ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN APOLIPOPROTEIN H (APOH) WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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Systemic lupus erythematosus (SLE) is a major public health problem in the United States. It is estimated that at least 500,000 Americans suffer from (SLE), which is a complex autoimmune disease of primarily unknown etiology. SLE is about three times more common in African Americans (about 1:250 incidence) than in Caucasian Americans (about 1:1000 incidence), and predominantly affects women of child-bearing age (female to male ratio of 9:1). SLE causes a variable amount of morbidity, shortened life expectancy, and substantial total health expenditures, largely due to complications such as thrombosis, atherosclerosis, renal disease, and antiphospholipid syndrome (APS). Genetics plays a significant role in the etiology of SLE; therefore, understanding the underlying genetic influence of this disease is of significant public health importance. This study dealt with the analysis of one of the genes that has been proposed to play a role in the pathogenesis of SLE: apolipoprotein H (APOH gene, apoH protein), also referred to as β_2-glycoprotein I (β_2-GPI). ApoH is thought to have anti-atherogenic properties and has been shown to be a major target antigen for antiphospholipid antibodies (APA) present in patients with APS. Twelve APOH SNPs (9 TagSNPs and 3 additional functional coding SNPs) were genotyped in 398 women with a clinical diagnosis of SLE and 496 healthy women as controls. The associations of allele and genotype distributions of these 12 SNPs with SLE, race, renal disease in white patients, and APA in black and white patients and controls were

analyzed. Significant allelic distribution differences were observed between whites and blacks for 9 of the 12 SNPs, indicating a race-dependent variation. No associations were found between genotype distributions in any of the 12 SNPs and SLE, renal disease, or APA status. However, haplotype analysis revealed six haplotypes that significantly differed in frequency between cases and controls. Of particular interest was one haplotype that was present in 16.2% of cases and 0.6% of controls, suggesting a potential risk factor for SLE. In conclusion, our study suggests that combined effects of APOH SNPs (haplotype) may be implicated in modifying the risk of SLE.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 BACKGROUND AND SIGNIFICANCE

1.1 INTRODUCTION TO LUPUS

Systemic lupus erythematosus (SLE or lupus) is a multifactorial, chronic autoimmune disorder characterized by complex pathophysiology involving many organ systems, including dermatologic, renal, central nervous system (CNS), hematologic, musculoskeletal, cardiovascular, pulmonary, the vascular endothelium, and gastrointestinal. Symptoms include malar or discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal complications, neurologic disorder, hematologic involvement, or the presence of antinuclear antibodies (ANA). SLE presents in periods of flares (an acute increase in symptoms) and remission (Trethewey, 2004).

Lupus is a relatively common disease, affecting an estimated 500,000 or more Americans (Trethewey, 2004). The overall incidence is in the range of 1.8-7.6 cases per 100,000 per year in the United States, with variable incidence worldwide. The epidemiology of SLE is striking in that 80% of cases are women between the ages of 15 and 45 (essentially those of childbearing age). Studies have shown that women taking oral contraceptives or hormone replacement are at increased risk to develop SLE, suggesting a hormonal role in disease etiology. The male to female ratio is 9:1. Although SLE appears in all ethnic groups, it is more common among certain populations – it is three times more common in African Americans (about 1:250 incidence) than in Caucasian Americans (about 1:1000 incidence) (Lamont, 2006).

A genetic component of lupus is supported by the fact that the disease is more likely to occur among first-degree relatives (familial prevalence of 10-12%), and concordance rates are

higher in monozygotic twins (24-58%) than in dizygotic twins (2-5%) (Brent, 2007). The risk of an SLE sibling compared to the general population risk has been reported between 20-40 (Nath et al. 2004). These concordance rates, however, are relatively low, suggesting that environmental factors also play a role in the onset of disease.

Autoantibodies, circulating immune complexes, and T lymphocytes all contribute to the expression of SLE in the different organs and cell types. Autoantibodies are produced against various antigens, including nuclear, cytoplasmic, and blood cell surface antigens, which in turn leads to accumulation of immune complexes within the blood vessel walls and subsequent symptoms (Trethewey, 2004).

Although the prognosis of SLE patients has improved over time, the 10-year survival rate is about 80% and the 20-year survival rate is about 70%. Early deaths are often caused by active disease, while atherosclerosis and acute myocardial infarction are among the most common causes of mortality later in the disease course (Urowitz, 1976). Lupus nephritis and infection are leading causes of death at any stage of SLE. Other causes of morbidity and mortality include antiphospholipid syndrome (APS), carditis, pneumonitis, pulmonary hypertension, stroke, myocardial infarction, and cerebritis – all of which may lead to thrombosis (Lamont, 2006).

