

AGE (2013) 35:1651–1662
DOI 10.1007/s11357-012-9467-x

Regulatory regions of the paraoxonase 1 (*PON1*) gene are associated with neovascular age-related macular degeneration (AMD)

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Received: 23 March 2012 / Accepted: 20 August 2012 / Published online: 7 September 2012
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Abstract Physiological stress response and oxidative damage are factors for aging processes and, as such, are thought to contribute to neovascular age-related macular degeneration (AMD). Paraoxonase 1 (*PON1*) is an enzyme that plays an important role in oxidative stress and aging. We investigated association of DNA sequence variants (SNP) within the upstream regulatory region of the *PON1* gene with neovascular AMD in 305 patients

and 288 controls. Four of the seven tested SNPs (rs705379, rs705381, rs854573, and rs757158) were more frequently found in AMD patients compared to controls ($P=0.0099$, 0.0295 , 0.0121 , and 0.0256 , respectively), and all but one (SNP rs757158) are in linkage disequilibrium. Furthermore, haplotype TGGCCTC conferred protection (odds ratio (OR)=0.76, (CI)=0.60–0.97) as it was more frequently found in control individ-

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Electronic supplementary material The online version of this article (doi:10.1007/s11357-012-9467-x) contains supplementary material, which is available to authorized users.

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uals, while haplotype CGATGCT increased the risk (OR=1.55, CI=1.09–2.21) for AMD. These results were also reflected when haplotypes for the untranscribed and the 5'untranslated regions (5'UTR) were analyzed separately. To assess haplotype correlation with levels of gene expression, the three SNPs within the 5'UTR were tested in a luciferase reporter assay. In retinal pigment epithelium-derived ARPE19 cells, we were able to measure significant differences in reporter levels, while this was not observed in kidney-derived HEK293 cells. The presence of the risk allele A (SNP rs705381) caused an increase in luciferase activity of approximately twofold. Our data support the view that inflammatory reactions mediated through anti-oxidative activity may be relevant to neovascular age-related macular degeneration.

Keywords Paraoxonase 1 · Gene regulation · SNP association · Age-related macular degeneration (AMD)

Introduction

Age-related macular degeneration (AMD) is a progressive degenerative disease, which in its advanced stages leads to severe visual impairment. It is the major cause of loss of vision in elderly patients in developed countries (Deangelis et al. 2011). Advanced stages of AMD manifest either as atrophic changes in the macula (dry/atrophic/nonexudative AMD) or as choroidal neovascularization (wet/neovascular/exudative AMD). Although the neovascular AMD represents only 10–20 % of all AMD cases, it accounts for the vast majority of cases with severe and rapid vision loss (~90 %) (Jager et al. 2008).

The etiologic complexity of AMD arises from an interplay of genetic and environmental factors. Recent progress in AMD genetics has established several important risk loci, among them the complement factor H (*CFH*) on chromosome 1 (1q31) (Edwards et al. 2005; Hageman et al. 2005, 2006; Hughes et al. 2006; Klein et al. 2005; Li et al. 2006) and the age-related maculopathy susceptibility 2 (*ARMS2/HTRA1*) on chromosome 10 (10q26) (Dewan et al. 2006; Jakobsdottir et al. 2005; Rivera et al. 2005; Schmidt et al. 2006; Tanimoto et al. 2007; Weger et al. 2007; Yang et al. 2006). Both loci combined are thought to account for more than 50 % of AMD cases (Edwards et al. 2005; Klein et al. 2005; Maller et al. 2006; Swaroop et al. 2007; Thakkinstian et al. 2006). Their association with AMD

has been replicated across multiple ethnic groups worldwide and further validated through a meta-analysis of whole genome scans (Fisher et al. 2005). The discovery of *CFH* as a genetic risk factor for AMD was supported by functional studies revealing a role of the alternative complement pathway in drusen formation (Hageman et al. 2005). Following the discovery of genetic susceptibility loci, an impressive number of further genes has been reported that either increase or lower the risk towards AMD development (Katta et al. 2009). Despite the strong associations established, it is still not possible to predict the onset or course of AMD (Swaroop et al. 2007). Hence, it is necessary to identify other genes involved in the pathogenesis and understand their interactions.

To date, several additional candidate genes have been studied for their association with AMD, including lipid metabolism and oxidative stress genes (*APOE* (Baird et al. 2004b; Pang et al. 2000), *VLDLR* (Conley et al. 2005; Haines et al. 2006), *LRP5* (Kloeckener-Gruissem et al. 2011), *LRP6* (Haines et al. 2006), *PON1* (Baird et al. 2004a; Brion et al. 2011; Esfandiary et al. 2005; Ikeda et al. 2001; Pauer et al. 2010), *LIPC* (Neale et al. 2010), and *SOD2* (Gotoh et al. 2008; Kimura et al. 2000). Discordant results across multiple studies indicate the existence of additional factors that influence the development of AMD in different populations.

