Understanding the Role of Novel Gene-Environmental and Gene-Gene Interactions in the Pathogenesis of Age Related Macular Degeneration

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UNDERSTANDING THE ROLE OF NOVEL GENE-ENVIRONMENTAL AND GENE-GENE INTERACTIONS IN THE PATHOGENESIS OF AGE RELATED MACULAR DEGENERATION

by

Juan A. Ayala-Haedo

A DISSERTATION

Submitted to the Faculty
Of the University of Miami
In partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2010
UNDERSTANDING THE ROLE OF NOVEL GENE ENVIRONMENTAL AND GENE GENE INTERACTIONS IN THE PATHOGENESIS OF AGE RELATED MACULAR DEGENERATION

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The purpose of the study was to assess single nucleotide polymorphisms (SNPs) in $NOS2A$, $ESR1$, $ESR2$ and $MMP-2$ genes that may affect the risk for age-related macular degeneration (AMD) and may interact with environmental factors such as estrogen exposure and smoking, thereby modifying their effect on AMD.

AMD is an ocular degenerative disease with known genetic and environmental factors. However, the disease risk genes identified so far account only for part of the genetic attributable risk and the role of new disease risk genes remain to be evaluated.

For non-genetic risk factors, the most extensively analyzed are smoking and estrogen exposure. Smoking increases the risk for development of AMD and estrogen exposure has a protective effect. Both of these factors have been linked to oxidative pathway activation and extracellular matrix homeostasis (ECM) through interactions with the $NOS2A$ and metalloproteinase ($MMP$) genes,
respectively. In addition, estrogen exerts its activity through the estrogen receptors ERα and ERβ.

We examined a Caucasian cohort of AMD cases and controls. Nine hundred and ninety-eight individuals (males and females) for the NOS2A gene and 777 females for both the ESR1/2 and MMP-2 genes were assessed.

We genotyped TagSNPs within these selected candidate gene regions using HapMap phase II or III. Multivariable logistic regression or generalized estimating equation (GEE) models containing SNP genotypes, age, sex, environmental factor and genotype/environmental interaction were constructed. In addition, because we previously reported interactions between the ARMS2 locus and estrogen exposure and smoking, we also analyzed interactions within ARMS2 locus.

We found that SNPs in NOS2A are associated with increased risk for AMD and might modulate the smoking effect on AMD. The synergistic interaction between NOS2A and smoking is independent of the ARMS2 locus.

No SNP in the MMP-2 gene was significantly associated with increased risk for AMD. We also detected no significant interactions with estrogen exposure or with the ARMS2 locus.

SNPs within the ESR1 gene are associated with an increased risk for developing AMD and the inverse association of AMD and HRT is dependent on SNP genotypes in ESR1 and ESR2 and independent of the ARMS2 locus.
To everyone that made this dream possible
ACKNOWLEDGEMENTS

Since my early years, I have always had the desire and dream of becoming a scientist. I was aware this goal would require hard work and dedication. Completing this doctoral dissertation, to me, is the first step towards that goal.

I have to thank all the people who made completing this dissertation possible; it has been an incredible journey since the beginning, and I feel blessed I had the pleasure and fortune to meet, work and interact with extraordinary individuals along the way.

My family has been key throughout my life, especially during this process. I have had a remarkable support system without which I would not have made it this far. My father, with his example, has taught me since my youth that only through hard work, enthusiasm and passion can one truly be a great person and, as a consequence, a great physician. Thank you Papi, Mami, Veronica and Carolina for always being there to encourage, nurture and guide me throughout my life.

In 2007, I started working with Dr. Margaret Pericak-Vance, my mentor, and I have to say, that this experience has changed my life in many ways. Her mentoring, from the beginning, was not only oriented to academic subjects; she also taught me by example invaluable life lessons. During this process, it is sometimes easy to be discouraged, especially when results are not what one is expecting, and it was especially during those moments, when her passion and
love for her job gave me the strength that I needed to continue, making me aware of the important role a mentor plays in the life of a student serving as an inspirational and transformational force. Thanking her in only one page probably will not be enough, but I hope she knows how much I appreciate everything she has done for me and how I will always be thankful to her.

To the Vance family, thank you for your support, generosity and for always having welcomed me to your house and having made me feel at home.

To Dr. William Scott, thank you for your invaluable guidance during my thesis writing process.

To the Pericak-Vance lab, Patrice, Anna, Chad, Paul, Monica and everybody that has worked with me, thank you for helping me make this project a reality.

To the attendings and patients that participated in the studies.

To my many friends around the world, thanks for all the support and encouragement, for that little e-mail, phone call or even text message that kept me going in difficult times; it is during those times that one realizes, distance is artificial and the connection we create with people surrounding us is what is real. Thanks for listening, advising, laughing, crying, enjoying and sharing your lives with me.

Nancy, Gus, Nahir, Dany, Martin and Adri, go Paraguayan team! Thank you a lot for being every step of the way there during this long process.

Carmen, I will miss you so much next year. Thanks for all the amazing cooking and extraordinary support! Noelia, thank you immensely for all your
support and encouragement, your friendship is invaluable to me. Ale thank you for having shared this journey with me. Steph, you are a precious friend.

Finally, to the past and current members of my dissertation committee, Dr. Vinata Lokeshwar, Dr. Richard Lee, Dr. John Gilbert and Dr. M. Elizabeth Fini, thank you for giving me the opportunity to discuss my work and also to reach one of my most valuable goals in life. Working with each of you has been one of the most extraordinary experiences in my life; your dedication and support are truly appreciated. Thanks again for all your guidance, which was much more than I could have asked for.

To everybody else who made this project possible, thank you immensely!
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PUBLICATION OF CHAPTERS AND ROLE OF AUTHOR

**Chapter one:** this chapter contains sections included in the different papers that were or will be submitted as original publications.

**Chapter two:** this work has been accepted for publication on February 2010 in Annals of Human Genetics.


I prepared and wrote this paper based on my work in Dr. Pericak-Vance’s laboratory. I participated in the Miami patient’s pre-selection and recruitment process, DNA plating, running assays and data interpretation. My work in the laboratory was supervised by Patrice P. Whitehead and the data analysis by Paul Gallins. My mentor and Dr. W.K. Scott supervised and edited the paper.

**Chapter three:** this work will be included as part of a next manuscript to be sent in the future.


**Chapter four:** this work is in submission process in August 2010 to Annals of Human Genetics.


I prepared and wrote this paper based on my work in Dr. Pericak-Vance’s laboratory. I participated in the Miami patient’s pre-selection and recruitment process, DNA plating, running assays and data interpretation. My work in the laboratory was supervised by Patrice P. Whitehead and the data analysis by Paul Gallins. My mentor supervised and edited the paper.

**Chapter Five:** this chapter contains sections included in the different papers that were or will be submitted as original publications.
Appendix


**Pharmacogenetics of beta blockers.**

I prepared and wrote this review. Drs. M.E. Fini and Drs. Schwartz supervised and edited the chapter.

II This section was prepared from data generated in Dr. M.E. Fini’s laboratory.

**Understanding the genetic and environmental mechanisms that influence the pathogenesis of glaucoma.**

I prepared and wrote this section based on my work in my previous laboratory under the supervision of Dr. M.E. Fini.
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CHAPTER ONE: INTRODUCTION. MATERIAL AND METHODS

OVERVIEW

Age Related Macular Degeneration (AMD) is a degenerative disease characterized by the loss of photoreceptor in the macula that is responsible for visual acuity.

Both genetic and environmental factors are associated with an increased susceptibility to AMD. The best replicated genetic risk factors include: complement factor H (CFH), complement factor B (CFB) and complement component 2 (C2) and 3 (C3), ARMS2 (LOC387715/HTRA1) and apolipoprotein E (APOE). Although, several genes and pathways are associated with AMD they can only explain part of the genetic risk for the disease. The remaining genetic effect is likely to correspond to a combination of multiple smaller effects on multiple genes, gene-gene and/or gene-environmental interactions. Of the modifiable or environmental risk factors described for AMD, cigarette smoking and estrogen exposure are the two best-studied that influence the disease.

Our study was designed to ask the question of whether there is an increased risk for AMD conferred by gene variations in certain candidate genes and whether there are gene-gene and gene-environmental interactions with the selected candidate genes that can explain part of remaining genetic risk. In this chapter we will discuss the components of the study design including: candidate gene selection, patient recruitment, DNA genotyping and statistical analysis.
BACKGROUND
AMD

Age-related macular degeneration (AMD) is a degenerative disorder that is characterized by loss of central vision due to the loss of the photoreceptors and retinal pigment epithelium (RPE) in the macula, an area in the central retina responsible for visual acuity (Johnson et al., 2004., Jager et al., 2008., Kliffen et al., 1997; Friedman et al., 2004; Klein et al., 1992). It is the main cause of irreversible visual loss in the elderly in developed countries (Resnikoff et al., 2004). Its prevalence increases with age affecting over 30 million people worldwide. The prevalence of early AMD changes ranges from 8 to 30 % in the age groups younger than 60 and over 75 years old, respectively. The prevalence of advanced AMD ranges from 0.1 to 7.1 % in the age groups younger than 60 and over 75 years old, respectively (Kliffen et al., 1997; Friedman et al., 2004; Klein et al., 1992). AMD affects over 8 million Americans. The overall disease prevalence is also expected to increase by at least 50% by 2020, due to the increasing age of the American population (Friedman et al., 2004). AMD affects Caucasians more than other races (Friedman et al., 2004; Congdon et al., 2004., De Jong et al., 2006; Green et al., 1999).

Although the AMD pathogenesis remains to be completely understood, several hypotheses have assigned a significant role in disease pathogenesis to an abnormal oxidative stress pathway activation, extracellular matrix homeostasis and an abnormal inflammatory response (Montezuma et al., 2007; Zarbin, 2004). Strong evidence has linked environmental exposure and genetic
predisposition to an increased risk for AMD (Age-Related Eye Disease Study Group 2000; van Newkirk et al., 2000).

Segregation analysis (Heiba et al., 1994), twin studies (Meyers et al., 1995; Klein et al., 1994; Hammond et al., 2002; Seddon et al., 2005) and familial aggregation analysis (Seddon et al., 1997; Klaver et al., 1998; De Jong et al., 1997) demonstrated a strong genetic component associated with the disease with high degree of heritability and concordance among siblings. First-degree relatives of AMD patients have an increased risk for developing the disease compared to the general population (Seddon et al., 1997; Klaver et al., 1998). The percentages for heritability and concordance among monozygotic twins range from 45-71% and from 37-100 %, respectively, suggesting an important genetic contribution to the disease (Seddon et al., 2005; Hammond et al., 2002; Klein et al., 1994; Gottfredsdottir et al., 1999; Meyers et al., 1995). In addition, Seddon et al., (2005) reported that the part of the variations in disease severity among individuals could be explained by both genetic factors (46-71 %) and unique environmental exposures (19-37%).

Linkage and association studies have reported genes that are risk factors for developing the disease; the most well-replicated of these are complement factor H (CFH) (Klein et al., 2005; Edwards et al., 2005; Haines et al., Hageman, et al., 2005; Despriet et al., 2006; Chen et al., 2006), complement factor B (CFB) and complement component 2 (C2) (Gold et al., 2006; Richardson et al., 2008; Spencer et al., 2007, ARMS2 (LOC387715/HTRA1) (Jakobsdottir et al., 2005; Rivera et al., 2005; Kanda et al., 2007), complement component 3 (C3) (Yates et
al., 2007; Maller et al., 2007; Spencer et al., 2008), and apolipoprotein E (APOE (Klaver et al., 1998; Schmidt et al., 2000; Baird et al., 2004). Fisher et al., (2005) conducted a meta-analysis including data from multiple genome-wide linkage studies and reported seven loci associated with AMD including: 1q, 2p, 3p, 4q, 10q, 12q, and 16q. The two loci with the strongest evidence for linkage corresponds to the 10q26 region (ARMS2 locus) followed by the 1q locus (CHF region).

However, although these genes are associated with AMD, specific mechanisms of action, as well as their roles in relation to the additional, yet unidentified genes merit further investigation.

In addition, several other non-modifiable factors such as age, female gender and race, as well as modifiable factors such as antioxidant intake, smoking, hypertension and obesity are associated with risk of AMD (Klein et al., 2004; Tomany et al., 2004). Of these, cigarette smoking and estrogen exposure are the two most well-established environmental factors influencing the disease.

There are also important gene-environmental interactions reported with smoking and estrogen exposure, e.g., cigarette smoking has a synergistic interaction with genotypes of the ARMS2 locus and a joint effect with genotypes of the APOE gene (Schmidt et al., 2006; 2005); estrogen exposure is also associated with genotypes of the ARMS2 locus (Edwards-Velez et al., 2010). Several groups established disease population-attributable risks (PAR)s for the different genes; CHF (~20-50%), ARMS2 locus (~20-50%), C3 (~10-20%) and C2-CFB (~18%) (Despriet et al., 2006; Schmidt et al., 2006; Xing et al., 2008; Tuo
et al., 2008; Tam et al., 2008; Spencer et al., 2008; Yates et al., 2007; Jakobsdottir et al., 2008). Maller et al., (2006) reported that a combination of gene variants in CHF, ARMS2 locus and CFB/C2 explained half of the disease relative risk in siblings of AMD patients. Gold et al., (2006) described that CHF and CFB/2 variants could predict disease outcome in up to 70% of individuals. Other groups reported that the combined effect of smoking, and gene variants in the ARMS2 locus and CHF could explain between 60-70% of the disease attributable risk (Schmidt et al., 2006; Tam et al., 2008).

In summary, in AMD even after extensive research only a part of the disease attributable risk could be elucidated by the gene variants, gene-gene and gene-environmental interactions discovered so far. Because multiple genome wide scans have not detected additional regions with genome wide significance, the remaining fraction will likely be explained by smaller effects of multiple genes (rather than bigger effects of single genes) and by the interaction of those genes with other known genetic and environmental risk factors.

Here we analyze the association between candidate genes and an increased risk for AMD and evaluate gene-environmental interactions that would also be associated with the disease and could explain part of the remaining genetic risk.

**Disease gene mapping**

Several methods were traditionally utilized for disease gene mapping in both monogenic and complex diseases. In monogenic diseases linkage analyses were frequently utilized to discover causative genes. In these cases disease traits are
linked to certain genetic markers distributed thorough different areas of interest. These studies employ multiple generation pedigrees with affected and unaffected individuals to establish the linkage. This allows the identification of the disease causing loci based on proximity to the genetic markers (Strachan et al., 1999; Griffiths et al., 2000; Gibbs et al., 2003; Haines et al., 2006).

In complex diseases such as hypertension, diabetes or AMD, as opposed to monogenic diseases, there is no single gene that is necessary and sufficient to manifest the disease phenotype. On the contrary multiple genes with smaller contributions in conjunction with environmental factors are thought to induce the disease phenotype (Griffiths et al., 2000; Gibbs et al., 2003; Haines et al., 2006; Roberts et al., 2010).

One of the most widely used study designs to evaluate complex diseases is the comparison between individuals with or without the disease to detect genetic differences between the two groups. This type of study design is called case-control association analysis. In these studies, the simple premise is to evaluate whether certain alleles or markers are distributed unevenly between cases and controls. Indicating a protective effect if it is present more frequently in controls and higher risk if it is present in cases more predominantly (Risch et al., 1996; Gibbs et al., 2003; Haines et al., 2006).