1.2 RENAL MANIFESTATIONS OF SLE

One of the more severe manifestations of SLE, lupus nephritis, usually develops within five years of a diagnosis based on the American College of Rheumatology classification criteria. The prevalence of clinical renal involvement in persons with SLE is estimated to be 30-90%, with severity and prevalence estimates of lupus nephritis tending to be higher in the Black and Asian populations and in children (Brent, 2007).

The pathogenesis of lupus nephritis is due largely in part to the production of autoantibodies directed against nuclear elements, which form immune complexes. In the kidneys, deposition of these immune complexes initiates an inflammatory response by activating the complement cascade and recruiting inflammatory cells. Histological evidence of lupus nephritis is present in most patients with SLE, even if they do not have clinical symptoms of renal disease (Brent, 2007).

Results from a study by Matsuda et al. (1993) suggested that patients with SLE were positive for apoH-dependent anticardiolipin (aCL), while lupus anticoagulant (LAC) and apoH-independent aCL were produced in patients with end-stage renal disease (not related to SLE), especially those on hemodialysis. A study by Frostegård et al. (2005) showed that SLE patients with cardiovascular and renal involvement had more oxidized epitopes on low-density lipoproteins (LDL) compared with controls. Furthermore, aCL in these patients recognized epitopes generated during lipid peroxidation. Therefore, "neo" self antigens on lipoproteins, generated during oxidation, are present in SLE and may be of importance for the development of premature cardiovascular disease and possibly also for other autoimmune phenomena observed in SLE.

1.3 CARDIOVASCULAR MANIFESTATIONS OF SLE

Cardiovascular involvement is frequently reported in SLE patients, with estimates ranging from 6.1 to 8.9 percent of cases (Manzi, 1997). In a study by Manzi et al. (1997), a sample of 35-44 year old women with SLE was found to have an incidence of myocardial infarction 50 times higher than the general population. Such an increase in premature cardiovascular disease is thought to be attributable to multiple factors, including underlying vascular inflammation and arterial wall injury, corticosteroid use, renal disease, hypertension, antiphospholipid antibodies and thrombosis – all of which are common in women with SLE.

Several studies have also reported atherosclerotic vascular disease as a major finding in SLE women with cardiovascular events. Treatment with corticosteroids, renal disease, and hypertension are thought to play a role in the atherosclerotic process in these women. One study, however, showed that these "Framingham risk factors," as they are called, failed to serve as a sufficient explanation for the accelerated atherosclerosis observed (Esdaile, 2001).

Atherosclerosis is a progressive condition that begins with the formation of macrophage-derived foam cells. Foam cells are created when circulating lipoproteins, mainly from pro-atherogenic low-density lipoproteins (LDL), are taken up by macrophages. Before these LDLs can be taken up by macrophages, however, they must be modified. One such modification process is peroxidation, or the conversion of LDL to oxidized LDL (oxLDL) which is taken up by scavenger receptors. Thus, excessive intracellular accumulation of oxLDL encourages plaque (atheroma) formation (Ross, 1999; Matsuura, 2006).

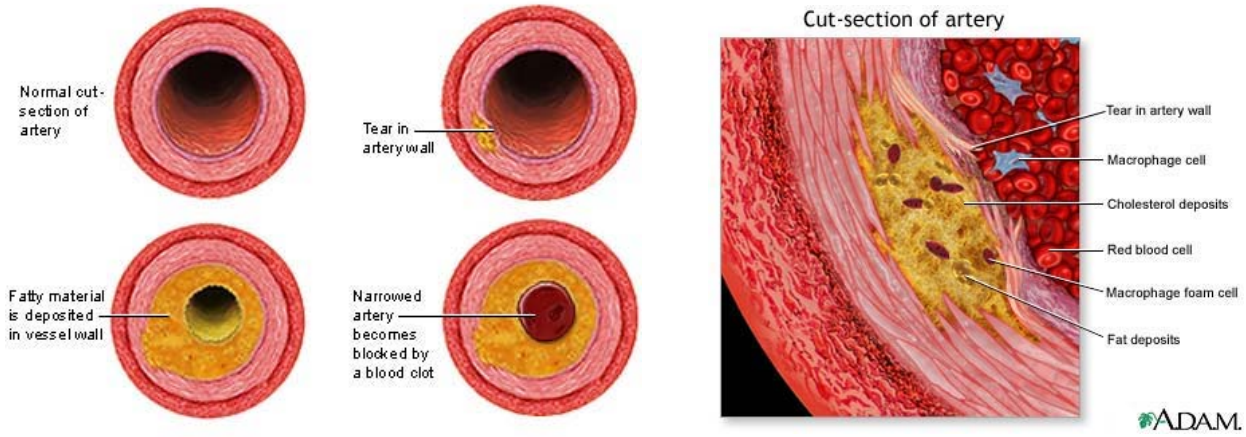


Figure 1. Enlarged views of atherosclerosis (Keller, 2004)