Oxidative stress is implicated in the development of AMD (AREDS 2001; Beatty et al. 2000; Khandhadia and Lotery 2010); thus, identification of enzymes conferring antioxidant protection is of interest. During the last decade, paraoxonase 1 (PON1), an enzyme with antioxidant properties, has been extensively investigated in several disorders, including age-related pathologies such as hyperlipidemia, atherosclerosis, coronary artery disease, metabolic syndrome, type 2 diabetes, as well as Alzheimer's disease (Androutsopoulos et al. 2011; Mackness et al. 1998b, c; Paragh et al. 1998; Senti et al. 2003). PON1 is a protein mainly synthesized in the liver, and most studies have focused their analysis on this fact. The enzyme is secreted into the blood where it binds high-density lipoprotein (HDL) (Aviram 2004). One of the functions of PON1 is to protect low-density lipoprotein (LDL) from oxidative damage and to inactivate oxidized LDL by hydrolyzing its oxidized phospholipids (Deakin and James 2004).

PON1 serum concentration and activity are highly variable across the general population (Deakin and James 2004). This variability is attributed to environmental factors, such as smoking, diet, alcohol consumption,

but also to the genetic polymorphisms in the *PON1* gene. There are two annotated missense SNPs within the *PON1* coding region: p.L55M (rs854560), where the major allele L leads to elevated PON1 protein levels, and p.Q192R (rs662), affecting substrate specificity as well as enzyme activity (Garin et al. 1997; Humbert et al. 1993). These two missense polymorphisms affect also the ability of HDL to protect LDL from oxidative modifications, with MM/QQ genotype being most effective (Mackness et al. 1998a). The level of PON1 concentration in serum is also affected by sequence variants within the promoter of the *PON1* gene. In particular, the C allele at position -107 (SNP rs705379) yields the highest level of PON1 in the serum among all alleles that have been investigated so far (Levieu and James 2000).

The antioxidant properties of PON1 stimulated research for association of DNA sequence variants within the *PON1* coding region and AMD (Baird et al. 2004a; Brion et al. 2011; Esfandiary et al. 2005; Ikeda et al. 2001; Pauer et al. 2010). Much focus has been on studying the effects of the two above-mentioned polymorphisms, p.L55M and p.Q192R. In a Japanese AMD patient cohort, the 55L and the 192R variants were found more frequently (Ikeda et al. 2001), while in one Caucasian cohort, the 55Q variant was associated with the disease (Pauer et al. 2010). This association could not be confirmed in further three Caucasian patient groups suggesting the influence of other factors as well (Baird et al. 2004a; Brion et al. 2011; Esfandiary et al. 2005). No information has been published on associations of sequence variants within upstream regulatory regions of the gene and diseases. It was our goal to expand the knowledge of effects of *PON1* gene sequences beyond protein isoforms and to investigate the influence of the upstream regulatory regions on AMD. We report here the identification of protective and risk haplotypes with effects on gene expression, which displays cell-type specificity.

Materials and methods

Patients

Patients with neovascular AMD were diagnosed at the Department of Ophthalmology at the University Hospital Zurich. Inclusion criteria regarding age, visual acuity, and lesion type (minimally classic, predominantly classic, and occult) were based on the

MARINA (Rosenfeld et al. 2006) and ANCHOR (Brown et al. 2006) trials. The patients were given a full explanation of the nature and purpose of the study. The study was conducted according to the Declaration of Helsinki and was approved by the local ethics committee. Clinical and demographic description as well as the analysis of several common AMD risk factors of this patient cohort has been published previously (Kloekener-Gruissem et al. 2011). Data from 305 neovascular AMD patients are reported in the current study. The average age of the patients was 79.41 years (± 6.97); female to male ratio, 1.95. All were of European descent, living in Switzerland. DNA of 288 individuals representing the general population was used as the control group (Moskvina et al. 2005). These individuals were not age-matched and did not undergo clinical assessment for the present study. They were of European descent, living in Switzerland. The female/male ratio was 2.1.

Genotyping

Venous blood was collected in EDTA tubes, and genomic DNA was extracted as described (Kloekener-Gruissem et al. 2011). DNA concentration was adjusted to 10 ng/ μ l. SNP genotyping was performed either by DNA sequencing (primer 1 CCTCCCCGACTGGACTAGG and primer 2 AGGGAGTGAGGAGGACGAAG were used for genotyping SNPs rs705379, rs705380, and rs705381; primer 3 CCAAAGCCTTGAGAAGGAA and primer 4 TGCTCTAGGTGATGCATGTG were used for genotyping SNPs rs854571 and rs854572) or by TaqMan technology (SNPs rs854571, rs854573, and rs757158), using ABI chemistry (Applied Biosystems, Inc. [ABI], Rotkreuz, Switzerland). Sequencing primers were designed by Primer3 (v. 0.4.0; <http://frodo.wi.mit.edu/primer3>, provided in the public domain by Massachusetts Institute of Technology, Cambridge, MA) and synthesized by Microsynth (<http://www.microsynth.ch>; Balgach, Switzerland). Annealing temperatures varied between 54 and 60 °C. For PCR, 50-ng genomic DNA and primers were cycled 35 times, each cycle lasting 1 min. For TaqMan assay, probes from ABI were used. Genotype calling was performed on SDS2.2 software, ABI. The allele calling of SNP assay was verified by DNA sequencing analysis for 10 % of all samples, yielding 100 % concordance. In addition, genotyping of 10 % of all samples was repeated for each SNP.

