-1.73) | 11 (0.44) | 18 (0.39) | 0.75 (0.27-2.08) | 0.585 |
| AA | 9 (0.21) | 20 (0.18) | 0.81 (0.32-2.03) | 9 (0.36) | 7 (0.15) | 0.33 (0.10-1.06) | 0.063 |
| G | 49 (0.57) | 132 (0.61) | 1.24 (0.75-2.04) | 21 (0.42) | 60 (0.65) | 2.37 (1.17-4.81)↑ | 0.017 |
| A | 37 (0.43) | 84 (0.39) | 0.81 (0.49-1.34) | 29 (0.58) | 32 (0.35) | 0.42 (0.21-0.86)↓ | 0.017 |

[a]Odds ratio adjusted for age and sex; [b]Chi-square test; data in boldface are $p < 0.05$.

compared the frequencies of genotypes and alleles in the dry *vs.* wet AMD groups to detect their association with the disease progression. We found that none of the genotypes/alleles altered the risk of progression to advanced wet form of AMD in comparison to the dry form for both SNPs (data not shown). Next, the groups were stratified depending on the risk factors for the detection of the potential gene-environment interaction. We found no association between the p.Gly142Ser polymorphism and any of the analyzed risk factors. However, for the –253G>A polymorphism the GG genotype and the G allele increased the risk (OR 2.05 and 1.9; $p < 0.05$), while the AA genotype and the A allele reduced the risk of developing AMD in females (OR 0.3 and 0.53, respectively, $p < 0.05$). The analysis also showed that none of the genotypes/alleles altered the risk for the development of AMD in males (Table 5). In the subgroup of rural inhabitants the AA genotype and the A allele reduced the risk of AMD (OR 0.34 and 0.5, respectively, $p < 0.05$), whereas the G allele increased the risk (OR 1.99; $p < 0.05$) (Table 6), however, no such association was observed among urban inhabitants. Among patients with BMI 26–30 the AA genotype was negatively correlated with AMD risk (OR 0.4; $p < 0.05$) (Table 7), however, we found no significant association in the remaining groups (BMI $< 26$ and BMI $> 30$). Among never-smokers the GG genotype and the G allele increased the risk of AMD (OR 2.36 and 1.8, respectively, $p < 0.05$,) and (OR 1.8; $p < 0.05$), whereas the AA genotype and the A allele decreased the risk (OR 0.48 and 0.56, respectively, $p < 0.05$). Moreover, in the group of current smokers the GA genotype correlated positively with AMD risk (OR 3.83, $p < 0.05$) (Table 8). We found no significant association in the groups stratified depending on smoking intensity (moderate/heavy smokers; data not shown). The G allele increased the risk of AMD (OR 1.79; $p < 0.05$) and the A allele reduced the risk in patients 72 years old

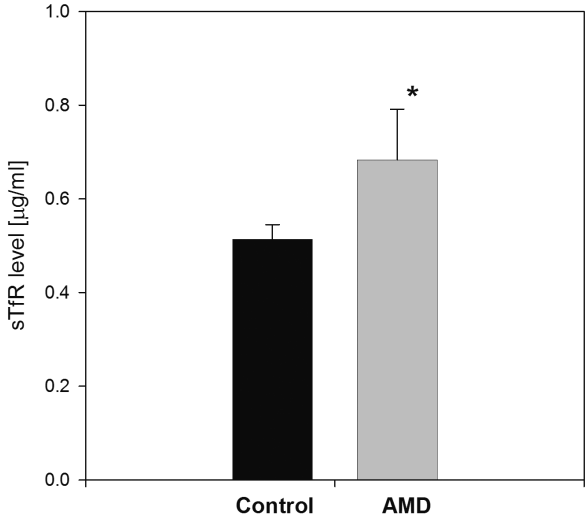


**Figure 3. Mean concentration of soluble transferrin receptor (sTfR) evaluated by the ELISA test in individuals without visual disturbances (control, black bar) and patients with age-related macular degeneration (AMD, gray bar).**
One hundred and seventy one persons were analyzed for the control, and 662 were analyzed for AMD. Error bars denote SEM, *$p < 0.05$ as compared with the controls.