1.4 ATHEROSCLEROSIS AND APOH

Apolipoproteins associate with lipoproteins and are integrated in lipoprotein particles such as LDL, high density lipoproteins (HDL), very low density lipoproteins (VLDL), and chylomicrons (CM). The focus of this study is on human apolipoprotein H (APOH gene, apoH protein), also referred to as β_2-glycoprotein I (β_2-GPI). This well-characterized apoH protein is a constituent of triglyceride rich lipoproteins in human lymph and plasma and is thought to play a role in triglyceride metabolism, via activation of lipoprotein lipase (Polz, 1979; Nakaya, 1980). ApoH is responsible for 17% of the composition of HDLs, 16% of CMs and VLDLs, and 2% of LDLs (Lozier et al. 1984).

There is also evidence that apoH binds to platelets and regulates adenylate cyclase activity (Schousboe, 1980). Moreover, apoH has been shown to be involved in cholesterol transport, foam cell formation, and consequently atherosclerosis. Lin et al. (2001) showed that apoH has an "antioxidant"-like effect on LDL oxidation; by increasing cholesterol efflux and decreasing cholesterol influx, apoH decreases cellular accumulation of cholesterol. In addition, Lin et al. (2001) reported that apoH inhibits the translocation of cholesterol from extracellular pools to macrophages, thus possibly lending to the prevention of atherosclerosis.

Further evidence that apoH may be an anti-atherogenic protein was provided by a study by Hasunuma et al. (1997) that suggested a dual role of apoH in the uptake of oxLDL by macrophages. This study demonstrated that apoH inhibits the uptake of oxLDL in the absence of anticardiolipin (aCL), while the binding of oxLDL was significantly increased by the simultaneous addition of human apoH and monoclonal aCL, indicating an atherogenic effect.

ApoH is known to be the actual target antigen for clinically relevant antiphospholipid antibodies such as aCL (Hunt et al. 1992). Certain groups of aCLs (anti-apoH antibodies) exert lupus anticoagulant (LAC) activity and disrupt the function of vascular endothelial cells. Lupus anticoagulants, antibodies against anionic phospholipids or associated proteins which inhibit phospholipid-dependent blood coagulation, are often found in patients with SLE. Takeya et al. (1997) showed that anti-apoH monoclonal antibodies (mAbs) enhance the binding of apoH to phospholipids, thus exerting LAC activity. This congregation of apoH molecules on the surface of phospholipids is thought to cause rigidness of the phospholipid surface and to subsequently reduce the lateral mobility of clotting factors toward the prothrombinase complex on this surface. Further evidence of this theory was provided by a study that showed antiphospholipid antibodies may promote thrombosis at the locations where fetal cells make contact with maternal blood and

where vascular endothelial cells are exposed to circulating blood by reducing the levels of annexin V and accelerating the coagulation of plasma on cultured trophoblasts and endothelial cells (Rand et al. 1997). This process may be relevant to thrombosis and pregnancy loss in patients with antiphospholipid syndrome (APS), a condition that occurs with relative frequency in patients with SLE.

1.5 ANTIPHOSPHOLIPID SYNDROME (APS) AND ApoH

Antiphospholipid syndrome (APS) is one of the most common causes of hypercoagulability in the general population (Ginsburg et al. 1992). Primary APS occurs in the absence of underlying disease, while secondary APS may occur in the context of autoimmune disorders such as SLE. APS is characterized by elevated serum levels of antiphospholipid antibodies (APA), venous or arterial thromboembolic events, or pregnancy morbidity (Hanly, 2003).

Antiphospholipid antibodies include a heterogeneous family of immunoglobins. They may be produced transiently and in low concentrations in up to 5% of healthy individuals. However, APA are found in higher frequency and concentration in individuals with autoimmune and other conditions such as syphilis, HIV, and other infections and malignancies (Levine et al. 2001). APA are present in up to 50% of SLE patients (Carsons, 2004). The most common APA that are clinically detected for APS are aCL and LAC, which studies have estimated to be present in 12%-30% and 15%-34% of SLE patients, respectively (Hanly, 2003). These APA recognize phospholipid binding proteins such as apoH and prothrombin. Further, the autoimmune aCL from patients with SLE and APS requires apoH as a cofactor in order to bind to cardiolipin (CL)

directly (as opposed to aCL binding directly to CL in patients with various infectious diseases and low incidence of thrombosis) – this is termed apoH-dependent aCL (Matsuura at al. 2006).

It has been shown by RT-PCR analysis that apoH mRNA is expressed in several cells which are targeted by autoantibodies in APS, such as endothelial cells, astrocytes, neurons, and lymphocytes. These cell types were found to be immunoreactive to anti-apoH mAbs in the same study by immunofluorescence technique, indicating an antigenic function of apoH in APS (Caronti et al. 1999). ApoH has also been found to be expressed in the liver and in enterocytes (gastrointestinal cells) at both the mRNA and protein levels (Averna et al. 1997).