The initial genetic markers that were utilized in linkage analyses were restriction length polymorphism (RFLP) and tandem repeats. RFLP result from single nucleotide substitutions at restriction enzyme sites that allow DNA of different fragment sizes to be generated depending on the presence or absence
of certain allele. The tandem repeats are repetition of nucleotides present throughout the genome and are classified based on the length of the repeat into: microsatellite (when repeat has $\leq 8$ bases) and minisatellite (when repeat has $> 8$ bases). Microsatellite sequences are frequently composed of di, tri or tetra nucleotide repeats. Single nucleotide polymorphisms (SNPs) analysis has become the method of choice for disease gene mapping, replacing RFLP and tandem repeats (Strachan et al., 1999; Haines et al., 2006; Nowrousian et al., 2010; Ku et al., 2010; Roberts et al., 2010). SNPs are commonly occurring variations present within the genome. It is estimated that each individual has, approximately, 3 millions of SNPs and there are a total of 17 million SNPs known across populations. Together it is believe that they are responsible for 80-90% of genetic variation between individuals (Kruglyak et al., 2001; Reich et al., 2003; Haines et al., 2006; Roberts et al., 2010). Additional genetic variations that are gaining popularity in studies and will play a more prominent role in the future include: indels, copy number variation (CNV), and copy neutral loss of heterozigosity (LOH) (Haines et al., 2006; Ku et al., 2010; Roberts et al., 2010; Nowrousian et al., 2010).

For current association analyses, including genome-wide scans, common SNPs that have a minor allele frequency (MAF) $\geq 5\%$ are usually selected; following the current common disease common variant (CDCV) hypothesis that states that common occurring diseases result from common allele variations present within the genome. The rare allele variants (MAF < 5%) can have greater effect, as seen in rare diseases but require additional techniques for
evaluation. Usually they are assessed by direct sequencing in individuals affected by certain conditions and later genotyping in other populations.

In our study we utilized a genetic case-control study design and only focused on common alleles with a MAF $\geq 5%$.

**Candidate gene selection**

We selected estrogen exposure and smoking as our environmental or modifiable factors and the genes interacting with those factors (*NOS2A, ESR-1/2, MMP-2*) as our candidate genes. Estrogen exposure and smoking are the most influential environmental factors for AMD susceptibility. Individuals that smoke have an increased risk for AMD as compared to non-smokers and estrogen exposure, on the other hand, is associated with a decreased risk for the disease (Klein et al., 2004; Tomany et al., 2004). Both these factors are linked to oxidative pathway activation and extracellular matrix homeostasis (ECM) through their interactions with the *NOS2A* and metallo-matrix proteinase (*MMP*) genes, respectively (Beauregard et al., 2004, Yu et al., 2003; Hoyt et al., 2003; Mazzo et al., 2005; Mershon et al., 2002; Cousins et al., 2003; Ruta et al., 2009). In addition, estrogen exerts its functions through its receptors, coded by *ESR1* and *ESR2*.

Smoking decreases blood antioxidant levels favoring a general oxidative state (Moriarty et al., 2003) and mitochondrial mediated apoptosis (Jiang et al., 2005). Fujihara et al., (2008) demonstrated that animals exposed to smoking develop dysfunction and degeneration of the retinal pigment epithelium (RPE) and Bruch’s membrane, as present in AMD.
In vitro studies demonstrate a direct interaction between smoking and the NOS2A gene, that codes for the inducible form of nitric oxide synthase (iNOS), which produces NO (Hoyt et al., 2003; Mazzio et al., 2005). RPE and choroidal cells, as well as AMD neovascularization membranes, show iNOS expression (Hattenbach et al., 2002). Cells exposed to NO have decreased proliferation and increased apoptosis (Ju et al., 2001; Osborne & Wood, 2004).

SNPs in the NOS2A gene modify the smoking effect and confer an increased risk for a neurodegenerative disease (Hancock et al., 2006; 2008). Smoking is linked to ECM homeostasis through MMP regulation. Ruta et al., (2009), reported that cells exposed to smoking condensates have an increase in MMP-2 activity that correlates with cellular apoptosis.

The protective effect of estrogen can be explained by its multiple functions on vascular and ECM homeostasis and regulation of oxidative pathway activation. Estrogen exerts its multiple functions through estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Both ERα and ERβ proteins are present in the human retina (Ogueta et al., 1999; Marin-Castano et al., 2003; Munaut et al., 2001). The Pvull_Xbal haplotype in the ESR1 gene is associated with lowered serum estradiol levels in post-menopausal women (Schuit et al., 2005) and with an increase risk for AMD (Boekhoorn et al., 2007).

Lastly, the lesions present in AMD are associated with alterations in ECM homeostasis. A dysfunction in MMPs dynamics induces lesions that could correspond to early AMD changes. Estrogen plays its role in ECM homeostasis by regulating the activity of MMPs. Estrogen promote MMP-2 activity (Cousins et
al., 2003) that degrades collagen type IV present in the RPE basement membrane and Bruch’s membrane. This could partially explain why post menopausal women with lower estrogen levels are more prone to develop AMD than men.

A decrease in MMP-2 activity in vivo, as seen in menopausal rats, leads to the accumulation of subepithelial deposits similar to those seen in AMD (Cousins et al., 2003). Recently, Seitzman et al., (2008) demonstrated that SNPs in MMP-2 confer an increase risk to developing AMD.

We have previously reported an interaction between both smoking and estrogen exposure and the ARMS2 locus (Schmidt et al., 2006; Edwards-Velez et al., 2010). For this reason we also included in our study a three way interaction analysis between ARMS2, the candidate genes (NOS2A, ESR1/2, MMP-2) and the correspondent environmental factor. Figure 1.1 shows a schematic representation of the environmental factors chosen as well as their possible role in AMD pathogenesis.

MATERIAL AND METHODS

Subjects

For the different studies, we recruited individuals from the Duke University Eye Center (DUEC), the Vanderbilt Eye Institute (VEI) and the Bascom Palmer Eye Institute (BPEI) at the University of Miami, Miller School of Medicine under research protocols approved by the Institutional Review Boards at each institution. We obtained written informed consent from all participants.
A retinal specialist examined all study subjects by slit-lamp biomicroscopy, dilated fundus examination and indirect ophthalmoscopy. In addition, we obtained fundus imaging in all patients. The pictures were graded using a modified grading system based on the Age-Related Eye Disease Study (AREDS) which has been previously described in detail (Schmidt et al., 2000). Briefly, the grading system was scored from 1 to 5. The 1 and 2 categories correspond to controls. The rest correspond to mild (grade 3) and advanced (grades 4 and 5) stages of AMD. Specifically, stage 4 corresponds to geographic atrophy or dry AMD and stage 5 correspond to neovascular or wet AMD (for the complete grading list, please see Table 1.1). AMD grades, however, are not static and can progress to more advanced stages and therefore increase the disease severity. As an example, Gehers et al., (2006) reports that 10-15% of geographic atrophy cases progress to neovascular AMD. The AMD grading sub stratification allows independent evaluations of the different grades by genetic and environmental risks factors.

In addition, we obtained information about environmental exposure. For the purpose of our studies we focused mainly on smoking history and estrogen exposure (through Oral Contraceptive Treatment [OCT] and Hormone Replacement Therapy [HRT]).

For the NOS2A analysis, we included a total of 998 non-Hispanic Caucasian participants, (712 AMD cases [grades 3, 4 and 5] and 286 unrelated controls [grades 1 and 2]. We assessed the total population for a main SNP effect. We included a subset of the population with environmental risk
information, including smoking history, (705 non-Hispanic Caucasian participants) in the analysis of SNP/smoking interaction (466 AMD cases [grades 3, 4 and 5] and 239 unrelated controls [grades 1 and 2]). A full description of the participants by clinical findings and smoking status is presented in Table 1.2.

For the *ESR-1/2, MMP-2* analysis, we included a total population of 777 Caucasian related and unrelated females (520 AMD cases [grades 3, 4, 5] and 257 controls [grades 1 and 2]); a full description of the subjects is provided in Table 1.3.

**DNA Analysis and Genotyping**

We processed the whole blood obtained for DNA extraction using a standard protocol (Puregene; Gentra Systems, Minneapolis, MN).

For the *NOS2A* analysis, we selected tagSNPs that captured the common (allele frequency ≥ 5%) variation among the phase II Hapmap SNPs using the Tagger function of the Haploview program (Lewis & Zaykin, 2000). We included tagSNPs that tagged other variants with an $r^2 \geq 0.67$ (or were tags only for themselves) in the study.

In addition, we also included in the analysis two previously validated coding sequence SNPs (rs1060826 and rs16966563) obtained from the NCBI data base. We genotyped 17 SNPs in the 998 subjects included in the study using TaqMan assays (Applied Biosystems, Foster City, CA).

For the *ESR-1/2* and *MMP-2* gene analysis, we selected tagSNPs that tagged only themselves or other variants with an $r^2 \geq 0.60$, capturing common variation (allele frequency ≥ 10%) among the phase III Hapmap SNPs.
We genotyped 30 SNPs for *ESR-1/2* and 4 SNPs for the *MMP-2* analysis in our cohort the 777 female subjects using Sequenom and TaqMan assays (Applied Biosystems, Foster City, CA).

We assessed pairwise linkage disequilibrium ($r^2$) in the *NOS2A, ESR-1/2* and *MMP-2* analysis to evaluate for residual correlations in both control and cases (Figure 1.2, 1.3, 1.4 and 1.5) for all pairwise marker combinations using the Haploview program.

For quality control purposes, we plated 2 different samples from the Fondation Jean Dausset-Centre d’etude de Polymorphisme Humain (CEPH) in quadruplicate on each 384-well plate. Additionally, we replicated internal controls throughout the sample list to ensure efficiency. Laboratory personnel were blinded to sample phenotypes and replicate sample locations. Each plate met a quality control efficiency of 100% and a genotyping efficiency of >95%.

**Statistical Analysis**

We evaluated deviations from Hardy-Weinberg equilibrium (HWE) using the Genetic Data Analysis program (GDA) (Barrett et al., 2005).

In the *NOS2A* analysis, we examined each SNP for association controlling for confounding by age at recruitment and sex. Then, in the subset for which smoking data were available, we fit models including genotype, age, sex, smoking status (ever/never) and a two-way interaction term between genotype and smoking.
We constructed a model coding for additive genotypic effects, and tested each term for significant association with AMD using a Wald chi-square test and a nominal significance level of \( p \leq 0.05 \).

In the \( ESR-1/2 \) and \( MMP-2 \) analysis, we utilized the population average generalized estimating equations (GEE) for the SNP association and interaction analysis with estrogen exposure (through HRT and OCT) and \( ARMS2 \) locus, with single marker tests of association using an additive genotypic model (minor allele was consider risk allele); the analysis was conducted in the Statistical Analysis Software version 9.1. (SAS Institute, Cary, NC, USA). The multivariate analysis included age, smoking status and estrogen exposure as covariates in the model. We included all cases (grades 3-5, \( n=520 \)) and controls (grades 1-2, \( n=257 \)) in the analysis.

The strength of the association was evaluated by the odds ratio and 95% confidence intervals. For models with significant interaction terms (\( p<0.05 \)), we assessed for a differential association of smoking or estrogen exposure by risk allele carrier status using a stratified analysis. No correction for multiple comparisons was utilized for the data analysis.
CHAPTER FIGURES

Figure 1. Schematic representation of smoking and estrogen exposure in the oxidative stress pathway and extracellular matrix homeostasis regulation and AMD pathogenesis.
Figure 1. 2. NOS2A tagSNPs pairwise linkage disequilibrium plots for 712 AMD cases. Pairwise linkage disequilibrium plots were generated in Haploview and are presented for 712 grade 3, 4 or 5 AMD cases used in the association study of NOS2A SNPs and AMD. Pairwise correlation coefficients ($r^2$) are presented. Standard color coding was used for the plots: $r^2=0$ is plotted white, $0<r^2<1$ is plotted as increasing shades of gray, $r^2=1$ is plotted in black. Blocks were defined according to the 95% confidence interval for the pairwise D’ value. The SNP location is visualized in the upper schematic representation of the gene with boxes corresponding to exons.
**Figure 1. 3. NOS2A tagSNPs pairwise linkage disequilibrium plots for 286 controls.** Pairwise linkage disequilibrium plots were generated in Haploview and are presented for 286 grade 1 or 2 unaffected controls used in the association study of NOS2A SNPs and AMD. Pairwise correlation coefficients ($r^2$) are presented. Standard color coding was used for the plots: $r^2=0$ is plotted white, $0<r^2<1$ is plotted as increasing shades of gray, $r^2=1$ is plotted in black. Blocks were defined according to the 95% confidence interval for the pairwise D' value. The SNP location is visualized in the upper schematic representation of the gene with boxes corresponding to exons.
Figure 1.4. LD patterns for the ESR1 gene in 520 cases with AMD (grade 3, 4, 5). The areas with stronger $r^2$ are represented by darker shadings. The SNP location is visualized in the upper schematic representation of the gene with boxes corresponding to exons. Standard color coding was used for the plots: $r^2=0$ is plotted white, $0<r^2<1$ is plotted as increasing shades of gray, $r^2=1$ is plotted in black. Blocks were defined according to the 95% confidence interval for the pairwise D’ value.
Figure 1. 5. LD patterns for the ESR1 gene in 257 female controls with AMD (grade 1 and 2). The areas with stronger $r^2$ are represented by darker shadings. The SNP location is visualized in the upper schematic representation of the gene with boxes corresponding to exons. Standard color coding was used for the plots: $r^2=0$ is plotted white, $0<r^2<1$ is plotted as increasing shades of gray, $r^2=1$ is plotted in black. Blocks were defined according to the 95% confidence interval for the pairwise D’ value.
CHAPTER TABLES

Table 1.1. AMD grading score modified from the Age-Related Eye Disease Study (AREDS). Groups 1 and 2 are classified as controls and groups 3, 4 and 5 as AMD cases

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No drusen or small nonextensive drusen (&lt; 63 µm in ø) without RPE abnormalities.</td>
</tr>
<tr>
<td>2</td>
<td>Multiple small drusen (&lt; 63 µm in ø) or nonextensive intermediate drusen (63 to 124 µm in ø) and/or RPE abnormalities associated with AMD.</td>
</tr>
<tr>
<td>3</td>
<td>Extensive intermediate drusen or large soft drusen (&gt;124 µm in ø) including drusenoid RPE detachment.</td>
</tr>
<tr>
<td>4</td>
<td>Geographic atrophy.</td>
</tr>
<tr>
<td>5</td>
<td>Exudative AMD, including nondrusenoid RPE detachment, choroidal neovascularization, subretinal hemorrhage or disciform scarring.</td>
</tr>
</tbody>
</table>

Table 1. 2. AGE, SEX, CLINICAL STATUS, AND CIGARETTE SMOKING HISTORY. 712 cases with age-related macular degeneration and 286 unaffected controls were included in the study.