Selection of SNPs and haplotype analysis

Seven SNPs, namely rs705379, rs705380, rs705381, rs854571, rs854572, rs854573, and rs757158 (Fig. 1 and Table 1), within *PONI* gene (NG_008779.1) were chosen for association studies based on the linkage disequilibrium (LD) data in the HapMap Caucasian (CEU) population panel, using a tagging criteria of $R^2 > 0.8$. Since there is no LD information available for SNPs in the 5'UTR (rs705379, rs705380, and rs705381), we included them in the analysis.

Individual haplotypes and their estimated population frequencies were inferred using PHASE program, v2.1.1 (<http://stephenslab.uchicago.edu/software.html>), with all parameters set at the default values (Stephens and Donnelly 2003; Stephens et al. 2001).

In silico analysis of 5'UTR variants on RNA folding

Putative RNA folding structures were predicted using the Mfold online software with standard settings (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>) (Zuker 2003). RNA structures were predicted for the *PONI* transcript (transcript: PON1-001 *ENST00000222381*) containing eight possible combinations of alleles at the SNPs within 5'UTR.

Cloning

A 400-bp fragment of *PONI* exon 1 containing the 5' UTR was PCR-amplified (primer 1 and primer 2) and cloned into the intermediate pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Fermentas International Inc, Glen Burnie, MD, USA). Fragments containing four of the eight possible haplotypes were obtained directly through PCR from patients' genomic DNA (CCA, CGA, CGG, and TGG; SNPs' order from left to right, rs705379, rs705380, and rs705381). DNA fragments containing the other four haplotypes were obtained by site-directed mutagenesis with the primer



Fig. 1 Schematic representation of the *PONI* gene. Positions of SNPs that were analyzed in this study are printed in *bold*. They map to the upstream untranscribed region (*UUR*) and the 5'untranslated region (5'UTR). SNPs in exon 3 (*Ex3*), exon 6 (*Ex6*),

extension approach. For all substitutions, primer 1 and primer 2 served as outside flanking primers. Each mutation was obtained using additional pairs of inside primers (sequences are written from 5' to 3') as primer 5 CCGACCCGGCGGGGAGGGGTGGGGCGG GCC with primer 6 GCAGCGCCGATTGGCCC GCCCCACCCCTCCC, primer 7 GGGGCTGACCGCAAGCCGCGCCTTCTGTGC with primer 8 GACCAGGTGCACAGAAGGCGCGGCT TGCGG, primer 9 GGGGCTGACCGCAAGC CACGCCTTCTGTGC with primer 10 GACCAGGTG CACAGAAGGCGTGGCTTGCGGTC, and primer 11 TGGTCGGCCAGCTAGCTGCCGACCCGGCGGG with primer 12 CCACCCCTCCCCGCCGGTTCGG CAGCTAGC. Subsequently, the *PONI* fragments representing each of the haplotype were cloned into pGL3-Control vector (Promega, Madison, WI, USA). In the first step, PCR reaction was performed to create a *HindIII* restriction site upstream of the three 5'UTR SNPs using primer 2 and primer 13 TATAAGCTTGTGGAAG GAGCAAATG. In the second step, 238-bp fragments spanning from the constructed *HindIII* site to the translational start codon ATG (*NcoI* site) were cloned into the pGL3-Control vector using *HindIII* and *NcoI* restriction enzymes (Fermentas). DNA sequences of all constructs were verified by Sanger sequencing.

Expression studies

HEK293 cells were seeded on 24-well plates (10^5 cells/well) in 1 ml DMEM, 10 % FBS, and 1 % penicillin/streptomycin and incubated under standard conditions (37 °C, 5 % CO₂). After 24 h, cells were transfected using 1.5- μ l branched polyethylenimine, PEI (1 μ g/ μ l), 600-ng pFirefly construct, and 15-ng pRenilla construct per well. Twenty-four hours later, the cells were harvested, and luciferase activities were analyzed using Dual-Glo Luciferase Assay System according to the protocol provided by Promega and measured on a Bio-Tek Synergy microplate reader (BioTek Instruments,

and exon 9 (*Ex9*), investigated by other groups, are printed in *italics* (Baird et al. 2004a; Brion et al. 2011; Esfandiary et al. 2005; Ikeda et al. 2001; Pauer et al. 2010)

Table 1 Allele frequencies of SNPs in the upstream regulatory region of *PON1*

Marker	Major/minor allele	Position	Region	MAF			<i>P</i> value	OR	CI (95 %)
				HapMap	AMD (<i>n</i> =305)	Controls (<i>n</i> =288)			
rs705379	T/C	-107	5'UTR	0.425	0.517	0.440	0.0099	1.36	1.08–1.73
rs705380	G/C	-126	5'UTR	0.042	0.056	0.044	0.3566	1.29	0.75–2.21
rs705381	G/A	-162	5'UTR	0.167	0.253	0.199	0.0295	1.37	1.03–1.81
rs854571	C/T	-831	UUR	0.302	0.284	0.257	0.3009	1.14	0.89–1.49
rs854572	C/G	-908	UUR	0.425	0.471	0.419	0.0759	1.23	0.98–1.56
rs854573	T/C	-1075	UUR	0.230	0.255	0.194	0.0121	1.42	1.08–1.88
rs757158	C/T	-1740	UUR	0.376	0.444	0.380	0.0256	1.30	1.03–1.64