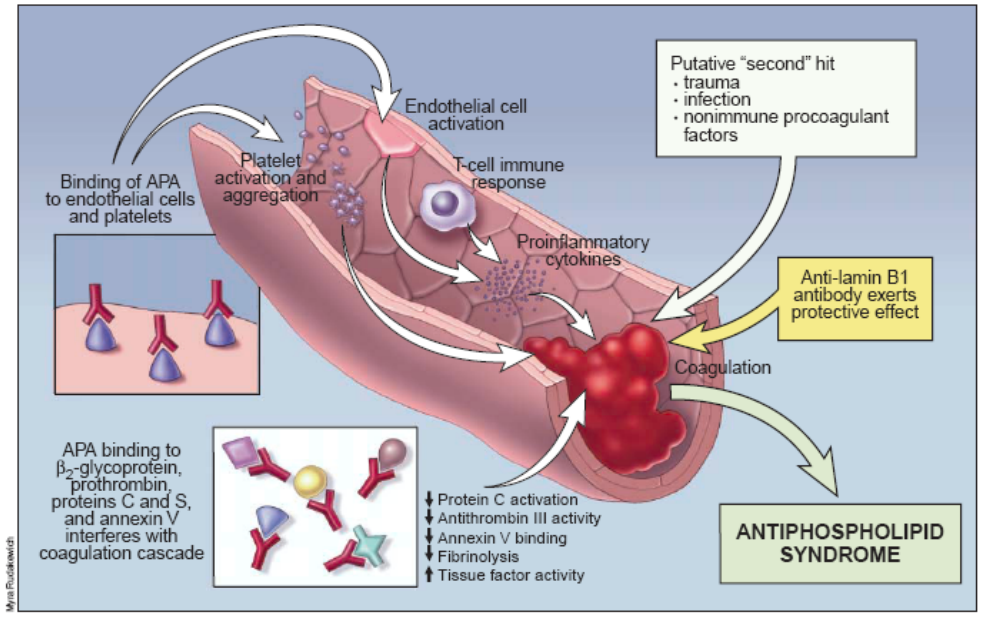


Figure 2. Pathogenic mechanism in antiphospholipid syndrome (Hanly, 2003)

1.6 MOLECULAR ASPECTS OF ApoH

ApoH is a single-chain 50-kDa glycoprotein composed of 326 amino acids that exists in both a free form and in combination with lipoprotein particles (Lozier et al. 1984). Schwarzenbacher et al. (1999) determined the crystal structure of β_2-GPI and revealed that there are five domains, including four complement control protein (CCP) modules and a positively charged fifth C-terminal domain, arranged like beads on a string to form a J-shaped molecule (Figure 3). This fifth domain is capable of binding negatively charged substances such as phospholipids, platelets, and viral proteins.

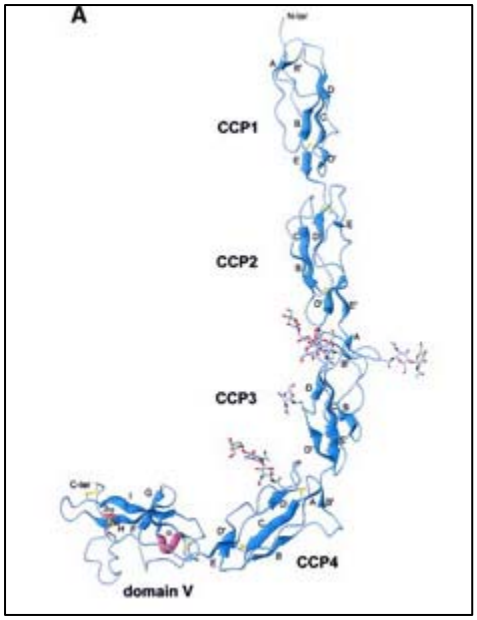


Figure 3. The secondary structure of apoH shown as a ribbon drawing (Schwarzenbacher et al. 1999)

The chromosomal location of APOH is 17q23-17q24. The gene is 18kb long and contains 8 exons. Exon 1 contains the 5′ untranslated region (5′UTR) and the signal peptide coding sequences. The transcriptional initiation site (said to be at +1) is located 31bp upstream

of the translational start codon. Exons 2 through 7 code for short consensus repeats (SCRs). SCRs 1, 3, 4, and 5 are coded by exons 2, 5, 6, and 7, respectively, while SCR2 is coded by exons 3 and 4. Exon 8 encodes the 3′UTR, stop codon, and C-terminus (Okkels et al. 1999).