<table>
<thead>
<tr>
<th>Status</th>
<th>Mean Age (SD)</th>
<th>Sex</th>
<th>Grade</th>
<th>Ever Smoked</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M (SD)</td>
<td>F</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Control</td>
<td>66.9 (8.3)</td>
<td>126</td>
<td>160</td>
<td>1</td>
</tr>
<tr>
<td>(N=286)</td>
<td>(44.1%)</td>
<td>(55.9%)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Case</td>
<td>76.4 (7.6)</td>
<td>257</td>
<td>455</td>
<td>4</td>
</tr>
<tr>
<td>(N=712)</td>
<td>(36.1%)</td>
<td>(63.9%)</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>
Table 1. Description of the sample (777 Caucasian females) by clinical grading and age. (520 cases (grades 1, 2 and 3) with age-related macular degeneration (AMD) and 257 controls (grades 1 and 2) participated in the study)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>187</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>172</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>287</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>520</td>
<td>257</td>
</tr>
<tr>
<td>Age (SD)</td>
<td>76.7 (8.3)</td>
<td>67.5 (8.2)</td>
</tr>
</tbody>
</table>
CHAPTER TWO: Analysis of single nucleotide polymorphisms in the NOS2A gene and interaction with smoking in age-related macular degeneration

OVERVIEW

Age-related macular degeneration (AMD) is a complex degenerative retinal disease influenced by both genetic and environmental risk factors. We assessed whether single nucleotide polymorphisms (SNPs) in the NOS2A gene increase risk and modulate the effect of smoking in AMD. We genotyped 998 Caucasian subjects (712 AMD cases and 286 controls) for 17 SNPs in NOS2A. We constructed multivariable logistic regression models containing SNP genotypes, age, sex, smoking status and genotype/smoking interaction. SNP rs8072199 was significantly associated with AMD (OR = 1.3; 95%; CI: 1.02, 1.65; \( P = 0.035 \)). We detected a significant interaction with smoking at rs2248814 (\( P = 0.037 \)). Data stratified by genotypes demonstrate that the association between AMD and smoking was stronger in carriers of AA genotypes (OR = 35.98; 95% CI: 3.19, 405.98) than in carriers of the AG genotype (OR=3.05; 95% CI: 1.36, 6.74) or GG genotype (OR=2.1; 95% CI: 0.91, 4.84). The results suggest a possible synergistic interaction of AA genotype with smoking. Our data suggests that SNPs in the NOS2A gene are associated with increased risk for AMD and might modulate the effect of smoking on AMD.

BACKGROUND

Cigarette smoking is the strongest, most well-established risk factor for AMD. Smoking 10 packs of cigarettes a week doubles the likelihood of developing AMD relative to non-smokers; among past smokers, only those who stopped 20 years before study have equivalent risks to non-smokers.
(Campochiaro, 2000; Zabin, 2004; She et al., 2007). Most recently, Fujihara et al., (2008) demonstrated that mice exposed to smoking develop signs of degeneration in the RPE and Bruch’s membrane, structures that are intimately involved in the AMD disease pathogenesis.

There are important environmental-gene interactions reported with smoking, e.g., cigarette smoking has a synergistic interaction with genotypes of the ARMS2 locus (Schmidt et al., 2006) and a joint effect with genotypes of the APOE gene (Schmidt et al., 2005). Other groups, however, have reported contradictory results (Conley et al., 2006; DeAngelis et al., 2007; Hughes et al., 2007). Although the pathophysiology of the disease is not clearly understood, it is well accepted that both oxidative stress and an abnormal inflammatory activation play important roles in disease pathogenesis (Montezuma et al., 2007, Zarbin, 2004). The nitric oxide synthase (NOS) enzyme group is known to participate in both phenomena and several groups suggested that it plays an active role in advanced AMD stages (Campochiaro, 2000; She et al., 2007). The NOS system is comprised of three distinct isoforms, the neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) nitric oxide synthases, being coded by the NOS 1, NOS3 and NOS2A genes respectively. These enzymes produce the lipophilic free radical nitric oxide (NO) an intracellular second messenger that is responsible for activation of multiple intracellular pathways, such as the oxidative stress pathway. Two of the enzymes are constitutively expressed (eNOS, nNOS) and iNOS upregulation is associated with cellular insult or stress (Osborne et al., 2004). iNOS expression can be detected in RPE cells, Muller cells and in
activated inflammatory cells such as macrophages present in AMD (Clemons et al., 2005).

In addition, *in vitro* studies of glial cell cultures and murine lung epithelium cells demonstrate a direct interaction between smoking and the NOS2A gene that codes for the inducible form of nitric oxide synthase (iNOS). Cells exposed to smoke condensates demonstrate a reduction in iNOS protein expression and enzymatic activity (Hoyt et al., 2003; Mazzio et al., 2005) decreasing oxidative stress pathway activation.

The NOS2A gene is an attractive AMD candidate gene given its interaction with smoking and its role in host defense, inflammation and neovascularization. The purpose of our study was to assess the main effect of SNPs in the NOS2A gene in AMD as well as a possible gene-environmental interaction with smoking.

**RESULTS**

We performed a multivariable logistic regression analysis adjusting for age and sex on all 998 subjects [286 controls (grades 1, 2) and 712 AMD cases (grades 3, 4, 5)]. SNP rs8072199 was significantly associated with AMD under the additive model (p= 0.035) (Table 2. 1).

We created models containing SNP/smoking interaction terms for all markers under the additive genetic model, for all 705 subjects with available smoking data [466 cases (grades 3, 4 and 5) and 239 controls (grades 1 and 2)]. No significant interactions were detected. However, when considering just grade 5 (273 cases) and grade 1 (169 controls), we detected a SNP/smoking
interaction for rs2248814 (p=0.039); rs1060826 presented a borderline significant value (p=0.061). Due to high LD ($r^2 = 0.95$ in cases and 0.96 in controls) between these two SNPs and based on the exonic location of rs1060826 we chose both SNP genotypes to stratify the data and examine the modification of the effect of smoking by allele carrier status.

Consistent with a recessive model, the stratified analysis demonstrates that the strength of the association between smoking and AMD increased in homozygous carriers of the rs2248814 and rs1060826 risk allele A (OR=35.98, 95% CI [3.19, 405.98], p=0.0038 and OR=35.63, 95% CI [3.12, 406.67], p=0.004, respectively) than in carriers of the AG genotype (OR=3.05, 95% CI [1.38, 6.74], p=0.0058 and OR=2.83, 95% CI [1.27, 6.31], p=0.011, respectively) or GG genotype (OR=2.1, 95% CI [0.911, 4.84], p=0.082 and OR=2.13, 95% CI [0.94, 4.85], p=0.072, respectively) (Table 2.2).

**DISCUSSION**

In our study, we demonstrated that a SNP present in the NOS2A gene, rs8072199, conferred an increased risk for AMD when controlling for age and sex. Prior studies of *in vitro* glial cell cultures and murine lung epithelium cells exposed to smoke condensates demonstrated decreased oxidative stress activation by a reduction in inducible nitric oxide synthase (iNOS) protein expression and enzymatic activity (Hoyt et al., 2003; Mazzio et al., 2005).

Therefore, we examined interactions between smoking and SNPs in NOS2A and detected a significant interaction with rs2248814 and a borderline interaction with rs1060826. To understand the nature of this interaction we
conducted an analysis of the effect of smoking when stratifying the sample by rs2248814 and rs1060826 genotype. Individuals carrying the AA genotype in both SNPs were much more sensitive to the effect of smoking on AMD and having one copy of the major allele reduced this effect considerably. AA homozygous individuals with AMD were 35 times as likely to have smoked as controls with the AA genotype. Individuals carrying one or no copies of the A allele were only 3 or 2 times as likely to have smoked as controls, respectively.

Additionally, we observed a similar trend when the smoking effect was evaluated using pack-years of exposure, comparing ‘heavier smokers’ (above the median pack-years) or ‘lighter smokers’ (below the median) versus ‘never smoked’. The smoking effect seemed to be stronger in the heavy smokers than in light smokers (data not shown). The pattern observed suggests a synergy between the effect of smoking and the AA genotype at rs2248814 and 1060826. The fact that we detected the SNP/smoking interaction when comparing the grade 1 (controls) and grade 5 subjects (neovascular cases) is in agreement with previous work indicating that both smoking and iNOS induction are individually associated with the neovascularization process that occurs in advanced neovascular AMD (Ando et al., 2002; Suner et al., 2004).

While these results are intriguing, they should be interpreted cautiously. SNPs rs8072199 and rs2248814, significant for SNP main effect and SNP/smoking interaction, respectively, are in low linkage disequilibrium ($r^2=0.07$) and neither has a known functional effect on iNOS, so that the effect seen in this sample might be due to a third, untyped SNP located in the gene that is in
moderate LD with both of these SNPs. The results presented here are from a single sample and do not withstand conservative corrections for multiple comparisons. However, the results generate interesting hypotheses about a possible role of iNOS in AMD and a gene-smoking interaction in the disease which bear examination in other data sets.

Because we had previously reported an interaction between smoking and ARMS2 (Schmidt et al., 2006), we also performed a two way and a three way interaction analysis in our subset of individuals with smoking history data. The two way analysis that included SNPs in NOS2A and ARMS2 and the three way analysis including SNPs in the two genes and smoking did not detect any significant interactions (data not shown).

In AMD several mechanisms are responsible for disease pathogenesis including oxidative stress related processes and abnormal inflammatory pathway activation. The nitric oxide synthase (NOS) enzyme system participate in both phenomena. The expression of NOS2A gene that codes for inducible nitric oxide synthase (iNOS) is detected in activated inflammatory cells in AMD as well as in RPE and Müller cells (Ando et al., 2002b) and in choroidal neovascularization membranes of AMD patients (Hattenbach et al., 2002): its blockade increases the formation of aberrant choroidal vessel in late stage AMD (Ando et al., 2002). Prior work on RPE and photoreceptor cells exposed to NO have shown a decrease in proliferation (Goureau et al., 1993) and increase in apoptosis (Ju et al., 2001; Osborne & Wood, 2004) respectively, that could also play a role in the
disease pathogenesis. Therefore, the fact that we found a main effect in a SNP in the NOS2A gene in our data set further implicates the gene in the disease.

Smoking has been traditionally identified as one of the strongest disease risk factors, increasing risk in a dose dependent fashion in smokers as compared to non smokers (Clemons et al., 2005; Khan et al., 2006; Tomany et al., 2004). Strong evidence exists for a possible causative role resulting in RPE dysfunction and death in an animal model of AMD (Fujihara et al., 2008). Smoking decreases blood antioxidants levels and to favor an oxidative state (Moriarty et al., 2003), which then results in increased mitochondrial mediated apoptosis that could partially explain the cell death that ensues in AMD (Jiang et al., 2005). However, the fact that the known attributed risk conferred by smoking can be so dramatically influenced by homozygous risk alleles constitutes a fascinating finding, with significant future implications not only for research but also in the public health arena.

Interestingly, the rs2248814 and rs1060826 AA genotypes are the same genotypes that have been previously reported to modify the smoking effect and confer an increased risk for Parkinson Disease (PD) (Hancock et al., 2006; Hancock et al., 2008). The main difference with the current study is that in PD smoking has a protective effect (Hague et al., 2004; Hancock et al., 2006; Levecque et al., 2003), partially attributed to the possible protective effect that nicotine may exert against neurotoxic insults (Fratiglioni & Wang, 2000; Preux et al., 2000) and a decreased iNOS induction and activity (Hoyt et al., 2003; Mazzio et al., 2005). Therefore, the presence of either the rs2248814 or rs1060826 AA
genotype in the NOS2A gene should remove or at least reduce the possible negative regulation of smoking on iNOS production and activity. In AMD, however, rs2248814/ rs1060826 AA genotype and smoking have a synergistic effect. These somewhat conflicting results may reflect varying degrees of linkage disequilibrium with one or more true functional variants, which remain to be identified. Further studies are necessary to determine if the presence of the AA genotype in either rs2248814 or rs1060826 is correlated with a modified protein expression, or activity, when cells are exposed to smoking, that could account for the effects observed in both PD and AMD.
### CHAPTER TABLES

**Table 2.1. RESULTS OF LOGISTIC REGRESSION ANALYSIS OF NOS2A AND AMD.** Association between single nucleotide polymorphisms (SNP) in the NOS2A gene and age-related macular degeneration (AMD) was assessed in 712 AMD cases and 286 controls, adjusting for age and sex. Odds ratios (OR) and 95% confidence intervals (95% CI) indicate the strength of association between AMD and increasing numbers of minor alleles at each SNP. Significant (p<0.05) results are noted in bold.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Base pair location on chromosome 17</th>
<th>Location in gene (amino acid change if exonic)</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1060826</td>
<td>23113994</td>
<td>Exon 22 (T919T)</td>
<td>1.16</td>
<td>(0.90, 1.49)</td>
<td>0.26</td>
</tr>
<tr>
<td>rs2297515</td>
<td>23117460</td>
<td>Intron 19</td>
<td>0.89</td>
<td>(0.64, 1.23)</td>
<td>0.48</td>
</tr>
<tr>
<td>rs2297516</td>
<td>23119857</td>
<td>Intron 17</td>
<td>0.82</td>
<td>(0.64, 1.04)</td>
<td>0.10</td>
</tr>
<tr>
<td>rs2297518</td>
<td>23120724</td>
<td>Exon 16 (S608L)</td>
<td>1.08</td>
<td>(0.80, 1.46)</td>
<td>0.62</td>
</tr>
<tr>
<td>rs2248814</td>
<td>23124448</td>
<td>Intron 12</td>
<td>1.17</td>
<td>(0.91, 1.50)</td>
<td>0.22</td>
</tr>
<tr>
<td>rs2314810</td>
<td>23128237</td>
<td>Intron 11</td>
<td>1.03</td>
<td>(0.61, 1.76)</td>
<td>0.91</td>
</tr>
<tr>
<td>rs1137933</td>
<td>23130059</td>
<td>Exon 10 (D385D)</td>
<td>1.04</td>
<td>(0.78, 1.38)</td>
<td>0.81</td>
</tr>
<tr>
<td>rs4795067</td>
<td>23130802</td>
<td>Intron 9</td>
<td>1.04</td>
<td>(0.81, 1.34)</td>
<td>0.75</td>
</tr>
<tr>
<td>rs944725</td>
<td>23133698</td>
<td>Intron 6</td>
<td>0.99</td>
<td>(0.79, 1.26),</td>
<td>0.97</td>
</tr>
<tr>
<td>rs17722851</td>
<td>23134963</td>
<td>Intron 5</td>
<td>0.75</td>
<td>(0.52, 1.10)</td>
<td>0.14</td>
</tr>
<tr>
<td>rs3794764</td>
<td>23135555</td>
<td>Intron 5</td>
<td>0.90</td>
<td>(0.68, 1.20)</td>
<td>0.48</td>
</tr>
<tr>
<td>rs16966563</td>
<td>23140076</td>
<td>Exon 4 (P68P)</td>
<td>1.21</td>
<td>(0.56, 2.53)</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>rs8072199</strong></td>
<td><strong>23140975</strong></td>
<td>Intron 2</td>
<td><strong>1.30</strong></td>
<td><strong>(1.02, 1.65)</strong></td>
<td><strong>0.035</strong></td>
</tr>
<tr>
<td>rs2072324</td>
<td>23141023</td>
<td>Intron 2</td>
<td>0.88</td>
<td>(0.67, 1.18)</td>
<td>0.41</td>
</tr>
<tr>
<td>rs3794766</td>
<td>23146048</td>
<td>Intron 2</td>
<td>1.24</td>
<td>(0.92, 1.66)</td>
<td>0.15</td>
</tr>
<tr>
<td>rs3730014</td>
<td>23149870</td>
<td>Exon 2 (A31A)</td>
<td>0.59</td>
<td>(0.19, 1.91)</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Table 2. 2. RESULTS FROM LOGISTIC REGRESSION MODELS OF NOS2A – CIGARETTE SMOKING INTERACTION AND AMD. Association between cigarette smoking and age-related macular degeneration (AMD), stratified by genotypes at NOS2A single nucleotide polymorphisms rs2248814 and rs1060826 was determined adjusting for age and sex. Odds ratios (OR) and 95% confidence intervals (95% CI) are presented for the association of “ever” smoking with AMD in 263 grade 5 cases and 169 grade 1 controls. Statistically significant associations (p<0.05) between cigarette smoking and AMD are noted in bold.