Comparison between patients with neovascular AMD (*n*=305) and control individuals (*n*=288). Three SNPs map to the 5'untranslated region while four of the SNPs lie within the upstream untranscribed region (Fig. 1). Minor allele frequencies for a Caucasian population were taken from the HapMap website (<http://www.hapmap.org>) and were calculated for our patient (AMD) and control groups. Statistical analyses are displayed as odds ratios (OR) with confidence intervals (CI) and *P* values, with $\alpha=0.05$

5'UTR 5'untranslated region, UUR upstream untranscribed region, MAF minor allele frequencies

Winooski, VT, USA). Data were normalized for transfection efficiency by Renilla luciferase activity. The experiment was performed three times with three replicates each. ARPE19 cells (ATCC, Manassas, VA, USA) were seeded on 24-well plates (8×10^4 cells/well) in 1 ml DMEM, 10 % FBS, 1 % penicillin/streptomycin, and 7.5 % Na_2CO_3 and cultured under standard conditions (37 °C, 5 % CO_2). Twenty-four hours later, transfection was performed; for each well, 0.75- μl X-tremeGENE 9 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA), 250-ng pFirefly construct, and 6.25-ng pRenilla construct were used. Luciferase activities were measured and analyzed 24 h after transfection as described above. The experiment was repeated four times. Three of these repeats included three technical replicates while one of them included eight.

Statistical analysis

Pairwise LD analysis was performed on phased haplotype data generated from the control group according to the following formulas: $R^2 = D^2 / (p_{A1}p_{A2}p_{B1}p_{B2})$, where $D = p_{A1B1}p_{A2B2} - p_{A1B2}p_{A2B1}$; $D' = D / D_{\max}$, with $D_{\max} = \min(p_{A1}p_{B2}, p_{A2}p_{B1})$ if $D > 0$ and $D_{\max} = \min(-p_{A1}p_{B1}, -p_{A2}p_{B2})$ if $D < 0$ (Mueller 2004). For association studies, odds ratios and significance were calculated using 2×2 contingency table provided on an open access Internet portal (<http://faculty.vassar.edu/lowry/odds2x2.html>). Odds ratios were displayed with 95 % confidence intervals and Chi-square and *P* values according to Pearson. Statistical significance was assumed at $P < 0.05$. For

expression studies, eight technical replicates were measured and averages with confidence intervals at 95 % were computed. Fold-change differences were determined after normalizing the data to those obtained for the TCG haplotype. This haplotype was chosen because it yielded the lowest luciferase activity in the ARPE19 cell line. The Shapiro–Wilk test was applied to assess normal distribution of the data. For statistical analysis, the two-tailed *t* test was used.

Results

Linkage disequilibrium revealed two DNA blocks within the upstream regulatory region of *PON1*

Linkage disequilibrium data are crucial for the design of association studies. If two SNPs share high linkage disequilibrium, with $R^2 > 0.8$ one of them can be used as tagged SNP. The region of *PON1* investigated in our study contains seven annotated SNPs (Fig. 1 and Table 1). LD data on the HapMap website are available only for four of them (rs854571, rs854572, rs854573, and rs757158). To complete this information, we performed LD analysis on phased genotype data of the control individuals (Table 2). Our analysis confirms already annotated data in a larger group of individuals (haplotype number *n*=500 in our study, *n*=224 in HapMap) and also provides pairwise R^2 and D' values for SNPs which are not included in HapMap. Among the seven analyzed SNPs, six belong to one haplotype block

as they revealed high pairwise D' values (rs705379, rs705380, rs705381, rs854571, rs854572, and rs854573). SNP rs757158 belongs to a separate haplotype block (Fig. 2). Among the analyzed SNPs, only two pairs (rs705381 with rs854573, and rs854572 with rs757158) fulfill tagging criteria of $R^2 > 0.8$ (Table S1).

Association studies showed risk and protection potential for AMD

Genotypes of seven SNPs within regulatory, non-coding regions of the *PON1* locus were determined from 305 patients with neovascular AMD and compared to those from 288 control individuals (Table 1). The patients' AMD phenotype has been reported in detail previously (Kloekener-Gruissem et al. 2011). Three SNPs within the 5'UTR and four SNPs within the upstream untranscribed region (UUR) (Fig. 1) were genotyped and analyzed. Note that as the transcriptional promoter region has not been defined experimentally, we chose to name this region "UUR", rather than "promoter region". Statistically significant association was found at two SNP loci within the 5'UTR and two within the UUR: rs705379 (OR=1.36, CI=1.08–1.73, $P=0.0099$), rs705381 (OR=1.37, CI=1.03–1.81, $P=0.0295$), rs854573 (OR=1.42, CI=1.08–1.88, $P=0.0121$), and rs757158 (OR=1.30, CI=1.03–1.64, $P=0.0256$) (Table 1).