1.7 APOH POLYMORPHISMS

Several studies have successfully identified functional polymorphisms in APOH, particularly in the coding regions. Sanghera et al. (1997b) identified two common structural missense mutations in the fifth domain which prevent apoH from binding to negatively charged phosphatidylserine (PS). These mutations are located at Trp316Ser (TGG → TCG), and Cys306Gly (TGC → GGC). Mehdi et al. (2000) went on to describe how these missense mutations disrupt binding of apoH to CL.

In 1993, Steinkasserer et al. described a missense mutation in APOH exon 7 at Val247Leu (GTA → TTA). Takada et al. (2003) investigated the impact of this 247Leu allele and a splice-site mutation of the LD receptor (LDLR) gene on familial hypercholesterolemia by performing an intra-familial correlation analysis in an eight-generation extended family, in which members were affected with either type IIa hyperlipoproteinemia (HLPIIa), type IIb hyperlipoproteinemia (HLPIIb), or no disease. All family members with HLPIIb were found to have both the deleterious LDLR allele and an apoH 247Leu allele, whereas all members with HLPIIa had the defective LDLR allele and the apoH 247Val allele. The results of this study indicate that the Val247Leu mutation in apoH plays a role in modifying the phenotype of FH in individuals with an LDLR mutation.

The APOH gene has been shown to have structural polymorphism with five alleles: APOH*1, APOH*2 (the most common), APOH*3^{W} and APOH*3^{B} (based on reactivity with an APOH monoclonal antibody in whites and blacks, respectively), and APOH*4 (only found in an African American population (Kamboh et al. 1988). Significant inter-racial differences in the frequencies of these alleles has been observed. The molecular basis for the apoH protein polymorphism has now been analyzed to some extent. Four mutations in exon 5, which codes for the third domain of apoH, were identified, including a point mutation at Ser140Ser (TCA → TCG) and missense mutations at Ile122Thr (ATC → ACC), Arg135His (CGT → CAT), and Ala141Asp (GCT → GAT). A point mutation at the intron 4/exon 5 junction at the location of -10 (A → C) was also identified (Kamboh et al. 2004). The three missense mutations were shown to decrease CL binding with apoH, suggesting that the third domain plays a role in this function. Interestingly, these mutant alleles at codons 122, 135, and 140 were found to be in significant linkage disequilibrium with the common polymorphism at codon 247.

Several missense mutations were found by Kamboh et al. (2004) to correspond to APOH alleles in haplotype combination: APOH*3 (122Thr/135His), APOH*3^{B} (122Thr/135His/141Asp), and APOH*3^{W} (122Thr/135His/316Ser). Further, two missense mutation in codon 3 (Ser88Asn) and codon 316 were found to correspond to APOH*1 and APOH*3, respectively (Sanghera et al. 1997a; 1997b). The previously identified mutations at codon 247 and codon 306 have not been found to be associated with a particular apoH protein polymorphism. Based on the results of an analysis of the evolutionary pathway of APOH haplotypes, Kamboh et al. (2004) proposed a revised nomenclature of the alleles. In this new naming system, the original APOH*3^{B} is now APOH*3 and the original APOH*4 is now APOH*3^{B}.

1.8 APOH TAG SNPs

Single nucleotide polymorphisms (SNPs) are single base pair differences in a genetic sequence that are relatively common (defined as more than 1% of individuals in a population). It is estimated by the International HapMap project that SNPs occur in our genome with a frequency of about one in every 1,200 bases.

SNPs that are in close proximity to each other tend to be inherited together. Such clusters of SNPs are termed haplotypes. "Tag" SNPs are a set of SNPs that uniquely identify a particular haplotype. This project aims to genotype select Tag SNPs in APOH in order to delineate any possible associations between our population of SLE patients, renal disease, race, APA involvement, and APOH haplotypes. If individuals with SLE, renal disease, or APA tend to share a particular haplotype, variants contributing to the disease might be somewhere within or near that haplotype.

The functional polymorphisms that have been identified in previous studies of APOH have been located in the coding regions of the APOH gene. However, it is also possible for polymorphisms in the non-coding regions of a gene to have an impact on phenotype. For example, recent findings have indicated that a polymorphism (G/A) in intron 4 of the programmed cell death 1 (PDCD1) gene may be associated with sporadic SLE risk (Sanghera et al. 2004). In addition, a SNP (T/G) at position -93 in the promoter region of the lipoprotein lipase gene was shown by Hall et al. (1997) to impact triglyceride levels – individuals who were homozygous for the T allele were reported to have lower triglyceride levels.