<table>
<thead>
<tr>
<th>SNP</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AA GENOTYPE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2248814</td>
<td>35.98</td>
<td>(3.19, 405.98)</td>
<td>0.0038</td>
<td>33</td>
<td>17</td>
</tr>
<tr>
<td>rs1060826</td>
<td>35.63</td>
<td>(3.12, 406.67)</td>
<td>0.004</td>
<td>33</td>
<td>17</td>
</tr>
<tr>
<td><strong>AG GENOTYPE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2248814</td>
<td>3.05</td>
<td>(1.38, 6.74)</td>
<td>0.0058</td>
<td>136</td>
<td>70</td>
</tr>
<tr>
<td>rs1060826</td>
<td>2.83</td>
<td>(1.27, 6.31)</td>
<td>0.011</td>
<td>132</td>
<td>68</td>
</tr>
<tr>
<td><strong>GG GENOTYPE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2248814</td>
<td>2.10</td>
<td>(0.91, 4.84)</td>
<td>0.082</td>
<td>95</td>
<td>60</td>
</tr>
<tr>
<td>rs1060826</td>
<td>2.13</td>
<td>(0.94, 4.85)</td>
<td>0.072</td>
<td>98</td>
<td>60</td>
</tr>
</tbody>
</table>
CHAPTER THREE: Analysis of interaction between SNPs in *MMP-2* gene, *ARMS2* locus, estrogen exposure and AMD.

OVERVIEW:

The purpose of the study was to assess association of AMD with SNPs in *MMP-2* gene and their interactions with exogenous estrogen exposure and the *ARMS2* locus. We genotyped 4 tagSNPs ($r^2>0.60$) in the *MMP-2* gene using a Sequenom assay, in 777 related and unrelated Caucasian non-Hispanic female subjects. As previously described, we classified individuals for AMD using a modified version of the age related eye disease study (AREDS) classification, where grades 1 & 2 were considered controls (n=257) and grades 3, 4 and 5 were considered AMD cases (n=520). We determined estrogen exposure through questions assessing hormonal replacement therapy (HRT) and oral contraceptive treatment (OCT) history. We constructed binary outcome variables [yes (1)/ no (0)] for each category (OCT and HRT).

We examined the association between AMD, tagSNPs and estrogen exposure through HRT and OCT and the *ARMS2* locus using generalized estimating equations (GEE), adjusting for age and cigarette smoking. We found a protective effect of HRT (OR=0.5, 95% CI [0.31, 0.81], p<0.01) and OCT (OR=0.41, 95% CI [0.24, 0.68], p<0.01) on AMD (grade 1 vs. 5).

No SNP in the *MMP-2* gene was significantly associated with increased risk for AMD. We detected no significant interactions with estrogen exposure and SNPs in *MMP-2* gene, as well as in the three-way interaction analysis between estrogen exposure, *ARMS2* and *MMP-2* SNPs.
These results suggest that SNPs in the *MMP-2* gene are not associated with an increased risk for AMD and might not interact with estrogen exposure or *ARMS2*.

**BACKGROUND**

In AMD, the loss in photoreceptors is a consequence of a dysfunction of the surrounding structures. The retinal pigment epithelium (RPE) that lies beneath the photoreceptor normally provides metabolic sustenance to the photoreceptor layer, and the Bruch’s membrane underneath the RPE serves as a component of the blood barrier; both of these structures are implicated in the pathogenesis of AMD (Johnson et al., 2004; Jager et al., 2008; de Jong, 2006). The disease is characterized in the early stages by the presence of deposits of polymorphous material between the retina and Bruch’s membrane, called drusen and/or hypo or hyperpigmented areas of the RPE without choroidal vessels. In late or advanced stages, AMD is characterized by the presence of geographic atrophy, choroidal neovascularization, or both (Johnson et al., 2004; Jager et al., 2008).

There are protective and risk factors reported for AMD; among the environmental factors, two of the best characterized include smoking and estrogen exposure. Individuals that smoke have an increased risk for AMD as compared to non-smokers; estrogen exposure has been described to decrease the risk for the disease (Klein et al., 2004; Tomany et al., 2004).

Both environmental factors participate in the oxidative stress pathway and ECM homeostasis regulation with opposite effects. Smoking might even
have a causative role in AMD; *in vivo* studies have demonstrated that animals exposed to smoking developed dysfunction and degeneration of the RPE and Bruch’s membrane (Fujihara et al., 2008). Smoking is linked to ECM homeostasis through its MMP regulation. Ruta et al. (2009) reported that cells exposed to smoking condensates had an increase in MMP-2 activity that correlated with cellular apoptosis.

Estrogen exposure on the other hand has antioxidant properties, decreasing lipid peroxidation, promoting RPE survival after oxidative stress exposure and decreasing synthesis of NO through its effect on iNOS activation regulation (Akçay et al., 2000; Subiah et al., 1993; Yu et al., 2005). In addition, estrogen participates in ECM homeostasis through its action on MMPs regulation. Specifically, estrogen promotes MMP-2 activity (Cousins et al., 2003) that normally degrades collagen type IV present in the RPE basement membrane and Bruch’s membrane. Decreased MMP-2 activity *in vivo*, as seen in menopausal rats, leads to the accumulation of subepithelial deposits similar to what is seen in AMD (Cousins et al., 2003). This could partially explain why post menopausal women with lower estrogen levels would be more prone to develop AMD than men.

Finally, SNPs in the *MMP-2* gene are associated with a decreased risk for AMD (Seitzman et al., 2008).

We hypothesize that the analysis of SNPs in the *MMP-2* gene and its interaction with estrogen exposure in our population might identify new pathways that are involved in AMD pathogenesis.
RESULTS

We performed GEE multivariate analyses on all 777 female Caucasian subjects (520 controls [grades 1 and 2] and 257 cases [grades 3, 4 and 5]. We constructed an additive genetic model including age at recruitment, smoking status as well as estrogen exposure in the model. We found an inverse association between estrogen exposure and AMD. OCT was inversely associated with AMD in the model using all cases and controls (OR=0.60, 95% CI [0.40, 0.89], p<0.01) and the association remained significant when only extreme cases were analyzed (grade 1 vs. 5) in our population of 777 related and unrelated females (520 cases and 257 controls). HRT was inversely associated only with neovascular AMD (OR=0.50, 95% CI [0.31, 0.81], p<0.01) (Table 3.1).

Because MMP-2 gene variants confer a protective effect on late AMD (Seitzman et al., 2008) we performed an association analysis between tagSNPs and increased risk for AMD under the additive genetic model. We found no evidence of an association between SNPs in the MMP-2 gene and an increase risk for AMD (data not shown). Since estrogen regulates MMP-2 activity (Cousins et al., 2003) we also evaluated the possible interaction between estrogen exposure and SNPs in the MMP-2 gene. We detected no interactions with either OCT or HRT and MMP-2 SNPs (Table 3.2).

Since our group described recently an interaction between estrogen exposure and the ARMS2 locus, we also performed a three way interaction analysis between SNPs in MMP-2, estrogen exposure and ARMS2 locus. MMP-2
had no interactions with either the ARMS2 locus and estrogen exposure (data not shown).

DISCUSSION

In AMD, several changes take place in the early stages before progressing to the advance stages that include geographic atrophy and neovascularization. Among the early manifestations of the disease, there is a deposit of extracellular material located between RPE and Bruch's membrane, called drusen (Jager et al., 2008).

ECM homeostasis is associated with AMD. MMPs are intimately involved in ECM turnover and are, therefore, linked to AMD (Montezuma et al., 2007; Zarbin, 2004). In addition, the AMD deposits that appear early in the disease pathogenesis seem to correspond to basement membrane components that get normally degraded by different MMPs, including MMP-2. MMP-2 degrades the collagen I, IV and laminin present in Bruch's and RPE membrane. Different groups have implicated MMP-2 in both early and late AMD (Cousins et al., 2003; Berglin et al., 2000; Kvanta et al., 2000); however, the role of MMP-2 in the disease seems to be controversial. MMP-2 is present in neovascular membranes of humans and animal models and seems to be involved in the neovascularization process itself since its blockade limits the abnormal neovascularization (Lambert et al., 2003; Berglin et al., 2000; Kvanta et al., 2000; Plantner et al., 1998); on the other hand decreased MMP-2 activity results in subepithelial deposits that resemble the changes observed in early AMD stages (Marin-Castano et al., 2003). The MMP-2 gene is located in the 16q13
chromosome and has a complex mechanism of regulation that includes transcriptional regulation, pro-enzyme activation and binding to inhibitors (Jones et al., 2003). It is a membrane bound protein and requires MMP-14 and the tissue inhibitor of metallo-matrix proteinases (TIMP-2) for its activation (Strongin et al., 1995; Kinoshita et al., 1998). Its activity regulation is associated with both smoking and estrogen exposure. Estrogen exposure is specifically associated with increased MMP-2 activity in RPE cells (Cousins et al., 2003). Smoking exposure is also associated with an increased MMP-2 activity but correlated with smoking induced apoptosis (Ruta et al., 2009). In addition, in vivo and in vitro studies report that cells exposed to sublethal levels of oxidative stress inducing agents have decreased MMP-2 activity (Marin-Castano et al., 2005). This decrease in MMP-2 activity can be avoided by maintaining an appropriate balance with MMP-14 and TIMP-2 (Elliot et al., 2006).

In the present study, we failed to replicate previous interesting findings that reported a decreased risk to develop AMD conferred by certain SNPs in the MMP-2 gene (Seitzman et al., 2008); in our cohort of Caucasian females we did not find any association between SNPs in the MMP-2 gene and altered risk for AMD.

We did confirm the previously described biological association between estrogen exposure and AMD (Velez-Edwards et al., 2010). We observed a significant protective effect of both OCT and HRT in our study group.

Since it has been well established that estrogen regulates MMP-2 activity in vitro and in vivo, we also evaluated for an interaction between MMP-2 and
estrogen exposure. We did not detect, however, an interaction between estrogen exposure and \textit{MMP-2} SNPs in our cohort either.

Because we recently reported an interaction between estrogen exposure and \textit{ARMS2} locus (Velez-Edwards et al., 2010) and since the nature of that interaction has not been clearly elucidated we also evaluated for a possible interaction between SNPs in \textit{MMP-2}, estrogen exposure and \textit{ARMS2} locus. We did not find any interactions in the three way analysis including all these variables in the model.
Table 3.1. Association of estrogen exposure (via Oral Contraceptive Treatment [OCT] and Hormone Replacement Therapy [HRT]) and age related macular degeneration (AMD), adjusted for age and smoking. GEE analysis using all cases (grades 3-5, n=521) and all controls (grades 1-2, n=257) and examining only extreme grades 5(n=287) and 1(n=187)

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Table 3.2. Analysis of interaction between *MMP-2* SNP genotypes and Hormone Replacement Therapy (HRT) and Oral Contraceptive Treatment (OCT), adjusted for age and smoking. GEE analysis using all cases (grades 3-5, n=520) and all controls (grades 1-2, n=257).

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*  p<0.05
**  p<0.01
CHAPTER FOUR: Analysis of interaction between SNPs in the \textit{ESR1} and \textit{ESR2} genes and estrogen exposure in age-related macular degeneration

OVERVIEW

The purpose of the study was to assess the association of AMD with SNPs in \textit{ESR1} and \textit{ESR2} genes and their interactions with exogenous estrogen exposure and the \textit{ARMS2} locus. We studied 777 Caucasian, non-Hispanic females (520 AMD cases and 257 controls). We genotyped 30 tagSNPs within the \textit{ESR1} and \textit{ESR2} genes. We examined the association between AMD, \textit{ESR1} and \textit{ESR2} tagSNPs and estrogen exposure (Hormonal Replacement Therapy [HRT] or Oral Contraceptive Treatment [OCT]) using generalized estimating equations adjusted for the \textit{ARMS2} genotype, age and cigarette smoking.

One SNP in \textit{ESR1} was significantly associated with increased risk of AMD. We detected significant SNP/HRT exposure interactions for six \textit{ESR1} and three \textit{ESR2} tagSNPs. The stratified analysis for \textit{ESR1} and \textit{ESR2} demonstrated that the inverse association of AMD and HRT is found only in carriers of one or two copies of the risk allele. No significant three-way interactions between tagSNPs, HRT, and \textit{ARMS2} SNPs that interact with HRT were detected. Our data suggests that SNPs within the \textit{ESR1} gene are associated with increased risk for developing AMD and that the inverse association of AMD and HRT is dependent on SNP genotypes in \textit{ESR1} and \textit{ESR2}. 
BACKGROUND

Among the disease-modifiable factors described for AMD, endogenous and exogenous estrogen exposure has been extensively analyzed in different populations, and results point towards an overall beneficial effect on AMD. Exposure to estrogen resulting from both hormone replacement therapy (HRT) and oral contraceptive treatment (OCT) is frequently associated with a lower prevalence of AMD (Snow et al., 2002). The Eye disease case-control study group reported a reduced risk of exudative AMD for women that had received HRT as compared to controls (TEDCCSG, 1992). The Los Angeles Latino Eye Study and Beaver Dam Eye study also found a protective estrogen effect on AMD early lesions (Klein et al., 1994.; Frasser-Bell et al., 2006). The Salisbury study found a lower prevalence of large drusen associated with HRT (Freeman et al., 2005). The Rotterdam study reported a higher risk for AMD in women with early menopause (Vingerling et al., 1995) and the Blue Mountain Eye study reported that the years elapsed between menarche and menopause had an inverse relationship with risk for early AMD (Smith et al., 1997).

Estrogen exerts its multiple functions through estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Both ERα and ERβ proteins are present in the human retina. Interestingly they are localized on the RPE and choroid, suggesting that estrogen may play a role in the pathogenesis of AMD (Ogueta et al., 1999; Marin-Castano et al., 2003; Munaut et al., 2001). Schuit et al., (2005) recently reported that the 

\[PvuII_XbaI\] haplotype (Rs2234693, Rs9340799) in the \(ESR1\) gene is associated with lowered serum estradiol levels
in menopausal women and Boekhoorn et al., (2007) that it increases the risk for AMD.

Estrogen pathway activation seems to exert its beneficial role through different processes including ECM homeostasis and oxidative pathway regulation, both processes that seem to be intimately involved in the disease pathogenesis. We hypothesize that SNPs in ESR1 and ESR2 might increase the risk for AMD and might interact with estrogen exposure.

Finally, our group has recently reported a synergistic interaction between SNPs in the ARMS2 locus and HRT in AMD. However, the biology underlying this interaction still remains to be elucidated. Here we propose to analyze a possible interaction with SNPs in ESR1 and ESR2, estrogen exposure and ARMS2 locus as well.

RESULTS

After testing main effects of each tagSNP, only RS3020410 in ESR1 was significantly associated with increased risk of AMD in the additive model (p=0.03; grade 3-5 cases vs. grade 1-2 controls) (data not shown).

There were no significant interactions detected with OCT and ESR1 or ESR2 SNPs.

Significant SNP/HRT interactions were detected for six ESR1 and three ESR2 tagSNPs (Table 4. 1 and 4.2). Of these SNPs, only rs827423 and rs2234693 are in strong linkage disequilibrium in cases and controls (\(r^2=0.76\); Figure 4.1 and 4. 2).