To strengthen the significance of the association, we included analyses of haplotypes. For the seven SNP haplotypes (rs705379 at -107, rs705380 at -126 and

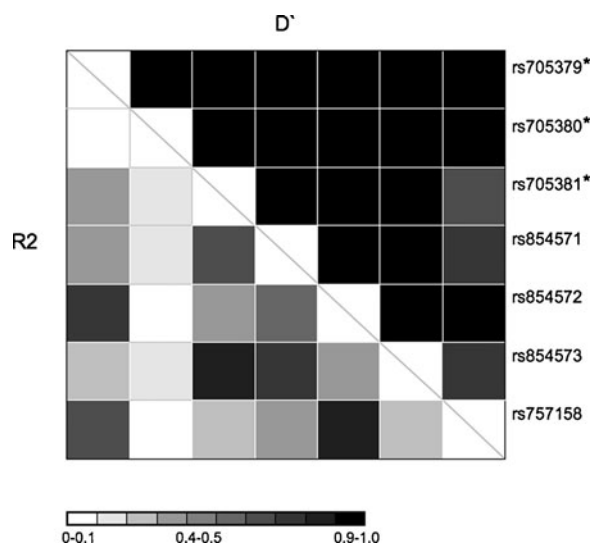


Fig. 2 Pairwise linkage analysis of SNPs within the *PON1* 5' UTR and upstream untranscribed region. R^2 and D' values are depicted by the grayscale color code according to the legend. Asterisks indicate SNPs for which no data are available on the HapMap website

rs705381 at -162, rs854571 at -831, rs854572 at -908, rs854573 at -1075, and rs757158 at -1740), PHASE predicted 18 different combinations. Haplotypes with fewer than five counts in PHASE (11 haplotypes) were not taken into account for statistical analyses. The haplotype TGGCCTC, comprising exclusively major alleles, was more frequently found in the control group, suggesting a protective function (OR=0.76, CI=0.60–0.97, $P=0.0293$), whereas the haplotype CGATGCT was more frequent in AMD patients (OR=1.55, CI=1.09–2.21, $P=0.0141$), posing a risk for the disease (Table 2). Similar results were found when SNPs from the 5'UTR and the upstream untranscribed region (UUR) were analyzed separately. The PHASE program revealed the presence of four possible haplotypes for SNPs in the 5'UTR (TGG, CGG, CGA, CCA; order of SNPs from left to right, rs705379 at -107, rs705380 at -126, and rs705381 at -162). Carriers of the haplotype TGG, representing the major alleles within a population, were more frequently found in the control group compared to the patient group, and, hence, this haplotype confers a protection against AMD (OR=0.72, CI=0.57–0.91, $P=0.0063$), while conversely the haplotype CGA indicates a risk for AMD (OR=1.35, CI=0.99–1.85, $P=0.0564$) (Table 3). For the four SNPs in the UUR, PHASE predicted seven different haplotypes. Three haplotypes showed statistically significant differences between patient and control group

Table 2 Haplotype analysis for all seven SNPs

Haplotype	AMD ($n=556$) n (%)	Controls ($n=500$) n (%)	P value	OR	CI (95 %)
TGGCCTC	264 (47.5)	271 (54.2)	0.0293	0.76	0.60–0.97
CGGCGTT	100 (18.0)	74 (14.8)	0.1637	1.26	0.91–1.75
CGATGCT	94 (16.9)	58 (11.6)	0.0141	1.55	1.09–2.21
CCATGCT	25 (4.5)	22 (4.4)	0.9203	1.02	0.57–1.84
CGGCCTC	26 (4.7)	18 (3.6)	0.3833	1.31	0.71–2.43
CGGTGTT	17 (3.1)	25 (5.0)	0.1069	0.60	0.32–1.12
CGATGCC	12 (2.2)	14 (2.8)	0.5023	0.77	0.35–1.67

SNP order for each haplotype, from left to right is as follows: -107, -126, -162, -831, -908, -1075, and -1740. Haplotype counts (n) and percentages (%) are given. Haplotypes with less than five counts in PHASE were not taken into account for statistical analysis. Odds ratios (OR) with confidence intervals (CI) and P values ($\alpha=0.05$) are shown

frequencies (Table 3). The haplotype TGCT (rs854571 at -831, rs854572 at -908, rs854573 at -1075, and rs757158 at -1740) was associated with the disease (OR=1.44, CI=1.07–1.95, $P=0.0165$), whereas the two haplotypes CCTC and TGTT were more frequently found in the control group (OR=0.79, CI=0.63–0.99, $P=0.0492$ and OR=0.48, CI=0.27–0.84, $P=0.0092$, respectively) (Table 3).

Sequence variants in the 5'UTR alter predicted structure of the *PON1* mRNA

It is known that SNPs within the 5'UTR of genes can affect its mRNA folding and consequently change translational efficiency (Zuercher et al. 2010). We simulated the influence of the 5'UTR polymorphisms on secondary RNA structures using the web server Mfold. RNA structures of the entire *PON1* mRNA (5'UTR, coding sequences, and 3'UTR) were predicted (Fig. 3). The predicted structure of the reference sequence carrying the minor alleles at three analyzed positions (-107C, -126C, and -162A) was not different from that containing only one major T allele at position -107 (Fig. 3a). In contrast, predicted folding of RNA containing the major alleles at position -126 and -162, singly, or in combination, assumed a strikingly different structure (Fig. 3b). Based on these bioinformatic predictions, we hypothesized that

SNPs within the 5'UTR of *PON1* may have different effects on protein synthesis.