For this study, nine Tag SNPs were analyzed, five of which are located in introns, one in the promoter region, one in the 3′UTR, one in the 3′ flanking region, and one in the coding region. Three additional non-synonymous coding SNPs were also included in this analysis. A

summary of information about these SNPs is included in Table 1. The University of Washington-Fred Hutchinson Cancer Research Center group has sequenced the APOH gene and identified 146 SNPs (http://pga.gs.washington.edu/data/apoh/). Information about the minor allele frequencies (MAF) of these SNPs in populations of European and African descent has been reported and is included for the SNPs relevant to this study in Table 1. The MAF for *APOH*/rs1801689 is marked with an '*' because these percentages were not available from the Seattle SNP database. MAF for *APOH*/rs1801689 was obtained from the International HapMap project (http://www.hapmap.org). For each SNP, the reference SNP (refSNP) ID from NCBI is also listed, along with the corresponding nucleotide substitution. The nine TagSNPs are marked in blue font (Table1).

Table 1. SNPs used for statistical analysis in this study

Db SNP ID	*APOH* Location	Seattle SNP Site	SNP	MAF in Whites	MAF in Blacks
rs3760292	Promoter	-643	T>C	17%	17%
rs8178826	Intron 2	3470	A>G	5%	0%
rs8178839	Intron 4	7381	T>G	7%	17%
rs8178847	Exon 5	10059 (Codon 135)	G>A (Arg>His)	7%	5%
rs8178848	Intron 5	11251	G>A	5%	3%
rs2873966	Intron 6	14905	G>A	22%	19%
rs8178858	Intron 6	15320	G>A	24%	5%
rs3176975	Exon 7	16121 (Codon 247)	G>T (Val>Leu)	26%	50%
rs1801689	Exon 7	(Codon 306)	T>G (Cys>Gly)	2.5%*	0%*
rs1801690	Exon 8	18593 (Codon 316)	G>C (Trp>Ser)	2%	0%
rs6933	3′UTR	18648	A>G	48%	18%
rs7211492	3′ Region	20246	A>G	12%	3%

1.9 SPECIFIC AIMS

The specific aims of this study include the following:

1) To determine the allele frequency and genotype distributions of APOH SNPs among white and black subjects from Pittsburgh.
    a. *H_0 – no significant differences will be observed in the allele frequencies of APOH SNPs between white and black subjects*
    b. *H_1 – a significant difference will be observed in the allele frequencies of APOH SNPs between white and black subjects*
2) To determine the association of APOH SNPs with SLE in white and black subjects.
    a. *H_0 – no association will be observed between APOH SNPs and SLE*
    b. *H_1 – an association will be found between APOH SNPs and SLE*
3) To determine the association of APOH SNPs with renal involvement in white patients and antiphospholipid antibodies (APA) in white and black patients and controls.
    a. *H_0 – no association will be observed between APOH SNPs and renal disease or APA in white patients with SLE*
    b. *H_1 – an association will be found between APOH SNPs and renal disease or APA in white patients with SLE*
4) To determine the association of haplotypes, including nine APOH TagSNPs with SLE in white patients
    a. *H_0 – no association will be observed between APOH haplotypes and SLE*
    b. *H_1 – an association will be found between APOH haplotypes and SLE*

2.0 PATIENTS AND METHODS

2.1 SUBJECTS

2.1.1 Sample Populations

DNA (obtained from leukocytes) and blood sera from 398 women with a diagnosis of SLE were used for analysis in this study. All subjects were recruited through a study by Dr. Susan Manzi, in which the prevalence of cardiovascular disease and other risk factors in women with SLE were evaluated. All of the study participants met the American College of Rheumatology criteria for the classification of SLE (Tan et al. 1982; Hochberg 1997). This cohort consisted of women ranging in age from 18 to 79 years (mean age 43.23 years +/– 11.43 SD). 350 of the women were Caucasian, while 48 were African American. Lupus nephritis was diagnosed in the SLE patients based on either renal biopsy, proteinuria (at least 2 measurements of >0.5 gm/24 hours or 3+ protein by dipstick), or the presence of red blood cell casts (Tripi et al. 2006).

A cohort of 496 control samples from the same geographic location as the SLE group was obtained from the Central Blood Bank of Pittsburgh. These individuals were described as "healthy" and had no apparent history of SLE or related rheumatologic disease. This cohort consisted of women ranging in age from 17 to 81 years (mean age 45.63 years +/– 13.47 SD). 454 of the women were Caucasian, while 42 were African American. The University of

Pittsburgh Institutional Review Board approved this study, and all participants underwent the informed consent process.

2.1.2 Antiphospholipid antibody quantification from patient samples

Serum samples from all patients and controls were screened in duplicate for the presence of aCL (both IgG aCL and IgM aCL), LAC, and anti-apoH as previously described (Sanghera et al. 2004; Tripi et al. 2006). All subjects who were positive for at least one of these antibodies were considered to be positive for antiphospholipid antibody (APA).