The stratified analysis for ESR1 demonstrates that the inverse association of AMD and HRT is found only in carriers of at least one copy of the minor allele
at SNPs RS3020375, RS9341052, RS2813543, RS2234693, and RS827423 and in carriers of two copies of the major allele for RS2813544. The stratified analysis was significant in carriers of two copies of the major allele for RS154455 and RS3020449 in ESR2 (Table 4.3).

No significant three-way interactions between tagSNPs, HRT, and previously described ARMS2 SNPs that interact with HRT were detected.

However, we detected two significant associations with ARMS2 and HRT, using RS10490924 as an interacting variable for ARMS2: one in ESR1, RS3020410 (p-value=<0.01) and the other in ESR2, RS1256061 (p-value=0.05) (data not shown).

DISCUSSION

In the present study we demonstrated that the Rs3020410 SNP in ESR1 confers an increased risk for neovascular AMD when adjusting for age and smoking status in our cohort. These results may suggest that the possible beneficial effect of estrogen exposure might be counteracted by the presence of the risk allele. We also confirmed a protective effect for both OCT and HRT in AMD and established an interaction between both ESR1 and ESR2 and HRT, showing that the protective effect seen with HRT in AMD derives from the presence of certain genotypes.

It is widely accepted that estrogen has a protective effect on AMD. This beneficial effect is partially associated with decreased oxidative pathway activation. Estrogen pathway activation also possesses an antioxidant effect by inhibiting lipid peroxidation by promoting RPE survival after oxidative stress exposure and through its effect on iNOS activation that induces the synthesis of
NO (Akçay et al., 2000; Subiah et al., 1993; Yu et al., 2005). Estradiol (E2) seems to block the lipopolysaccharide (LPS) or IL-1 mediated iNOS activation (Vegeto et al., 2001; Beauregard et al., 2004). However, the opposite effect is true in naïve cells (Yu et al., 2003; Mershon et al., 2002), probably because it might exert different functions in normal or oxidative stress induced environments.

Estrogen is linked to ECM homeostasis and it regulates activity and expression of MMPs in RPE. Decreased functional estrogen levels, as seen in post menopausal women, is reported to be associated with decreased matrix metalloproteinase 2 (MMP-2) expression and activity which results in ECM deposition in the Bruch membrane (Cousins et al., 2003), since the target for MMP-2 is collagen type IV, present in the RPE basement membrane and Bruch membrane. This abnormal ECM deposit could represent the early changes seen in AMD.

Estrogen functions are exerted by the intranuclear receptors ERα and ERβ that are widely present in different tissues. Usually one receptor type is predominant in the tissue; in the eye several groups reported both receptors to be present (Ogueta et al., 1999; Marin-Castano et al., 2003; Munaut et al., 2001). ERα and ERβ might exert different or opposite functions in tissue where both are present. Cignarella et al., (2009), demonstrated a reduction in iNOS induction with ERα stimulation and an opposite effect with ERβ activation (Cignarella et al., 2007); ERβ can also inhibit the ERα dependent transcriptional activity (Hall et al., 1999; Lindberg et al., 2003).
In addition, the Pvull-XbaI haplotype contained within the \textit{ESR1} gene is associated with lower estrogen levels in postmenopausal women and increased risk for AMD (Schuit et al., 2001; Boekhoorn et al., 2007). RS2234693 T allele contained within the haplotype could be responsible for eliminating a transcription binding site that could in turn lead to decreased \textit{ESR1} mRNA levels, resulting in lower functional levels of estradiol that could markedly influence the disease pathogenesis.

Our findings are particularly interesting, especially since RS2234693 T allele decreases the protection conferred by HRT in our data set, following the same direction as previously described studies.

Because we have previously reported a synergistic interaction between HRT and the \textit{ARMS2} locus we also included in our analysis an interaction assessment for SNPs in the estrogen receptor, estrogen exposure and SNPs in the \textit{ARMS2} locus that were previously associated with HRT. No significant three way interactions, however, were found with SNPs in both \textit{ESR1} and 2, HRT and SNPs in \textit{ARMS2} locus.

In summary, our data suggests that SNPs in \textit{ESR1} may increase the risk of AMD and that the inverse association of AMD and HRT is dependent on SNP genotypes present in both \textit{ESR1} and \textit{ESR2}. This interaction is independent of the \textit{ARMS2} locus.
### CHAPTER TABLES

Table 4.1. Analysis of interaction between *ESR1* SNP genotypes and Hormone Replacement Therapy (HRT), adjusted for age and smoking. GEE analysis using all cases (grades 3-5, n=520) and all controls (grades 1-2, n=257).

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*  p<0.05  
** p<0.01
Table 4. 2. Analysis of interaction between ESR-2 SNP genotypes and Hormone Replacement Therapy (HRT), adjusted for age and smoking. GEE analysis using all cases (grades 3-5, n=520) and all controls (grades 1-2, n=257).

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<td>0.02*</td>
</tr>
<tr>
<td>14</td>
<td>ESR2</td>
<td>63785526</td>
<td>RS17766755</td>
<td>Intronic</td>
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<td>RS7154455</td>
<td>Intronic</td>
<td>0.04*</td>
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<td>RS17179740</td>
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<td>14</td>
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<td>63843145</td>
<td>RS3020449</td>
<td>Intronic</td>
<td>0.05*</td>
</tr>
</tbody>
</table>

* p<0.05  
** p<0.01
Table 4.3. Association between Hormone Replacement Therapy (HRT) and age related macular degeneration (AMD); stratified by genotype at *ERS1/ESR2* genes. GEE analysis containing all cases (grades 3-5, n=520) and all controls (grades 1-2, n=257).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Subgroup</th>
<th>p-value for HRT association</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS827423</td>
<td>TT</td>
<td>0.2</td>
<td>1.68</td>
<td>0.76, 3.71</td>
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<td></td>
<td>TC&amp;CC</td>
<td>&lt;0.01**</td>
<td>0.53</td>
<td>0.33, 0.85</td>
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<tr>
<td>RS2234693</td>
<td>TT</td>
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<td>1.8</td>
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<tr>
<td></td>
<td>TC&amp;CC</td>
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<td>0.28, 0.75</td>
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<tr>
<td>RS3020375</td>
<td>AA</td>
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<td>0.93</td>
<td>0.52, 1.66</td>
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<tr>
<td></td>
<td>AC&amp;CC</td>
<td>0.02*</td>
<td>0.52</td>
<td>0.30, 0.91</td>
</tr>
<tr>
<td>RS9341052</td>
<td>AA</td>
<td>0.48</td>
<td>0.86</td>
<td>0.56, 1.31</td>
</tr>
<tr>
<td></td>
<td>AG&amp;GG</td>
<td>&lt;0.01**</td>
<td>0.16</td>
<td>0.05, 0.59</td>
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<tr>
<td>RS2813543</td>
<td>GA&amp;AA</td>
<td>0.01*</td>
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<td>0.23, 0.85</td>
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<tr>
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<td>0.29, 0.84</td>
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<tr>
<td>RS2813544</td>
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<tr>
<td></td>
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<td>0.83</td>
<td>0.53, 1.29</td>
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<tr>
<td>RS7154455</td>
<td>GG</td>
<td>0.01*</td>
<td>0.48</td>
<td>0.26, 0.86</td>
</tr>
<tr>
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<td>GC&amp;CC</td>
<td>0.9</td>
<td>0.97</td>
<td>0.56, 1.67</td>
</tr>
<tr>
<td>RS3020449</td>
<td>TT</td>
<td>0.04*</td>
<td>0.49</td>
<td>0.25, 0.96</td>
</tr>
<tr>
<td></td>
<td>TC&amp;CC</td>
<td>0.57</td>
<td>0.86</td>
<td>0.53, 1.42</td>
</tr>
</tbody>
</table>

* p<0.05
** p<0.01
CONCLUSION

Age-related macular degeneration (AMD) is one of the leading causes of irreversible blindness in developed countries, and since population demographics in western countries indicate an increasingly aging population, AMD will become an even more important health concern in future years (Resnikoff et al., 2004; Friedman et al., 2004).

AMD is a neurodegenerative ocular disease that affects the macula and leads to irreversible loss of central vision. The disease is believed to be a continuum of early manifestations, such as drusen and pigment abnormalities, to the late stages with geographic atrophy and neovascular AMD (Johnson et al., 2004., Jager et al., 2008., Kliffen et al., 1997; Friedman et al., 2004; Klein et al., 1992).

Hypotheses for disease pathogenesis include oxidative stress, extracellular matrix homeostasis and abnormal inflammatory activation (Montezuma et al., 2007; Zarbin, 2004). The pathognomonic lesions of early AMD stages, drusen, are composed of elements from the alternative inflammatory pathway cascade (CFH, CFB/C2, C3) and components of the basement membrane among others. This suggests a disruption in extracellular matrix (ECM) homeostasis and inflammatory/immune balance (Cousins et al., 2003; Berglin et al., 2000; Kvanta et al., 2000).

Early twin and familial aggregation studies have established a strong genetic component for the disease (Meyers et al., 1995; Klein et al., 1994; Hammond et
In addition, epidemiological studies established the role of non-genetic risk factors, including environmental exposure, on AMD susceptibility. Estrogen exposure and smoking are the most influential environmental factors for AMD susceptibility. Smoking doubles the risk for AMD and estrogen decreases the risk by half (Klein et al., 2004; Tomany et al., 2004).

In recent years, several risk factor genes were discovered in replicated studies through regional linkage and association and genome-wide linkage and association scans including: CFH, ARMS2 locus, CFB/C2, C3, APOE (Klein et al., 2005; Edwards et al., 2005; Haines et al., Hageman, et al., 2005; Despriet et al., 2006; Chen et al., 2006; Gold et al., 2006; Richardson et al., 2008; Spencer et al., 2007; Jakobsdottir et al., 2005; Rivera et al., 2005; Kanda et al., 2007; Yates et al., 2007; Maller et al., 2007; Spencer et al., 2008; Klaver et al., 1998; Schmidt et al., 2000; Baird et al., 2004). Calculated Population Attributable Risk (PAR) values for the two most important genetic risk factors (CFH and ARMS2 locus) range between 20-50% depending on the population analyzed (Despriet et al., 2006; Schmidt et al., 2006; Xing et al., 2008; Tuo et al., 2008; Tam et al., 2008). However, only 60-70% of the overall disease attributable risk is explained by the combination of the most important genetic and environmental risk factors (Schmidt et al., 2006; Tam et al., 2008). The remaining effect is likely to correspond to smaller effects of multiple genes or to gene-environmental interactions between known and yet to be discovered candidate genes.
We undertook this research with the purpose of evaluating two new gene pathways activated through environmental exposures that could play a role in the AMD pathogenesis and explain part of the remaining disease risk. We evaluated associations between selected candidate genes (NOS2A, ESR1 & 2 and MMP-2 genes) and AMD, the interactions between these genes and environmental factors (estrogen exposure and smoking) and gene-gene interactions with one of the strongest genetic risk factors, the ARMS2 locus, for which the function remains unknown.

We selected smoking and estrogen exposure as our environmental risk factors because of the extensive body of literature supporting their role in AMD susceptibility. Both these factors are linked to oxidative pathway activation and ECM homeostasis through their interactions with the NOS2A and metallo-matrix proteinase (MMP) genes (Beauregard et al., 2004, Yu et al., 2003; Hoyt et al., 2003; Mazzio et al., 2005; Mershon et al., 2002; Cousins et al., 2003; Ruta et al., 2009). In addition, estrogen exerts its functions through its receptors, coded by the ESR1 and 2 genes.

We demonstrated that gene variants of NOS2A increase the risk for AMD and that other gene variants act synergistically with smoking to increase the risk of AMD 35-fold.

We also demonstrated that SNPs in the ESR1 gene increase the risk for AMD and that the protective effect seen by estrogen exposure on AMD is conferred by genotypes present in ESR-1 and 2 genes.
Our data does not support, however, an association of MMP-2 gene variants and increased risk for AMD or an interaction with the gene and estrogen exposure.

In summary, we demonstrate two novel gene pathways are associated with AMD. More importantly, we also show that certain gene variants in the candidate genes selected can significantly modify the effect of environmental exposure on AMD. These results are particularly interesting and important since they suggest that while exposure to environmental factors alone can modify the risk of AMD; both extrinsic environmental and intrinsic genetic components are required to manifest the full environmental effect on AMD. Our findings also partially explain the lack of consensus across studies regarding the extent of protection or risk conferred by either estrogen exposure or smoking, because these studies did not account for genetic variations in relation to such environmental factors.

Our data have potential applications in current medical practice. For instance, individuals identified to be at greater genetic risk should be counseled about their specific options to reduce the likelihood of developing the disease by modifying their exposure to environmental risk factors. As we demonstrated, the effects of only certain gene variants are protective in the presence of estrogen, and it may not be necessary to administer exogenous estrogen to individuals without these variants. On the other hand, certain individuals with risk factors such as smoking that also have disease risk-increasing gene variants could be selected, and specific interventions can be designed to help them refrain from smoking. Consequently, the implications of these findings on public health include the
possibility of individually-specialized interventions and treatments: once the
genetic risk for certain diseases is fully understood, the modification of individual
risk factors become the next step, with the tailoring of diagnostic procedures and
treatment for each patient to improve care delivery and clinical outcomes.

FUTURE DIRECTIONS

Next generation sequencing (NGS)

With the rapidly progressing field of genome sequencing newer techniques
are becoming available for high-throughput studies at lower cost. These large –
scale sequencing techniques have started to revolutionize the field because they
have enabled direct exploration of causative variants of the disease and not only
allele markers.

We will continue research in AMD utilizing these techniques to evaluate
specific pedigrees that do not posses any of the current genetic risk factors and
have, however, developed the disease phenotype. In AMD, as well as in other
diseases much of the attention has been centered on the Common Disease
Common Variant (CDCV) hypothesis evaluating common alleles and their role in
the disease. NGS will allow us to search for the rare variants and to assess their
role in AMD. This analysis will shed light into additional genes/gene variants that
might be involved in the disease pathogenesis and explain more of the remaining
genetic disease risk.
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Green WR. Histopathology of age-related macular degeneration. Mol Vis. 1999 Nov 3;5:27.


Hall JM, McDonnell DP. The estrogen receptor beta isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology. 1999 Dec; 140(12): 5566-78.


APPENDIX I: Pharmacogenomics of ophthalmic β-blocker

OVERVIEW

It is biologically plausible that β-AR polymorphisms may correlate to some degree with level of IOP, development and/or clinical expression of primary open angle glaucoma (POAG) and allied diseases, and pharmacogenomic effects (efficacy and toxicity) of topical beta blockers. Given the significant interpatient variability in "safe" levels of intraocular pressure (IOP), expression of POAG, and response to topical beta antagonists, a better understanding of these relationships appears clinically relevant.

BACKGROUND

Primary open-angle glaucoma (POAG) remains a major cause of worldwide preventable blindness. POAG is caused in part by chronic elevation of intraocular pressure (IOP), leading to characteristic and progressive damage to the optic nerve head and visual field loss (Wolfs et al., 2000). POAG and allied diseases are notable for significant interpatient variability. For example, some patients with elevated IOP do not develop optic nerve damage or visual field loss; these patients are diagnosed with ocular hypertension. Similarly, some patients develop typical nerve damage at apparently normal IOP; these patients are diagnosed with normal-tension glaucoma (NTG).