AMD risk haplotype altered reporter gene activity in cell culture

To correlate our association data and the structural predictions with potential effects on gene expression, we designed experiments to test whether different *PON1* 5'UTR haplotypes influence luciferase reporter gene expression in cell culture. We generated the eight possible haplotypes in DNA constructs, containing the *PON1* 5'UTR region fused to the *Luciferase* coding region, both transcribed from the SV40 promoter. Since tissue-specific effects on gene expression are likely to occur, we included two different cell lines in this test: the embryonic kidney-derived cell line HEK293 and the retinal pigment epithelium-derived cell line ARPE19. Both cell lines were transiently transfected with the various *PON1* 5'UTR haplotypes in front of the luciferase reporter. In ARPE19 cells, we found statistically significant differences in luciferase levels (Fig. 4). To assess contribution of each individual SNP to increased protein expression, we performed a pairwise comparison of luciferase levels between haplotypes differing at only one position (Table 4). This analysis revealed that SNP rs705381 at position -162 is mostly responsible for

Table 3 Haplotype analysis of the upstream untranscribed and untranslated region

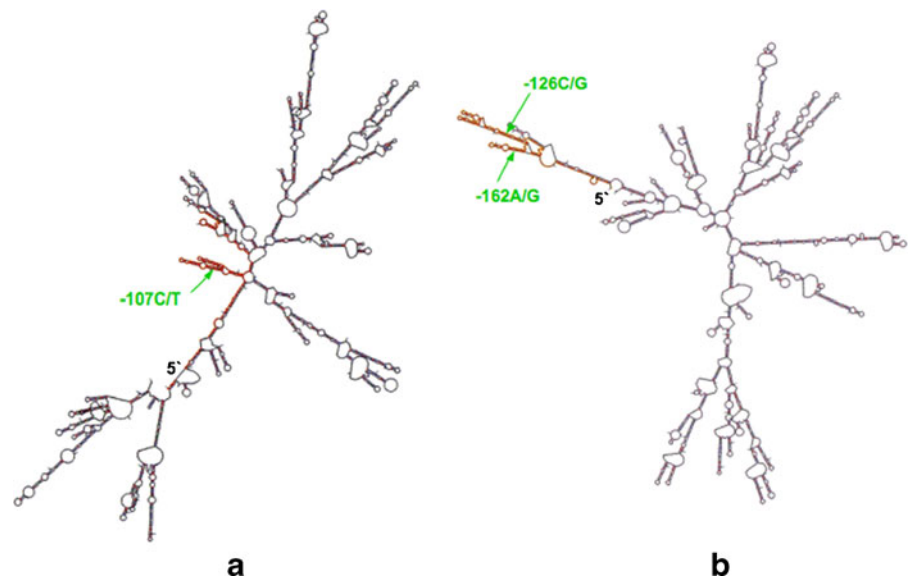
Region	Haplotype	AMD <i>n</i> (%)	Controls <i>n</i> (%)	<i>P</i> value	OR	CI (95 %)
5'UTR ^a	TGG	280 (48.4)	297 (56.7)	0.0063	0.72	0.57–0.91
	CGG	150 (26.0)	121 (23.1)	0.2713	1.17	0.89–1.54
	CGA	116 (20.1)	82 (15.6)	0.0564	1.35	0.99–1.85
	CCA	32 (5.5)	24 (4.6)	0.4708	1.22	0.71–2.10
UUR ^b	CCTC	308 (52.4)	320 (58.2)	0.0492	0.79	0.63–0.99
	TGCT	128 (21.8)	89 (16.2)	0.0165	1.44	1.07–1.95
	CGTT	109 (18.5)	87 (15.8)	0.2253	1.21	0.89–1.65
	TGTT	19 (3.2)	36 (6.5)	0.0092	0.48	0.27–0.84
	TGCC	19 (3.2)	17 (3.1)	0.8875	1.05	0.54–2.04

Four haplotypes in the 5'untranslated region (5'UTR) and five haplotypes in the upstream untranscribed region (UUR) were analyzed in our patient and control population. The order of SNPs, from left to right is -107, -126, and -162 (5'UTR) and -831, -908, -1075, and -1740 (UUR). Haplotype counts (*n*) and percentages (%) are given. Odds ratios (OR) with confidence intervals (CI) and *P* values (alpha=0.05) are shown. Haplotypes with fewer than five counts in PHASE were not taken into account for statistical analysis

^a AMD, *n*=578; controls, *n*=524

^b AMD, *n*=588; controls, *n*=550

Fig. 3 Predicted *PON1* mRNA foldings. **a** The structure represents folding of the *PON1* mRNA containing the minor alleles CCA in the 5'untranslated region. It was indistinguishable from the allele combination TCA. **b** The structure depicts mRNA folding in the presence of the other six allelic combinations: CGA, TGA, CCG, TCG, CGG, and TGG (SNP order from left to right, -107,-126, and -162)



the observed difference in luciferase activity. The presence of the minor allele A leads to increase in the reporter activity up to 2.1-fold. Small contribution to the protein expression regulation has been found for two other SNPs (rs705379 at -107, and rs705380 at -126), with minor allele C at position -107 and minor allele C at position -126, causing up to 1.6- and 1.4-fold increase in luciferase level, respectively. Interestingly, in HEK293 cells, we did not find statistically significant differences in luciferase activity between the different constructs (data not shown), supporting our initial assumption of cell-specific effects on gene expression.