2.2 DETERMINATION OF GENOTYPES

2.2.1 Pyrosequencing

Polymerase chain reaction (PCR) was used to amplify DNA samples for two TagSNPs (*APOH*/rs8178848 and *APOH*/rs8178826) prior to genotyping by Pyrosequencing. For each sample, DNA was combined with 10X PCR buffer II (Applied Biosystems), 50mM $MgCl_2$ (Applied Biosystems), 1.25mM dNTPs (GE Healthcare), 1.25 units of Ampli*Taq* DNA Polymerase (Applied Biosystems), 20μM forward primer (biotin-labeled) and 20μM reverse primer (Invitrogen). For each primer set, the forward primer was labeled with biotin. Quantities of reagents used for optimum amplification of each fragment are listed in Table 2.

Table 2. PCR Reaction Mixtures

	***APOH*/ rs8178848**	***APOH*/rs8178826**
	SLE/CBB (WGA)	SLE/CBB (WGA)
DNA	2.0µl	4.0µl
10X Buffer	5.0µl	5.0µl
$MgCl_2$	5.0 µl	4.0µl
dNTPs	3.5µl	3.5µl
Forward Primer	0.5µl	0.4µl
Reverse Primer	0.5µl	0.4µl
Taq Polymerase	0.25µl	0.25µl

A thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with heated lid was used to regulate the temperature cycles required for PCR. The PCR conditions for these two TagSNPs are outlined in Table 3, along with the primers used (written in the 5' to 3' direction):

Table 3. PCR Conditions

	***APOH*/rs8178848**	***APOH*/rs8178826**
PCR Conditions	95°C for 5 min. – 1 cycle 95°C for 30 sec. 60°C for 30 sec. } 45 cycles 72°C for 40 sec. 72°C for 5 min. – 1 cycle	95°C for 5 min. – 1 cycle 95°C for 30 sec. 60°C for 20 sec. } 45 cycles 72°C for 30 sec. 72°C for 5 min. – 1 cycle
Primer Sequences	Forward Primer - Biotin Labeled: CAGCATGGAGGAGCTACCAATAA Reverse Primer: TGCCTGGCCCATAGTTGAG	Forward Primer – Biotin Labeled: CCTTTGTCAAAGTTATGCATGTTC Reverse Primer: GGCTCTTTAAGCAAAAAAGCATAG

The success of PCR was confirmed via gel electrophoresis of approximately 30% of the samples. To a gel consisting of 2% agarose in TBE buffer (tris, boric acid, and disodium EDTA dihydrate) with 0.8% ethidium bromide (Amresco), 5µl of marker dye (0.25% Orange G, Sigma) and 7µl of PCR product was added for each sample. The samples were subjected to electrophoresis for about 35 min at 250V.

The genotypes of *APOH*/rs8178848 and *APOH*/rs8178826 were analyzed using the Pyrosequencing technology designed and produced by Biotage. Pyrosequencing is a fairly novel method of genotyping SNPs and insertion/deletion polymorphisms (InDels) either individually or in multiplex analysis. Although multiplex analysis was attempted for this study, the particular Pyrosequencing assays for these TagSNPs were not appropriate for duplex analysis; therefore, simplex (individual) analysis was performed. Table 4 lists the conditions of these assays:

Table 4. Pyrosequencing Assay Conditions

	PYROSEQUENCING ASSAYS
***APOH*/rs8178848**	Sequencing Primer: 5'TGGCCCATAGTTGAG Sequence Analyzed: TA/GCCCAGA AAGGTACAAG TTACTACTG
***APOH*/rs8178826**	Sequencing Primer: 5'GCAAAAAAGCATAGTAAAAT Sequence Analyzed: CAACTTGCA/ GTGAACATGC ATAACTTTGA CAAAGG

A TagSNP in the promoter region, *APOH*/rs3760292 (position -643), was also previously analyzed via Pyrosequencing in the sample populations (Erin Jacobs, 2005, data not yet published). This data was utilized for the statistical analyses performed in this study.

The biochemical principle of Pyrosequencing is outlined briefly as follows: proceeding PCR amplification of the SNPs and agarose electrophoresis with ethidium bromide staining as a quality control check, PCR products are processed in order to render them single-stranded, which is necessary for the sequencing primer to anneal (Ronaghi et al, 1996). This single-stranded DNA (ssDNA) is obtained by mixing the PCR product (in this case, 18µl was used per sample) with streptavidin-coated beads, which bind to the biotinylated strand of the DNA double helix. Alkalai solutions are then used to neutralize the pH and separate the non-biotinylated from the biotinylated strands that are attached to the beads. A Vacuum Prep Tool is used to capture the

beads and transfer them to annealing buffer containing 10mM sequencing primer diluted in annealing buffer. The primer is annealed to the ssDNA template via heating to 90°C for 2 minutes, then cooling at room temperature (Biotage, 2006; Gharizadeh, 2002).