IOP is at least partly mediated by the beta-adrenergic system. In the iris and ciliary body, beta-2 adrenergic receptors (β<sub>2</sub>-ARs) predominate over beta-1 adrenergic receptors (β<sub>1</sub>-ARs) by about a 9:1 ratio (Wax et al., 1987). β<sub>2</sub>-ARs are also found in the trabecular meshwork (Wax et al., 1989) and optic nerve
(Mantyh et al., 1995; Feher et al., 2005). Therefore, it is reasonable to suspect that beta-adrenergic receptor (β–AR) polymorphisms may have some relation to the development of POAG and allied diseases.

Topical beta blockers are a major class of medication used to treat POAG and ocular hypertension. Their proposed mechanism of action is a decrease in the rate of aqueous fluid formation by the ciliary body (Yablonski et al., 1978; Coakes et al., 1978). In the US, there is currently one FDA-approved selective beta-1 antagonist and several non-selective beta-1 and beta-2 antagonists. The selective agent, betaxolol hydrochloride 0.5% (Betoptic, Alcon Laboratories, Ft. Worth, TX) is clinically less effective than the non-selective agent timolol maleate 0.5% in patients with POAG (Allen et al., 1986). Furthermore, there is a variable and unpredictable rate of non-responders to all topical beta-blockers. The non-response rate of the current formulation, Betoptic S 0.25%, a suspension, has not been directly evaluated but it is assumed to be similar to the previous formulation, Betoptic 0.5%. It is possible that some of this interpatient variability is related to individual differences in pharmacokinetics. However, it is also reasonable to suspect that β–AR polymorphisms may have some relation to the interpatient variability in response to topical beta blockers.

This chapter briefly summarizes the current literature on the pharmacogenomics of the beta-adrenergic system as they relate to the eye.

GENETIC INFORMATION AND IN VITRO MODELS

The β–AR belongs to the family of G-protein coupled receptors and is a seven trans-membrane protein located on the cell surface (Strader et al., 1994).
There are three subtypes of the β–AR: beta-1, beta-2 and beta-3. Only the beta-1 and beta-2 receptors are clinically relevant for ophthalmology.

The β1–AR gene, \textit{ADRB1}, is located on chromosome 10q24-26 and the β2–AR gene, \textit{ADRB2}, is located on chromosome 5q31–32 (Hoehe et al., 1995; Ligget et al., 2000). \textit{ADRB1} and \textit{ADRB2} are intronless genes encoding proteins of 413 and 477 amino acids, respectively (Liggett et al., 2000; Small et al., 2003). Overall, 54% of their amino acid sequence is shared (Frielle et al., 1989). The β1 and β2–AR were initially characterized based on their different affinities for catecholamines (epinephrine and norepinephrine). They were also shown to have differing agonist-promoted adenylate cyclase (AC) activities (Frielle et al., 1989; Green et al., 1992; Rousseau et al., 1996).

The beta-adrenergic pathway uses catecholamines as the extracellular signal (primary messenger). The β–AR interacts with a G-protein comprised of α, β, and γ subunits (transducer); AC (primary effector); cyclic adenosine monophosphate, (c-AMP) (secondary messenger); and c-AMP dependent protein kinases (secondary effectors). Upon stimulation by catecholamines, a conformational change occurs in the receptor that induces binding to the G-protein which allows the exchange of GDP to GTP, resulting in a dissociation of the α subunit, that in turn activates the adenylate cyclase. AC then induces the production of c-AMP, which leads to the activation of c-AMP dependent protein kinases, which induce a series of intracellular signaling cascades (Hein et al., 1995; Neves et al., 2002) (Figure 5.1).
Numerous SNPs have been associated with the $ADRB1$ region. The two most common non-synonymous coding polymorphisms are associated with the N- and C-terminal regions and are located at the nucleotide position 145 and 1165 (Liggett et al., 2000; Small et al., 2003) (Figure 5.2 and Table 5.1).

At nucleotide 145, there is an A→G substitution that results in a serine→glycine (Ser→Gly) substitution at the amino acid located at position 49. This Gly49 substitution leads to an enhanced downregulation of the receptor after prolonged exposure to the agonist. This substitution is associated with an altered N-glycosylation pattern, but it shows no difference in ligand binding affinity or in basal or agonist induced AC activity (Rathz et al., 2002). The Gly 49 phenotype demonstrates higher basal and agonist-promoted AC activities and ligand affinities (Levin et al., 2002).

At nucleotide 1165, there is a C→G substitution that results in an arginine→glycine (Arg→Gly) substitution at the amino acid located at position 389. This exchange takes place in the G-coupling domain of the $\beta_1$-AR. The Arg389 variant has increased basal and maximal agonist-stimulated levels of AC activities (Mason et al., 1999), as well as agonist-promoted desensitization (Rathz et al., 2003).

$ADRB2$ has multiple SNPs located in the coding and in the promoter regions. In addition, the 5’ UTR region of $ADRB2$ codes for a leader cistron, a 19-amino-acid peptide that is involved in the regulation of gene expression (Parola et al., 1994). The best-characterized non-synonymous polymorphisms are located in the region corresponding to the amino acid residues 16, 27, 164 and
19 from the ADR protein and 5’ leader cistron, respectively (Figure 5.2 and Table 5.1).

At nucleotide 46, there is a G→A substitution that results in a glycine → arginine (Gly→Arg) substitution at the amino acid located at position 16. The Gly16 variant exhibits a relatively enhanced isoproterenol-mediated down-regulation of the β2-AR in different culture systems (Green et al., 1994; Green et al., 1995).

At nucleotide 79, there is a C→G substitution that results in a glutamine→glutamic acid (Gln→Glu) substitution at the amino acid located at position 27. The Glu27 variant results in absent down-regulation of the β2-AR, while the combination of the Gly16 and Glu27 variants exhibits a relatively enhanced isoproterenol-mediated down-regulation of the β2-AR (Green et al., 1994; Green et al., 1995).

At nucleotide 491, there is a C→T substitution that results in a threonine→isoleucine (Thr→Ile) substitution at the amino acid located at position 164, in the ligand-binding pocket of the β2-AR. The Ile164 variant is associated with decreased receptor coupling to the stimulatory Gs protein, resulting in lower basal and agonist-promoted AC activities in vitro (Green et al., 1993, Green et al., 1994) and in vivo (Turki et al., 1996). The Ile164 variant also results in reduced desensitization after agonist stimulation and decreased affinity for certain ligands (both agonists and antagonists) (Green et al., 1993, Rathz et al., 2003).
At nucleotide -47, there is a T→C substitution that results in a cysteine→arginine (Cys→Arg) substitution at the amino acid located at position 19. The Cys19 variant increases β2-AR density in a cellular culture system. Airway smooth muscle cells containing the Cys19 polymorphism express higher protein levels, despite similar mRNA levels, suggesting as translational regulation of the protein (McGraw et al., 1998).

**PHARMACOGENOMIC STUDIES: SYSTEMIC HYPERTENSION**

Conflicting results have been reported with respect to β1-AR polymorphisms and systemic hypertension. In different studies, both the Arg389 polymorphism (Bengtsson et al., 2001) and the Gly389 polymorphism (McCaffery et al., 2002; Liang et al., 2004) have been associated with increased blood pressure and/or increased risk of systemic hypertension. Other studies have found no association between these polymorphisms and the risk of systemic hypertension (Filigheddu et al., 2004; Iacoviello et al., 2006; Gjesing et al., 2007).

There is some evidence of an association between β2-AR polymorphisms and blood pressure. The Gly16 polymorphism is associated with increased blood pressure and/or an increased risk of systemic hypertension in multiple studies (Kotanko et al., 1997; Gratze et al., 1999; Bray et al., 2000; Ranade et al., 2001; Pereira et al., 2003; Filigheddu et al., 2004). Other associations have been found with the Arg 16 polymorphism (Timmerman et al., 1998; Busjahn et al., 2000; Bengtsson et al., 2001; McCaffery et al., 2002; Rana et al., 2007), the Gln27 polymorphism (Bray et al., 2000; Binder et al., 2006), and the Arg16Gly+Gln27Gln haplotype (Wallerstedt et al., 2005). However, several other
studies have found no association between β2-AR polymorphisms and systemic blood pressure (Candy et al., 2000; Herrmann et al., 2000; Jia et al., 2000; Xie et al., 2000; Kato et al., 2001; Lin et al., 2001; Tomaszewski et al., 2002; Liang et al., 2004; Galletti et al., 2004; Iacoviello et al., 2006; Gjesing et al., 2007).

β1-AR polymorphisms may correlate with the efficacy of systemic beta-antagonists. The Arg389 polymorphism is correlated with increased magnitude of response to systemic atenolol (Sofowora et al., 2003) and metoprolol (Johnson et al., 2003; Liu et al., 2003; Liu et al., 2006). Other studies, however, have found no association with various agents (O’Shaughnessy et al., 2000; Filigheddu et al., 2004; Karlsson et al., 2004; Beitelshees et al., 2006).

Thus far, no evidence has been found of a correlation between β2-AR polymorphisms and the clinical efficacy of systemic beta blockers (Jia et al., 2000; Filigheddu et al., 2004).

**PHARMACOGENOMIC STUDIES: GLAUCOMA AND OCULAR HYPERTENSION**

The current literature has focused on β-AR polymorphisms in three ophthalmologic contexts (Table 5.2 and Table 5.3):

- IOP of normal volunteers;
- Presence or expression of POAG and allied diseases;
- Pharmacogenomics of topical beta blockers.

There is some evidence of a relationship between β-AR polymorphisms and IOP in normal volunteers. In normal volunteers, Arg389 homozygotes have a higher baseline IOP than Gly389 carriers (Schwartz et al., 2005). Normal
volunteers with the Gly16Gly variant of the β2–AR demonstrate a greater and more prolonged decrease in IOP following exercise than do normal volunteers with the Arg16Gly variant (Güngör et al., 2002).

Likewise, there is some evidence of a relationship between β–AR polymorphisms and the clinical expression of various types of glaucoma. There are no significant correlations between the Gly16, Glu27, or Ile164 polymorphisms in the β2–AR and the diagnosis of POAG in several series from North America, Europe, and Asia (Güngör et al., 2003; Inagaki et al., 2006; McLaren et al., 2007). However, among patients with POAG and NTG, there do appear to be some statistically significant correlations between β–AR polymorphisms and clinical disease expression.

Among Japanese patients with POAG, individuals with the Gly16 allele of the β2–AR were younger at diagnosis than individuals without the Gly16 allele. In the same population, POAG patients with the Glu27 allele of the β2–AR had higher IOP at diagnosis than individuals without the Glu27 allele (Inagaki et al., 2006). In Japanese patients with NTG, the Arg389 allele of the β1–AR is significantly more common, and the Gly389 allele is significantly less common, than among controls, suggesting a possible protective effect of the Gly389 variant against NTG in this population (Inagaki et al., 2006).

The research with perhaps the most direct clinical applications relates to the pharmacogenomic studies of topical beta blockers, both in terms of efficacy and toxicity (McLaren et al., 2003). Two reports have studied the clinical efficacy of topical beta blockers in normal volunteers. Among normal volunteers treated
for six weeks with the selective β1–antagonist betaxolol, individuals homozygous for the Arg389 variant of the β1–AR demonstrate a greater magnitude of response than do Gly389 carriers (Schwartz et al., 2005). Among normal volunteers treated with one drop of the nonselective antagonist timolol, three β2–AR receptor haplotypes do not correlate with magnitude of response of timolol (Fuchsjager-Marl et al., 2005).
### Table 5.1. β-adrenergic receptor polymorphisms location and functional consequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI SNP</th>
<th>Base substitution</th>
<th>Amino acid change</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRB2</td>
<td>rs1042711</td>
<td>47T/C</td>
<td>Cys19Arg</td>
<td>Cys19 increases receptor expression</td>
<td>Mc Graw et al, 1998</td>
</tr>
<tr>
<td></td>
<td>rs1042714</td>
<td>79C/G</td>
<td>Gln27Glu</td>
<td>Glu27 exhibits reduced downregulation of the receptor</td>
<td>Green et al, 1994, Green et al, 1995</td>
</tr>
<tr>
<td></td>
<td>rs1800888</td>
<td>491C/T</td>
<td>Thr164Ile</td>
<td>Ile164 decreases coupling to Gs and AC activity and agonist-promoted desensitization in vitro</td>
<td>Green et al, 1993, Green et al 1994a, Rathz et al, 2003</td>
</tr>
</tbody>
</table>

**ADRB1** = β-1 adrenergic receptor; **ADRB2** = β-2 adrenergic receptor; **AC** = adenylate cyclase enzyme; **Gs** = G stimulatory protein; **SNP** = single nucleotide polymorphism; **NCBI** = National Center for Biotechnology Information.

### Table 5.2. Summary of findings in ocular β-adrenergic receptor polymorphisms association studies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphisms</th>
<th>Sample</th>
<th>Measurements</th>
<th>Findings</th>
<th>P-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRB2</td>
<td>Arg16Gly, Gln27Glu, Thr164Ile</td>
<td>19 healthy volunteers</td>
<td>Δ IOP induced by exercise</td>
<td>Greater and more prolonged IOP reduction in Gly16 homozygous</td>
<td>&lt; 0.02</td>
<td>Güngör et al, 2002</td>
</tr>
<tr>
<td>ADRB2</td>
<td>Arg16Gly, Gln27Glu, Thr164Ile</td>
<td>30 PCG, 105 POAG, 92 healthy volunteers</td>
<td>Association between genotype and disease phenotype</td>
<td>No association was found</td>
<td></td>
<td>Güngör et al, 2003</td>
</tr>
<tr>
<td>ADRB1</td>
<td>Ser49Gly, Arg389Gly</td>
<td>294 NTG, 211 POAG, 240 healthy volunteers</td>
<td>Association between genotype and disease phenotype</td>
<td>Different allele and genotype frequencies between NTG and controls in Arg389Gly variant Gly16 carriers were younger at diagnosis Glu27 carriers had higher baseline IOP</td>
<td>0.004 and 0.006</td>
<td>Inagaki et al, 2006</td>
</tr>
<tr>
<td>ADRB2</td>
<td>Arg16Gly, Gln27Glu</td>
<td>299 POAG, 284 healthy volunteers</td>
<td>Association between genotype and phenotype stratified by ancestry</td>
<td>No association was found</td>
<td></td>
<td>McLaren et al, 2007</td>
</tr>
</tbody>
</table>

**ADRB1** = β-1 adrenergic receptor; **ADRB2** = β-2 adrenergic receptor; **PCG** = primary congenital glaucoma; **POAG** = primary open angle glaucoma; **NTG** = normal tension glaucoma; **IOP** = intraocular pressure; **Δ IOP** = variation.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphisms</th>
<th>Sample</th>
<th>Drug Treatment</th>
<th>Measurements</th>
<th>Findings</th>
<th>P-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRB1</td>
<td>Ser49Gly Arg389Gly</td>
<td>19 Glaucoma patients, 18 Healthy Volunteers</td>
<td>0.5% aqueous and 0.1% hydrogel of timolol for 2-4 weeks</td>
<td>Head tilt up and and maximum exercise tests evaluation</td>
<td>Higher SAP and and DAP upon head tilt up in Ser49 carriers.</td>
<td>0.03 and &lt; 0.01</td>
<td>Nieminen et al, 2005</td>
</tr>
<tr>
<td>ADRB1</td>
<td>Ser49Gly Arg389Gly</td>
<td>48 Healthy Volunteers</td>
<td>Betaxolol hydrochloride 0.25% for 6 weeks</td>
<td>Δ IOP</td>
<td>Higher baseline IOP and increased Δ IOP after tx. in Arg389 homozygous</td>
<td>0.03</td>
<td>Schwartz et al, 2005</td>
</tr>
<tr>
<td>ADRB2</td>
<td>Arg16Gly Gln27Glu</td>
<td>270 healthy volunteers</td>
<td>Timolol for 8 hs.</td>
<td>Δ IOP</td>
<td>No association found</td>
<td></td>
<td>Fuchsberger-Mayr et al, 2005</td>
</tr>
</tbody>
</table>

ADR1 = β₁ adrenergic receptor; ADRB2 = β₂ adrenergic receptor; IOP = intraocular pressure; Δ IOP = variation in intraocular pressure; SAP = systolic arterial pressure; DAP = diastolic arterial pressure; tx = treatment
Fig 5. 1. Schematic representation of the β-adrenergic pathway. Upon agonist stimulation of the β-AR/G-protein system, the adenylate cyclase (AC) enzyme gets activated inducing the production of c-AMP that in turn activates protein kinase A (PKA) leading to an intracellular signaling cascade.