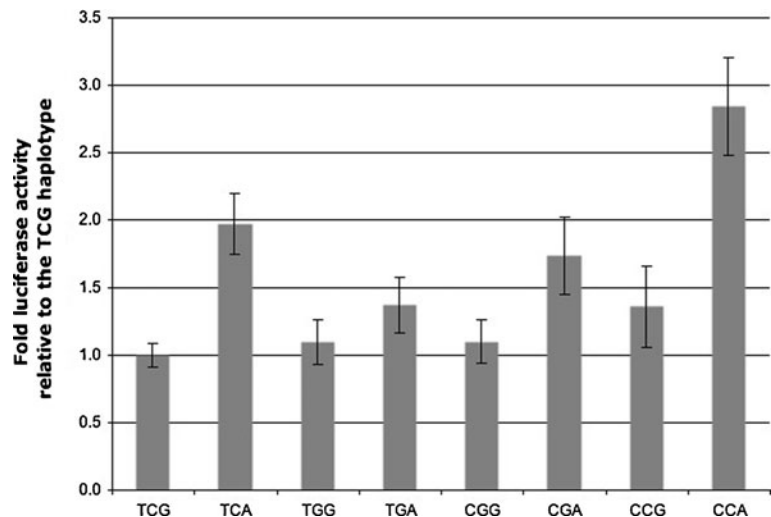
Discussion

This is the first report to show association of SNPs in the upstream regulatory region of *PON1* with neovascular AMD. Previous studies have investigated association between SNPs within the *PON1* coding region and AMD (Brion et al. 2011; Baird et al. 2004a; Esfandiary et al. 2005; Pauer et al. 2010; Ikeda et al. 2001). The focus was on two missense polymorphisms p.L55M and p.Q192R. Conflicting results were obtained when comparing different patient cohorts; while association with wet AMD has been found for both variants in a Japanese population (Ikeda et al. 2001), only the p.Q192R variant was associated in only one of the four Caucasian cohorts (Pauer et al. 2010). Furthermore, in the Japanese study, the arginine at position 192 of the PON1 protein has been found more frequently in the patient group,

whereas in Caucasians, the glutamine (192Q) variant has been associated with the disease. Possible explanations encounter different allele frequencies in the different ethnic groups but also a diverse phenotype of AMD (Bird 2003; Oshima et al. 2001). Specifically for SNP p.Q192R in Caucasians, the minor variant is R with a frequency of 0.332, whereas in Japanese, the arginine is the major variant with an allele frequency of 0.706 (HapMap). As there is no linkage disequilibrium between p.Q192R variant and the promoter polymorphisms (Leviev and James 2000) (HapMap), a direct comparison between our data and those reported from other Caucasian patient cohorts, which examined p.Q192R (Baird et al. 2004a; Brion et al. 2011; Esfandiary et al. 2005; Pauer et al. 2010) is not meaningful.

In contrast to p.Q192R, the variant 55L is in linkage disequilibrium with the allele -107C (Leviev and James 2000), which is associated with AMD in our study. It has been shown that these two alleles lead to increased levels of PON1 in serum (Leviev and James 2000). Thus, it may be difficult to understand how higher levels of a protein that is known to have antioxidant properties can be associated with AMD. For a possible explanation, we would like to emphasize that variant p.L55M has been reported to affect PON1 activity towards lipid peroxides, with the LL genotype being less effective than MM at protecting LDL against oxidation (Mackness et al. 1998a). Since alleles at position -107C and -162A are in linkage disequilibrium with variant 55L, they are likely to be also associated with the less effective protection against oxidation, thus can confer the risk for AMD and

Fig. 4 Fold increase of luciferase activity for the eight *PONI* 5'UTR haplotypes in ARPE19 cells. The luciferase activity of the reporter construct containing the TCG haplotype was set to 1. SNP order in haplotypes from left to right: -107, -126, and -162. Error bars show confidence intervals, CI=95 %. Displayed are results from a representative experiment with eight technical replicates



be responsible for increased oxLDL levels (Ikeda et al. 2001) in AMD patients.

To strengthen the significance of the association found with individual SNPs, we performed haplotype association analysis. We discovered two seven-SNP haplotypes

Table 4 Effects of 5'UTR haplotypes on luciferase reporter activity

SNP	Haplotype ^a		Fold increase in luciferase activity ^b	P value ^c
	1	2		
-107	CCA	TCA	1.4	<0.001
	CCG	TCG	1.4	0.016
	CGA	TGA	1.3	0.030
	CGG	TGG	1.0	0.984
-126	CCA	CGA	1.6	<0.001
	CCG	CGG	1.2	0.094
	TCA	TGA	1.4	<0.001
	TCG	TGG	0.9	0.242
-162	CCA	CCG	2.1	<0.001
	CGA	CGG	1.6	<0.001
	TCA	TCG	2.0	<0.001
	TGA	TGG	1.2	0.030