**Table 7. Distribution of genotypes, frequency of alleles of the -253G>A polymorphism of the TFRC gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration (AMD) patients and controls with respect to their BMI (<26; 26-30; >30).**

| Genotype/allele | BMI < 26 | | | |
|---|---|---|---|---|
| -253G>A | Control (52) Number (%) | AMD (122) Number (%) | OR[a] (95% CI) | $p$[b] |
| GG | 17 (0.33) | 52 (0.43) | 1.69 (0.84-3.40) | – |
| GA | 22 (0.42) | 48 (0.39) | 0.86 (0.44-1.69) | – |
| AA | 13 (0.25) | 22 (0.18) | 0.60 (0.27-1.34) | – |
| G | 56 (0.54) | 152 (0.62) | 1.45 (0.93-2.26) | – |
| A | 48 (0.46) | 92 (0.38) | 0.69 (0.44-1.08) | – |
| Genotype/allele | BMI 26-30 | | | |
| -253G>A | Control (31) Number (%) | AMD (114) Number (%) | OR[a] (95% CI) | $p$[b] |
| GG | 9 (0.30) | 41 (0.36) | 1.31 (0.54-3.15) | 0.549 |
| GA | 11 (0.35) | 52 (0.46) | 1.44 (0.63-3.33) | 0.391 |
| AA | 11 (0.35) | 21 (0.18) | 0.40 (0.16-0.97)↓ | 0.043 |
| G | 29 (0.47) | 134 (0.59) | 1.64 (0.94-2.85) | 0.082 |
| A | 33 (0.53) | 94 (0.41) | 0.61 (0.35-1.07) | 0.082 |
| Genotype/allele | BMI > 30 | | | |
| -253G>A | Control (35) Number (%) | AMD (60) Number (%) | OR[a] (95% CI) | $p$[b] |
| GG | 13 (0.37) | 31 (0.52) | 1.93 (0.83-4.51) | – |
| GA | 15 (0.43) | 17 (0.28) | 0.50 (0.21 -1.21) | – |
| AA | 7 (0.20) | 12 (0.20) | 0.94 (0.33-2.73) | – |
| G | 41 (0.59) | 79 (0.66) | 1.34 (0.78-2.29) | – |
| A | 29 (0.41) | 41 (0.34) | 0.75 (0.44-1.29) | – |

[a]Adjusted for age and sex; [b]Chi-square test; Data in boldface are $p < 0.05$.

and more (OR 0.56; $p < 0.05$). None of the genotypes/alleles altered the risk for AMD incidence in patients aged less than 72 years (not shown). Then, we compared iron and soluble transferrin receptor concentrations among controls and AMD patients. The total serum iron concentration did not differ between both groups ($p > 0.05$) (not shown). However, the transferrin receptor level was higher in the AMD group ($p < 0.01$) (Fig. 3). One-way ANOVA showed that there was no significant association of either polymorphisms in the *TFRC* gene with the sTfR or Fe level (not shown).

## DISCUSSION

Oxidative damage in the macula could be enhanced by the excess of iron within this organ (Eaton & Qian, 2002). To avoid the generation of ROS, iron should occur in an appropriate sequestered form. This may be ensured by iron binding to proteins, transferrin in the plasma or ferritin inside the cell (Brisson *et al.,* 2012). Non-transferrin bound iron (NTBI) was identified in the plasma of patients with various pathological conditions. The transit iron pool is often referred to the labile iron pool (LIP) (Jacobs 1976; Kakhlon & Cabantchik, 2002). It corresponds to the iron species, which constitute an intermediate iron pool between the storage, vesicular and functional iron compartments and have the ability to engage in redox cycling. LIP is not bound to the major iron storage protein, ferritin, which can be classified as an "intracellular NTBI" form (Brisson *et al.,* 2012). The increased presence of the NTBI and LIP forms in the retina may occur due to deregulation in the expression of iron homeostasis proteins, such as transferrin (Tf) and its two receptors (TfR, TfR2). Immunohistochemical analysis demonstrated the presence of transferrin and transferrin receptor in different layers of the retina, including inner photoreceptors segments, RPE and the choroidal tissue (He *et al.,* 2007).