PKA-C = (catalytic subunit); PKA-R = (regulatory subunit); ATP = adenosine triphosphate; c-AMP = cyclic adenosine monophosphate; β-AR = β-adrenergic receptor; Gs (α, β and γ) = G stimulatory protein with α, β and γ subunits
Fig 5. 2. Schematic representation on the β-adrenergic receptor protein with its seven trans-membrane domains and the most relevant SNPs associated with the β1 (orange) and β2 (blue) receptor.
APPENDIX II: Understanding the genetic and environmental mechanisms that influence the pathogenesis of glaucoma.

OVERVIEW

Glaucoma comprises a complex group of disorders characterized by a progressive and irreversible loss of retinal ganglion cells accompanied by subsequent visual impairment. It is one of the leading causes of blindness in the world and constitutes a significant public health concern. Its most common subtype in North America is Primary Open Angle Glaucoma (POAG). The disease pathogenesis generally involves an increase in intraocular pressure (IOP) due to an abnormally high resistance to the drainage of aqueous humor, leading to retinal ganglion cell death.

It has been long determined that the disease has a genetic component based on epidemiological and association genetic studies, and a positive family history has been therefore regarded as a significant risk factor.

Several loci have been associated with the disease, but only few causative genes have been yet identified. Although the disease can be inherited following an autosomal dominant or recessive Mendelian pattern, the vast majority of cases seem to correspond to a complex pattern of inheritance where environmental influence might play an important role in the phenotype expression.
We hypothesize that an analysis of the association between genetic and environmental factors with POAG might lead to the discovery of possible mechanisms that could be involved in the pathogenesis of glaucoma.

**BACKGROUND**

Glaucoma comprises a complex group of disorders characterized by a progressive and irreversible loss of retinal ganglion cells accompanied by subsequent visual impairment, (Wolf et al., 2000; Quigley and Green, 1979; Quigley et al., 1983). It affects over 70 million people worldwide and is one of the leading causes of blindness in the world. It is frequently associated with an increased intraocular pressure (IOP), but there are cases that present with IOP within normal ranges (Drance et al., 2001; Anderson et al., 2003; Anderson, 2003). The nomenclature used in defining glaucoma is based on etiology and anatomy. The disease can be classified into closed angle, open angle and congenital glaucoma. Closed angle glaucoma is associated with a decrease in the outflow facility due to an anatomical obstruction of the angle of the anterior chamber, whereas open angle glaucoma is associated with a drainage obstruction without any anatomical blockade (Anderson 2003). Congenital glaucoma often ensues as a result of developmental abnormalities of the anatomical structures of the eye.

Glaucoma resulting from the presence of other ocular conditions is termed secondary glaucoma, and if it is of unknown etiology, primary glaucoma (Anderson, 2003). The most common subtype in North America is the primary open angle glaucoma (POAG) which accounts for approximately half of all cases.
(Tielsch et al., 1991). Classically, POAG was associated with the triad of increased IOP (> 21 mmHg), optic nerve atrophy and loss of peripheral field vision (Hoskins et al., 1982), but this concept has evolved to currently consider high IOP as an important risk factor but not a disease-defining criterion.

The prevalence of the disease can range between 0.7- 5.6% in the general population depending on the ethnic group studied (Quinley, 1996). Epidemiological studies have identified certain populations as being at an increased risk of developing the disease (Sung et al., 2006). For example, in the Afro-Caribbean population the prevalence of the disease is 4 to 6 times higher than in the Caucasian population (Tielsch et al., 1991; Leske et al., 1994).

Additionally, the prevalence of the disease is 7–10 times higher in an individual with a first degree relative with glaucoma compared with the general population (Wilson et al., 1987; Shin et al., 1977; Perkins et al., 1974; Wolf et al., 1998; Sung et al., 2006).

Studies involving monozygotic twins have also shown a high degree of concordance of the Glaucoma among siblings, which argues strongly for a genetic component of the disease (Goldschmidt, 1973; Teikari 1987; Stöilova et al., 1996; Gottfredsotti et al., 1999).

Several genes showing a Mendelian pattern of inheritance have been associated with the disease; however mutations in these genes account for a small fraction of the total glaucoma population. The vast majority of cases seem to correspond to a complex multi-gene pattern of inheritance, the same as
observed for diseases such as in hypertension (Perkins et al., 1974; Shin et al., 1977; Wilson et al., 1987; Wiggs et al., 1995).

Presently, there is no cure for this disease, and the medical and surgical therapies are palliative at best, aimed at slowing the natural progression of the disease by reducing the IOP. Current medical therapy includes beta blockers and prostaglandin analogs that target the beta adrenergic receptors (ADR) and prostaglandin F2α receptors (PTGFR), resulting in a decreased aqueous fluid production and increased outflow drainage, respectively.

**Candidate Gene selection**

The *IL-1* gene family and interacting genes *ADR* and *PTGFR* were selected for our study because their important function in the aqueous fluid dynamics. IL-1 induction was observed to increase ocular outflow in an organ culture system; this action would facilitate acutely the drainage of the aqueous fluid (Kee et al., 1997). Also, the upregulation of IL-1 and ELAM-1 in glaucomatous tissue made these proteins interesting targets since they resulted in an increased protection against oxidative stress (Wang et al., 2001). IL-1 stimulation activates a series of intracellular signaling pathways, one of which involves ELAM-1, the MAPK pathway, and could mediate the effects of IL-1 by functioning as an activator of that pathway.

IL-1 is involved in PTGFR dynamics; it promotes synthesis of the PTGFR and induces the expression of COX-2, generating the ligand of the receptor (Tsuzaki et al., 2003; Zaragoza et al., 2006). Further activation of PTGFR induces expression of IL-1 in a positive feedback loop. Current glaucoma therapy
targets this receptor, stimulating it with prostaglandin analogs, promoting an increase in aqueous outflow.

ELAM-1 is a direct target of one of the downstream effectors of the beta-1 and 2 adrenergic receptor pathways. These receptors activate adenyl cyclase II that produces cAMP, an intracellular signal that activates a series of phosphorylating enzymes and downregulates the production of ELAM-1 (Ghersa et al., 1994; Zhang et al., 2000). Since adenyl cyclase II has been previously described as being downregulated in glaucomatous tissues, this observation provides an additional mechanism for ELAM-1 upregulation in those tissues, besides the known IL-1 induction (Ghersa et al., 1994; Zhang et al., 2000). Blocking the action of the beta adrenergic receptors, as in the treatment of glaucoma, should increase the levels of ELAM-1 even further, activating the common pathway shared with IL-1 (Fig 5.3).

The purpose of our study was to assess the association between SNPs within the chosen candidate genes (IL-1 gene family, ADR and PTGFR) and an increase risk for POAG in our Caucasian population.

**CHAPTER MATERIAL AND METHODS**

**Patient recruitment:** Patients were recruited as they came for follow-up visits to the institutions participating in the study. Study participants signed an Institutional Review Board (IRB) approved consent form. Glaucoma specialists selected POAG patients and controls following the inclusion and exclusion criteria.
**Inclusion and exclusion criteria:** Only POAG patients and normal controls over 40 were recruited for this study. Participating patients of the control group had a normal eye exam without signs or risk factors for glaucoma; whereas the POAG patient participants had documented evidence of the disease assessed by an altered cup to disc ratio, an abnormal visual field test on more than one occasion and elevated intraocular pressure.

For the *IL-1* analysis, we enrolled and genotyped 156 patients with POAG and 180 normal controls. The characteristics of the subjects are shown in Table 4.4. The control group was significantly younger than the POAG group (p<0.001, t-test), and there was no difference between the control and POAG groups in terms of gender.

Since polymorphisms may be distributed differently in different populations, further analyses were stratified by race and ethnicity. In the largest group in our cohort (Caucasian not of Hispanic or Latino ethnicity), there were 132 control subjects with a mean age of 64 + 13 years and 72 POAG patients with a mean age of 73 + 11 years. There was also no difference in this sub-cohort between the control and POAG groups in terms of gender (p=0.23, Fisher’s exact test, Table 5.5).

For the *PTGFR* gene analysis, 188 Caucasian subjects (66 POAG and 121 controls), participated in the study.

For analysis of the *ADRB1 & 2*, 189 Caucasian subjects were enrolled and genotyped (63 POAG and 121 controls).
Sample gathering: Capacitated personnel obtained the blood samples from peripheral venopuncture and handled them following the established rules of confidentiality. Demographic information was gathered from the patient’s chart and attached to the blood samples.

DNA extraction: DNA was obtained from blood cells using the PUREGENE™ DNA purification system by GENTRA SYSTEM, following the manufacturer’s protocol. DNA samples were stored at –20 °C to ensure preservation quality.

Genotyping and statistical analysis: SNPs were assessed using a Polymerase Chain Reaction (PCR) amplification and restriction enzyme (RE) digestion for the IL-1 gene family and Sequenom assay for the ADRB1 & 2 and PTGFR genes. The data generated was evaluated using chi-square and logistic regression analysis, comparing the POAG population with the control population.

For the IL-1 analysis, we selected three polymorphisms within the IL-1 gene from the two variants α and β for analysis: -889C>T located in the promoter region of the IL1A gene; -551 C>T and +3953C>T located in the promoter region and exon 5 of the IL1B gene, respectively (Fig 4.4). These SNPs are implicated in altered IL-1 expression. For example: the IL1A (889 C>T) SNP increases IL-1α secretion in vitro, whereas the IL1B (-511C>T and +3953 C>T) are associated with an increase in IL-1β secretion in vivo (Pociot et al., 1992; Dominici et al., 2002; Hall et al., 2004; Maury et al., 2004).
The *IL-1* SNPs were examined by PCR amplification and RE treatment to differentiate the wild type versus the variant allele. The genotypes were also confirmed by sequence analysis.

The primers and conditions determined to generate the PCR products are given in Table 4.6. Statistical analysis used chi-square and logistic regression in the three selected SNPs.

The PCR product generated for the *IL1A* (-889 C/T) SNP is 150 base pairs (bps) in length. The RE (Ncol) recognizes and digests the PCR SNP products containing the Ncol recognition sequence CCATGG, generating two fragments of 100 and 50 bps. Therefore, digestion patterns identify both homozygous and heterozygous individuals. Homozygous individuals with the C allele would generate two bands of 100 and 50 bps, whereas homozygous individuals with the T allele would generate a single band of 150 bps. The heterozygous individual with both allele types would generate three bands of 150, 100 and 50 bps (Figure 5.5 and Picture 1).

The *IL1B* (-511 C/T) amplified SNP product is 304 bps and can be recognized and digested by the RE (Bsu36I), producing two bands of 190 and 114 bps when the sequence CCTNAGG is present. As stated above, heterozygous and homozygous individuals would be identified based on digestion patterns. Homozygous individuals with the T allele would generate two bands of 190 and 114 bps, whereas homozygous individuals with the C allele would generate a single band of 304 bps. The heterozygous individual with both allele types would generate all three fragment sizes (Figure 5.6, Picture 2).
The \textit{IL1B} (+3953 C/T) amplified SNP product contains 249 bps and can be recognized and cut by the RE (Taq I), producing two bands of 135 and 114 bps when the sequence TCGA is present. (Figure 4. 7 and Picture 3) show the homozygous and heterozygous digestion patterns generated. Homozygous individuals with the C allele would generate two bands of 135 and 114 bps, whereas homozygous individuals with the T allele would generate a single band of 294 bps. The heterozygous individual with both allele types would generate all three fragment sizes.

Digestion data generated has been supported by sequencing randomly chosen PCR products confirming the results obtained using this technique (Figure 5.8).

\textbf{For the PTGFR gene analysis}, we genetically analyzed for thirteen SNPs: Rs3766355 G/T, Rs3766354 C/T, Rs3766353, Rs1073611 C/T, Rs1073610 C/T, Rs12093097 C/T, Rs3753380 A/G, Rs3766331 C/T, Rs12731181 A/G, Rs898 A/C, Rs12094298 A/C, Rs12082394 A/G and Rs3766351A/G (Figure 5.9).

We performed statistical analysis using chi-square and logistic regression on the three SNPs (rs3766355, rs3753880, rs3766354) that were reported to alter the receptor function and to influence the IOP lowering response to prostaglandin analogs.

\textbf{For the genes ADRB1 & 2 analyses}, 13 well characterized SNPs in the \textit{ADRB1} & 2 families that alter the receptor properties (Small et al., 2003) were selected. For the \textit{ADRB1} gene two SNPs were analyzed: +145A/G (Ser49Gly)
SNP and +1165G/C (Arg389Gly) SNP. For the ADRB2, eleven SNPs were analyzed:-1429T/A, -1343G/A, -1023G/A, -654G/A, -468C/G, -367T/C, -47T/C, -20C/T, +46G/A (Arg16Gly), +79C/G (Gln27Glu), +491C/T (Thr164Ile). Five corresponded to coding region of the receptor (2 from ADRB1 and 3 from ADRB2), whereas the remaining 8 are located in the promoter region of the ADRB2, potentially influencing promoter activity. The resulting data was analyzed using chi-square and logistic regression.

RESULTS

Analysis of polymorphisms in IL-1 gene family (IL1A, IL1B)

IL1A (-889T). The frequency of the IL1A (-889T) allele was higher in the control group than in the POAG group but was not statistically significant (36% versus 28%, p=0.18; Table 4.7). Homozygosity for the IL1A (-889T) allele (TT genotype) also showed no statistical significance (age-adjusted odds ratio (95% CI) = 1.82 (1.12, 2.96), p=0.23; Table 5.8).

IL1A (-889T) shows no preferential association with normal or POAG subjects.

IL1B (-511T): The frequency of the IL1B (-511T) allele was the same in normal controls and in POAG patients (33% p=0.96; Table 4.7). Homozygosity for the IL1B (-511T) allele (TT genotype) was also similar (age-adjusted odds ratio (95% CI) = 0.99 (0.63, 1.57), p=0.98; Table 4.8). IL1B (-511T) has the same frequency in normal and POAG subjects.

IL1B (+3953T): There were no statistically significant differences between the POAG and the control groups in terms of allele, genotype, or allele carriage
frequencies at the *IL1B* (+3953C/T) locus (Tables 4-5). *IL1B* (+3953) shows no preferential association with normal or POAG subjects.

The results were similar in the Caucasian Hispanic and African American populations, showing no statistically significant differences between the POAG and the control groups in terms of allele, genotype, or allele carriage frequencies at the *IL1B* (-511C/T), *IL1B* (+3953C/T) and *IL1A* (-889C/T) loci (data not shown).