The comparison is based on haplotypes that differ at a single position. Displayed are results for a representative experiment shown in Fig. 4

^a SNP order in haplotype, from left to right is -107, -126, and -162

^b Fold increase in luciferase activity, haplotype 1 versus haplotype 2

^c Two-tailed *t* test

associated with AMD. The most abundant haplotype (54.2 % in controls), TGGCCTC, was more frequently found in controls conferring a protection against AMD, whereas haplotype CGATGCT was a risk factor. Interestingly, the two haplotypes CGATGCC and CCATGCT, each differing at only one position in comparison to CGATGCT, appeared not to be associated with the disease. These data imply a possible interplay between the analyzed loci and underlines the importance of haplotype analysis to assess risk factors. Similar results were obtained when analyzing haplotypes within the 5'UTR and UUR separately.

It is known that sequence variants within the 5'UTR can regulate gene expression at the level of protein translation. We asked whether this would also apply to the *PONI* gene. To support our notion, we performed in silico RNA folding predictions for the different allelic combinations and found that the presence of the major alleles for two SNPs in the 5'UTR would lead to a striking difference in the folding properties of the entire *PONI* transcript. Furthermore, these alleles also appear to have an effect on reporter gene expression as measured by luciferase enzyme activity. Specifically, in the retinal pigment epithelium-derived cell line ARPE19, the protective TGG haplotype yielded the lowest level of luciferase activity, while the CCA haplotype resulted in the highest reported expression levels. When comparing those haplotypes differing at only one position, we discovered that the SNP at position -162 provided the largest contribution, with the A nucleotide causing up to 2.1-fold increase in luciferase reporter activity. Previous reports have shown that this allele

caused up-regulation of transcription in cultured kidney and liver cell lines (Brophy et al. 2001). These data, together with our findings, indicate that the position at –162 has regulatory function at both steps of gene expression, transcription, as well as translation. Alternatively, a sole effect on transcription of the luciferase reporter cannot be excluded. In our study, the two other investigated SNPs at positions –107 and –126 caused only minor changes in luciferase activity. In the literature, no effect of the SNP at –126 has been reported, but the minor allele C at position –107 has been shown to increase transcription of a luciferase reporter gene in HepG2 and HEK293 cells (Brophy et al. 2001). Taking these data together, we conclude that the three SNPs support different activities for *PON1* gene expression.

It is important to note that the luciferase reporter assays did not yield the same results in the two different cell lines tested. As cell and tissue-specific regulation of gene expression is a well-known phenomenon, it is not surprising that gene expression control potential within the 5'UTR of *PON1* is manifested in ARPE19 cells, but not in HEK293 cells. The latter cells, derived from kidney, do not seem to be implicated in the AMD pathology, while ARPE19 cells, derived from retinal pigment epithelium, do (Kinnunen et al. 2012). In this context, it is plausible that the relevant SNP at position –162 exerts a cell type-specific effect in favor of AMD. Since *Pon1* is expressed in the retinal pigment epithelium in mice (data not shown), further studies will provide crucial information to clarify these assumptions.

A number of factors such as blood pressure, usage of antihypertensive medication, blood cholesterol, or blood levels of high-density lipoprotein were shown to be associated with the development of neovascular AMD (Hyman et al. 2000). As the mean age of the population studied here is 79.5 years, it can be expected that some of the factors (e.g., increased arterial blood pressure) are present in the study population—assumably randomly distributed across the participants. An influence of the proposed cardiovascular risk factors on the outcome of the treatment of neovascular AMD using ranibizumab has not yet been shown. Hence, it remains speculative whether or not such risk factors may have had an impact in the current study population and the reported outcomes.

Many open questions remain on the involvement of PON1 in AMD pathology. As our patients were diagnosed with the end stage neovascular AMD, our results cannot account for a potential role of *PON1* as a trigger

for the onset of AMD or as an accelerator during the progression of the disease, or maybe both. Our and other previous association studies imply the involvement of *PON1* at the late stage of the disease. An alternative mechanism may exist, where PON1 enzyme changes are secondary to other events, present at early stages of AMD. For example, paraoxonase activity in serum is decreased in AMD patients. As AMD is considered to be a chronic inflammatory disease and the *PON1* transcription levels in liver are known to be down-regulated in response to infections, it seems feasible that *PON1* down-regulation and decreased activity are the outcome of primarily inflammation events. Cross talk between PON1 levels and inflammatory response could be mediated by the oxLDL, which can induce expression of genes involved in an inflammation by stimulating various transcription factors (Maziere and Maziere 2009). Our results provide insights in the action of PON1, but further investigation is required for a deeper understanding of the contribution of PON1 in the pathology of AMD.

Acknowledgments We would like to thank Marijana Samardzija for help with Excel macros to ease data entry in PHASE. We appreciate the genomic DNA preparations by Esther Glaus. Financial support by a cooperative project grant by the Zurich Center for Integrative Human Physiology (ZIHP) of the University of Zurich, Zurich, Switzerland, is greatly acknowledged. DB was supported by a grant from the Swiss National Foundation (SNF/SSMBS), a grant from the Holcim Foundation, and the Walter and Gertrud Siegenthaler Foundation Zürich, Switzerland.

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