Genetic factors may play an important role in AMD development (Hammond *et al.*, 2002; Montezuma *et al.,* 2007; Klein *et al.,* 1994). Family study performed on a genetically isolated population confirmed that a significant proportion of the variation of transferrin, iron and ferritin saturation can be influenced by genetic factors, independently of demographic factors (Njajou *et al.,* 2006). The variability of iron homeostasis genes may lead to a disturbance of the iron homeostatic mechanisms, re-

**Table 8. Distribution of genotypes, frequency of alleles of the –253G>A polymorphism of the *TFRC* gene and odds ratios (OR) with 95% confidence intervals (95% CI) in age-related macular degeneration (AMD) patients and controls among never smokers, former smokers and current smokers.**

| Genotype /allele | NEVER SMOKERS | | | |
|---|---|---|---|---|
| -253G>A | Control (70) Number (%) | AMD (169) Number (%) | OR[a] (95% CI) | $p$[b] |
| GG | 17 (0.24) | 79 (0.47) | 2.71 (1.44–5.09)↑ | 0.002 |
| GA | 32 (0.46) | 60 (0.36) | 0.64 (0.36–1.14) | 0.128 |
| AA | 21 (0.30) | 30 (0.18) | 0.48 (0.25–0.92)↓ | 0.026 |
| G | 66 (0.47) | 218 (0.64) | 1.90 (1.30–2.77)↑ | <0.001 |
| A | 74 (0.53) | 120 (0.36) | 0.53 (0.36–0.77)↓ | <0.001 |
| Genotype /allele | FORMER SMOKERS | | | |
| -253G>A | Control (38) Number (%) | AMD (74) Number (%) | OR[a] (95% CI) | $p$[b] |
| GG | 15 (0.39) | 30 (0.41) | 1.15 (0.51–2.60) | – |
| GA | 16 (0.43) | 30 (0.41) | 0.95 (0.42–2.16) | – |
| AA | 7 (0.18) | 14 (0.18) | 0.86 (0.30–2.46) | – |
| G | 46 (0.61) | 90 (0.61) | 1.11 (0.64–1.92) | – |
| A | 30 (0.39) | 58 (0.39) | 0.90 (0.52–1.56) | – |
| Genotype /allele | CURRENT SMOKERS | | | |
| -253G>A | Control (17) Number (%) | AMD (56) Number (%) | OR[a] (95% CI) | $p$[b] |
| GG | 10 (0.59) | 21 (0.38) | 0.37 (0.11–1.21) | – |
| GA | 5 (0.29) | 24 (0.43) | 2.22 (0.63–7.78) | – |
| AA | 2 (0.12) | 11 (0.19) | 1.63 (0.30–8.76) | – |
| G | 25 (0.74) | 66 (0.59) | 0.54 (0.23–1.28) | – |
| A | 9 (0.26) | 46 (0.41) | 1.86 (0.78–4.44) | – |

[a]Adjusted for age and sex; [b] Chi-square test; Data in boldface are $p < 0.05$.

sulting in increased level of toxic iron pools (NTBI and LIP). In the previous papers we reported an association of transferrin gene (TF) variants with AMD risk. Also, we found that the serum level of transferrin was higher among AMD patients than in control group (Wysokinski *et al.,* 2011; Wysokinski *et al.,* 2013).