**Analysis of polymorphisms in PTGFR gene**

In the *PTGFR* gene, for the rs3766355 G/T SNP, the genotype GG was present in 73% of the POAG patients and 71% of the controls; genotype GT in 24% of the POAG patients and 26% of the controls; and TT in 3% of the POAG patients and controls (*p*=0.68, Table 5.7).

For the rs3753880 G/A SNP, the genotype GG was present in 47% of the POAG patients and 50% of the controls; genotype GA in 44% of the POAG patients and 42% of the controls; and AA in 9% of the POAG patients and in 8% of controls (*p*=0.83, Table 5.7).

For the rs3766355 C/T SNP, the genotype CC was present in 79% of the POAG patients and 77% of the controls; genotype CT in 21% of the POAG patients and 22% of the controls; and TT in none of the POAG patients and in 2% of controls (*p*=0.94, Table 5.9).

**Analysis of polymorphisms in ADRB1 & 2 genes**

In the *ADRB1* gene, for the +145A/G SNP: the genotype AA was present in 68% of the POAG patients and 81% of the controls; genotype GA in 29% of the POAG patients and 19% of the controls; and GG in 3% of the POAG patients
and none of the controls (p=0.025, Table. 9). The frequency of each genotype was similar in POAG patients and controls for the +1165G/C SNP: CC in 53% POAG vs. 46% controls; GC in 8% POAG vs. 16% controls; and GG in 39% POAG vs. 39% controls (p=0.73, Table 4.10). For the eleven SNPs in the ADRB2 gene, the frequency of each genotype was remarkably similar in the POAG patients and normal controls. For six of the SNPs the frequency of each genotype was within 5% between POAG and controls; for four the frequency was within 10%; and for one the largest difference was 13% (p-values ranged from 0.22 to 0.97).

DISCUSSION

Analysis of polymorphisms in IL-1 gene family (IL1A, IL1B)

IL-1 treatment has been associated with an increase in ocular outflow mediated through ECM reorganization via matrix metallo-matrix proteinases (MMPs) production (Bradley et al., 1998) and to protection against oxidative stress inducing agents in TM cells (Wang et al., 2001). However, a chronic activation of the IL-1 pathway, as present in glaucoma, may create a deleterious effect as seen in other chronic inflammatory processes (Ross, 1995). Genetic polymorphisms within the IL-1 gene alter its expression and/or activity, potentially affecting the risk for glaucoma and its progression. In fact, IL-1 polymorphisms have been associated with a large variety of diseases across numerous organ systems, including rheumatoid arthritis, cardiovascular disease, ulcerative colitis, and Alzheimer’s disease (Haukim et al., 2002). Previous studies have reported a possible association between IL-1 polymorphisms and POAG. For example, in a
published study by Lin, et al. (2003) examining a Chinese cohort of 58 patients and 105 healthy volunteers, they reported that the \textit{IL1B} (+3953T) allele was associated with an increased risk of POAG. Wang, et al. (2006), also reported on an association between an \textit{IL1A} polymorphism at position (-889) C>T and an increased risk of POAG in a Chinese population. The same results were obtained in an Indian population (personal communication). However, How et al. (2007), reported no association between the \textit{IL1A} (-889C/T), \textit{IL1B} (+3953C/T), and \textit{IL1B} (-511C/T) and glaucoma in his sample of Chinese subjects.

Our results showed no statistically significant differences between the POAG and the control groups in terms of allele, genotype, or allele carriage frequencies at the \textit{IL1B} (-511C/T), \textit{IL1B} (+3953C/T) and \textit{IL1A} (-889C/T) loci in the Caucasian non-Hispanic, Hispanic, or African American populations. The difference between our results and what has been previously reported might be explained due to genetic variation between the different races. Because IL-1 may have both positive and negative effects on the progress of glaucoma, differences in genetic background and modifying genes would be expected to influence the way polymorphisms affect disease risk.

The findings suggest that the \textit{IL-1} cluster might not be a universal susceptibility locus, because the results differ based on the population studied. However, several other SNPs in the \textit{IL-1} cluster that have been reported to be associated with other diseases need to be considered before arriving at a final conclusion.
Analysis of polymorphisms in PTGFR gene.

Targeting the PTGFR with prostaglandin analogs has been fairly useful in lowering the IOP and increasing aqueous fluid drainage. This action, like IL-1, is thought to be dependent on ECM reorganization, with MMPs being upregulated after the drug administration (Lindsey et al., 1996, 1997; Weinrab et al., 1998; el-Shabrawi et al., 2000). The SNPs identified in the PTGFR have been reported to alter the receptor function and to influence the IOP lowering response to prostaglandin analogs (Higashide et al., 2006). In our investigation 13 SNPs were examined in the PTGFR gene, with 5 polymorphisms in the promoter region, 2 in the coding region and 6 in intronic regions of the gene.

We found no evidence of an association between POAG with any of the three PTGFR polymorphisms analyzed in this Caucasian population. The remaining PTGFR SNPs will be examined to exclude any associations with POAG.

Analysis of polymorphisms in ADRB 1 & 2 gene

The ciliary body is responsible for aqueous fluid production, and this action is mediated by the adrenergic receptor activation present on its surface (Wax et al., 1987; 1989). The predominant type of beta receptor present on the surface is the Beta 2 receptor. The beta adrenergic receptors are members of the G couple receptor (Gs) family that have adenyl cyclase as their effector and induce intracellular signaling cascades (Johnson et al., 2002; Small et al., 2003, Brum P.C. et al., 2006). They are targets in anti-glaucomatous treatment because their activation can be blocked via beta blockers, resulting in a decrease
in IOP due to a decreased production of aqueous fluid (Small et al., 2003). Our study included SNPs that have been extensively characterized in the literature.

Our results support an association between POAG and the ADRB1 gene polymorphism +145A/G but not +1165G/C. We found no evidence of an association of POAG with any of the ADRB2 polymorphisms in this Caucasian population.
APPENDIX II: TABLES AND FIGURES

Table 5. 4. Demographic characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>POAG</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race and Ethnicity</td>
<td>(n=156)</td>
<td>(n=180)</td>
</tr>
<tr>
<td>Caucasian, no Hispanic or Latino</td>
<td>72 (46%)</td>
<td>132 (73%)</td>
</tr>
<tr>
<td>Black or African American</td>
<td>42 (27%)</td>
<td>21 (12%)</td>
</tr>
<tr>
<td>Caucasian, Hispanic or Latino</td>
<td>35 (22%)</td>
<td>23 (13%)</td>
</tr>
<tr>
<td>Asian</td>
<td>7 (5%)</td>
<td>4 (2%)</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>72±11 (41-96)</td>
<td>63±13 (41-92)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>81/75</td>
<td>87/93</td>
</tr>
</tbody>
</table>

Table 5. 5. Demographic characteristic of Caucasian non-Hispanic individuals 40 and older

<table>
<thead>
<tr>
<th></th>
<th>POAG</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=72</td>
<td>n=132</td>
<td></td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>73±11</td>
<td>64±13</td>
<td>&lt;.001*</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>42/30</td>
<td>64/68</td>
<td>.23**</td>
</tr>
</tbody>
</table>

* t-test
** Chi-square
Table 5. 6. Primer sequences, PCR conditions and enzymes used for the genotyping tests for *IL-1* gene analysis

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer sequence</th>
<th>Annealing Temp. (°C)</th>
<th>Enzyme</th>
<th>Expected DNA products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL1A (-889 C/T)</strong></td>
<td>Forward 5'-GCATGCCATCACACCTAGTT-3'</td>
<td>58</td>
<td>Nco1</td>
<td>C: 178,16</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TTACATATGAGCCTCCATG-3'</td>
<td></td>
<td></td>
<td>T: 194</td>
</tr>
<tr>
<td><strong>IL1B (+3953 C/T)</strong></td>
<td>Forward 5'-GTTGTCATCAGACTTTGACC-3'</td>
<td>60</td>
<td>TaqI</td>
<td>C:135,114</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TTCAGTTCATATGGACCAGA-3'</td>
<td></td>
<td></td>
<td>T:249</td>
</tr>
<tr>
<td><strong>IL1B (-511 C/T)</strong></td>
<td>Forward 5'-TGGCATTGATCTGGTTCATC-3'</td>
<td>60</td>
<td>Bsu36I</td>
<td>C:304</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTTTAGGAATCTTCCACTT-3'</td>
<td></td>
<td></td>
<td>T:190,114</td>
</tr>
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</table>

Table 5. 7. Allele Frequency among Caucasian non-Hispanic 40 and older

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>POAG (%)</th>
<th>Control (%)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL1A (-889)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>103 (72%)</td>
<td>170 (64%)</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>41 (28%)</td>
<td>94 (36%)</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>IL1B (-511)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>96 (67%)</td>
<td>178 (67%)</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>48 (33%)</td>
<td>86 (33%)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>IL1B (+3953)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>111 (77%)</td>
<td>196 (74%)</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>33 (23%)</td>
<td>68 (26%)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

*Chi-square
Table 5. 8. Genotype Frequency and Logistic Regression analysis for risk of POAG among Caucasian non-Hispanic 40 and older

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>POAG (%)</th>
<th>Control (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=204</td>
<td>n=72</td>
<td>n=132</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL1A (-889)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>89 (44%)</td>
<td>37 (51%)</td>
<td>52 (39%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>95 (47%)</td>
<td>29 (40%)</td>
<td>66 (50%)</td>
<td>1.35 (0.83, 2.20)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>20 (10%)</td>
<td>6 (8%)</td>
<td>14 (11%)</td>
<td>1.82 (1.12, 2.96)</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>IL1B (-511)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>93 (46%)</td>
<td>33 (46%)</td>
<td>60 (46%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>88 (43%)</td>
<td>30 (42%)</td>
<td>58 (44%)</td>
<td>1.00 (0.63, 1.57)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>23 (11%)</td>
<td>9 (13%)</td>
<td>14 (11%)</td>
<td>0.99 (0.63, 1.57)</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>IL1B(+3953)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>41 (57%)</td>
<td>71 (54%)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>83 (41%)</td>
<td>29 (40%)</td>
<td>54 (41%)</td>
<td>1.13 (0.66, 1.92)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>9 (4%)</td>
<td>2 (3%)</td>
<td>7 (5%)</td>
<td>1.27 (0.75, 2.17)</td>
<td>0.65</td>
</tr>
</tbody>
</table>
### Table 5.9. Genotype Frequency and Logistic Regression analysis for risk of POAG among Caucasian non-Hispanic 40 and older.

<table>
<thead>
<tr>
<th></th>
<th>Total n=188</th>
<th>POAG (%)</th>
<th>Control (%)</th>
<th>Odds Ratio *</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=67</td>
<td>n=121</td>
<td></td>
<td>(95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>rs3766355</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>135 (72%)</td>
<td>49 (73%)</td>
<td>86 (71%)</td>
<td>1</td>
<td>0.68</td>
</tr>
<tr>
<td>GT</td>
<td>48 (26%)</td>
<td>16 (24%)</td>
<td>32 (26%)</td>
<td>1.11</td>
<td>(0.61, 2.14)</td>
</tr>
<tr>
<td>TT</td>
<td>5 (3%)</td>
<td>2 (3%)</td>
<td>3 (3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rs3753880</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>90 (49%)</td>
<td>31 (47%)</td>
<td>59 (50%)</td>
<td>1</td>
<td>0.83</td>
</tr>
<tr>
<td>GA</td>
<td>78 (43%)</td>
<td>29 (44%)</td>
<td>49 (42%)</td>
<td>1.06</td>
<td>(0.64, 1.76)</td>
</tr>
<tr>
<td>AA</td>
<td>15 (8%)</td>
<td>6 (9%)</td>
<td>9 (8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>rs3766354</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>144 (77%)</td>
<td>52 (79%)</td>
<td>92 (77%)</td>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>TC</td>
<td>40 (22%)</td>
<td>14 (21%)</td>
<td>26 (22%)</td>
<td>1.03</td>
<td>(0.49, 2.16)</td>
</tr>
<tr>
<td>TT</td>
<td>2 (1%)</td>
<td>0 (0%)</td>
<td>2 (2%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*age-adjusted from logistic regression.

**P-value from logistic regression analysis where genotypes were coded as 0, 1, 2. First (most common) genotype is the reference group for each locus.
Table 5.10. Genotype Frequency and Logistic Regression analysis for risk of POAG among Caucasian non-Hispanic whites 40 and older.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>POAG (%)</th>
<th>Control (%)</th>
<th>Odds Ratio *</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=184</td>
<td>n=63</td>
<td>n=121</td>
<td>(95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>+145A/G</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>141(77%)</td>
<td>43(68%)</td>
<td>98(81%)</td>
<td>1</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.61, 2.14)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>41 (22%)</td>
<td>18 (29%)</td>
<td>23 (19%)</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.61, 2.14)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>2 (1%)</td>
<td>2 (3%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>POAG (%)</th>
<th>Control (%)</th>
<th>Odds Ratio *</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=93</td>
<td>n=36</td>
<td>n=57</td>
<td>(95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>+1165G/C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>45(48%)</td>
<td>19(53%)</td>
<td>26 (46%)</td>
<td>1</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>12 (13%)</td>
<td>3 (8%)</td>
<td>9 (16%)</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.61, 2.14)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>36 (39%)</td>
<td>14 (39%)</td>
<td>22 (39%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5. 3. IL 1/ELAM pathway interacting genes analyzed.

MAPK

ELAM-1

\( \text{cAMP} \)

\( \text{NF Kappa B} \)

Adenyl cyclase II

\( \beta 1 \& 2 \)

ADR

\( \beta \)-blockers

IL-1

PTGFR

COX-2

Prostaglandins

Prostaglandin Analogs

Catecholamines

Figure 5. 4. Schematic representation of IL-1 gene with location of SNPs analyzed.

Adapted from van Rietschoten, J. G. I. et al.. Blood 2006;108:2143-2149
Fig. 5. 5-7. Pictures taken from the 2% agarose gel of each of the polymorphism analysis after RE digestion. (Picture 1,2,3).

Pic. 1  PCR product of 150 base pairs (bps) containing the \textit{IL1A} (-889 C/T) SNP. If the C allele is present the restriction enzyme (NcoI) recognizes and digests the product generating two fragments of 100 and 50 bps. Lanes 1-12 are representative samples from the cohort.

Pic. 2  PCR product of 304 base pairs (bps) containing the \textit{IL1B} (-511 C/T) SNP. If the T allele is present the restriction enzyme (Bsu36I) recognizes and digests the product generating two fragments of 190 and 114 bps. Lanes 13-23 are representative samples from the cohort.

Pic. 3  PCR product of 249 base pairs (bps) containing the \textit{IL1B} (+3953 C/T) SNP. If the C allele is present the restriction enzyme (Taq I) recognizes and digests the product generating two fragments of 135 and 114 bps. Lanes 24-35 are representative samples from the cohort.
Figure 5.8: Direct sequencing data of one segment amplified performed to confirm the RE digestion results

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Signal Strengths</th>
</tr>
</thead>
<tbody>
<tr>
<td>89-3-ca</td>
<td>A = 444, C = 450, G = 388, T = 463</td>
</tr>
<tr>
<td>Mobility</td>
<td>Lane/Cap: 25</td>
</tr>
<tr>
<td>D137300POP7</td>
<td>Matrix: n/a</td>
</tr>
<tr>
<td>D3 Gersten, Sabrina</td>
<td>Direction: Native</td>
</tr>
</tbody>
</table>

Figure 5.9. Schematic representation of PTGF2 with location of SNPs analyzed.

Adapted from Anderson, L. E. et al.. Biol Reprod 2001;64:1041-1047 Biology of Reproductio