In the present work, we determined the role of sTfR, serum iron concentration and two polymorphisms in the *TFRC* gene in AMD. The p.Gly142Ser polymorphism is a missense mutation located in the exon 4. This change may influence the primary structure of TfR, and its function – activity, stability and affinity for ligands. An association of this polymorphism with type 2 diabetes mellitus (T2DM) was reported (Fernández-Real *et al.,* 2010) and another study showed that an increased iron storage was associated with a higher risk of that disease (Bao *et al.,* 2012). The –253G>A polymorphism is located in the 5'-upstream region of the *TFRC* gene, hence that change may affect the regulation and level of the gene expression. This locus is found to be linked with the erythrocyte phenotype (mean hemoglobin level) (Ganesh 2009). Therefore, we searched for a difference in serum TfR levels in AMD and control groups, as well as a correlation with the genotypes/alleles of these polymorphisms in the *TFRC* gene. The gene-independent analysis of AMD risk factors showed no association with tobacco smoking, although a number of previous reports indicated strong correlation (Cong *et al.,* 2008; Tan *et al.,* 2007). This discrepancy may be a result of false responses to the surveys, or considerable majority of non-smokers among patients enrolled in the study resulting in a decreased power of the test. Our study showed a correlation between the age and family history of AMD and the occurrence of the disease, which confirms an important role of genetic factors in AMD development (Sanfilippo *et al.,* 2010; Scholl *et al.,* 2007). We did not detect any association of serum Fe level with AMD occurrence, which suggests that the increase of iron level in AMD had a tissue-specific, local character, and the iron level fluctuations in the retina might be too small to be detected in the serum (Chen *et al.,* 2009). However, we found that the sTfR level was significantly higher in AMD patients than in controls, suggesting its potential utility in the disease prognosis. The nature of this association is unclear and requires further research. Our study revealed no association between serum iron and sTfR levels and genotypes of analyzed polymor-

phisms. Moreover, there was no direct association between the genotypes of the p.Gly142Ser and –253G>A polymorphisms either with AMD or dry/wet form occurrence. Moreover, there was no association for any of these polymorphisms with AMD progression. This finding indicates that neither polymorphism is an independent genetic marker for AMD development. Stratification analysis depending on sex, living environment, BMI, tobacco smoking status and age showed significant interaction of female sex, rural ancestry, BMI in 26–30 range and tobacco smoking with the –253G>A polymorphism in the risk of AMD modulation. Moreover, we observed an association between age and AMD occurrence. Our study group has much greater male/female ratio among AMD patients than in controls, but it does not justify a conclusion on a higher risk of AMD among males. Although we were unable to check any association between sex and AMD independently from genotypes, we found that female sex together with the G variant of the –253G>A polymorphism might modulate AMD risk. Females were reported to have a higher risk of AMD than males in several studies (Finger *et al.*, 2011; Owen *et al.,* 2012), and this is consistent with our finding. We found a similar association between the –253G>A polymorphism and rural inhabitation. Numerous differences in the conditions, lifestyle and medical care exist between individuals living in rural and industrial environment, but the real nature of such association is still unknown. Moreover, we found the protective effect of the AA genotype of the –253G>A polymorphism against AMD, but only among individuals with BMI 26–30, and not in the BMI 30+ group. Since high BMI values were reported to play a role in AMD (Seddon *et al.,* 2006b; Chiu *et al.,* 2001), it can be expected, that this association should appear primarily in the 30+ group. We speculate, that this result might be interfered by significant differences in each group numbers, e.g. the AMD group is much more larger than the control for the BMI 26–30 group, in the contrary to the 30+ group. Therefore, this result should be interpreted carefully and seems to be worth verification on another independent population of Central Europe ancestry. Lastly, the genotypes/alleles of the –253G>A polymorphism were associated also with AMD in individuals aged 72 and more. It suggests that since among younger patients other factors may be primary responsible for the disease, in the advanced age group this polymorphism may also play a role.

The results obtained indicate that a functional mechanism may operate within transferrin receptor, being significant in AMD pathogenesis, probably through iron-dependent oxidative stress. A significantly higher level of sTfR in AMD patients may be potentially considered as a prognostic marker for AMD development, but the mechanism responsible for such dependency needs further research approach. Variants of the –253G>A polymorphism of the *TFRC* gene may be considered as a genetic risk marker of AMD. In summary, the data obtained suggest the important role of iron homeostasis and oxidative stress in AMD pathogenesis.

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