Next, enzymes including DNA polymerase, ATP sulfurylase, luciferase, and apyrase are added by the PSQ-96MA apparatus. The substrates, adenosine 5' phosphosulfate and luciferin, are then added. The first deoxyribonucleotide triphosphate (dNTP) in the dispensation order is released; if it is complimentary to the first unpaired base in the template strand, then DNA polymerase facilitates its incorporation and pyrophosphate (PPi) is released in quantities proportionate to the number of that particular dNTP available in sequence (Figure 3).

The next step in this chain of reactions is the conversion of PPi to ATP by ATP sulfurylase in the presence of adenosine 5' phosphosulfate. The ATP, which is produced in equal proportion to the amount to PPi, fuels the conversion of luciferin to oxyluciferin by luciferase – a reaction that produces light in an amount proportional to the amount of ATP. A camera detects this quantity of light and the computer program produces a diagram of peaks that correspond to the addition of each dNTP (referred to as a Pyrogram). Between the addition of dNTPs, apyrase degrades the unincorporated nucleotides and ATP so that the next peak can be measured from the same initial baseline. This process generates a record of nucleotide sequence, and thus the genotype of interest (Figure 4).

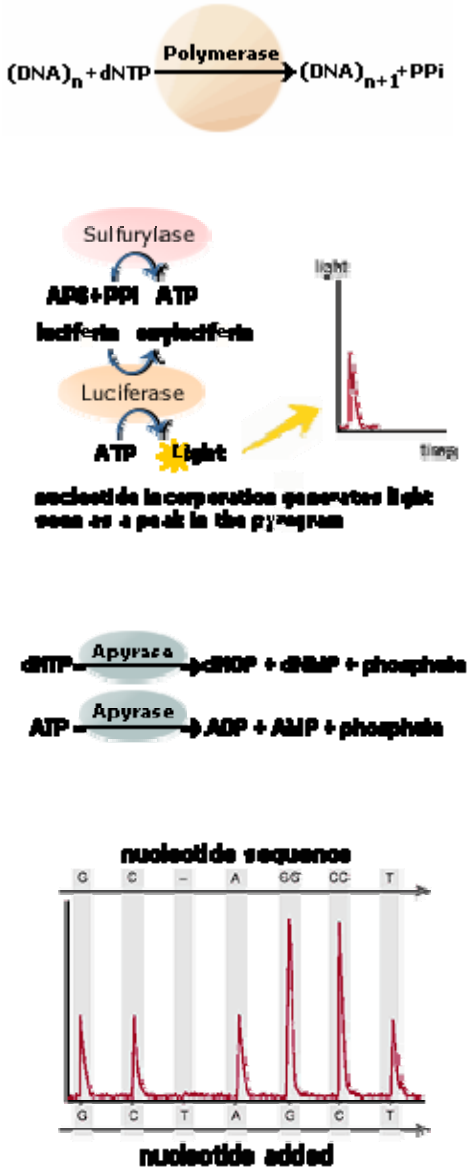
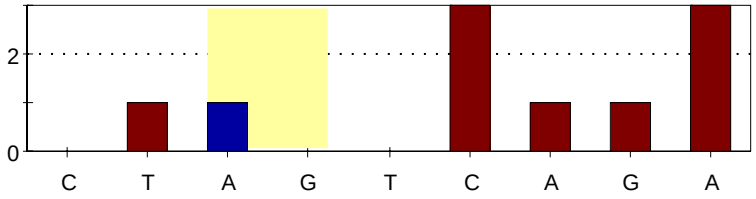


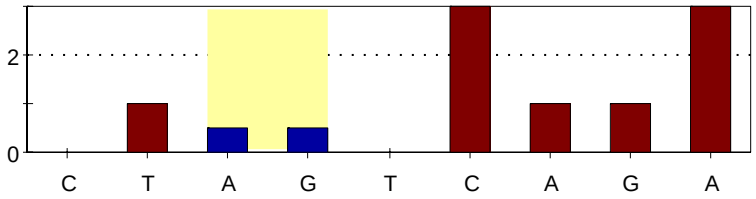
Figure 4. Schematic representation of the principle of pyrosequencing (Biotage, 2006)

Pyrosequencing data is produced and analyzed with software that works in concert with the PSQ-96MA. Following automatic data analysis, manual changes may be made at the user's discretion. Included here are the theoretical histograms corresponding to the two Pyrosequencing assays performed in this study, along with sample Pyrograms for each TagSNP (Figures 5, 6, 7, and 8).

A/A



A/G



G/G

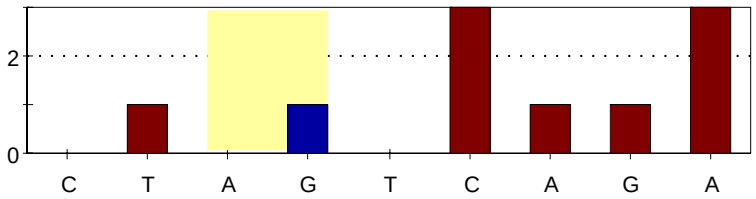
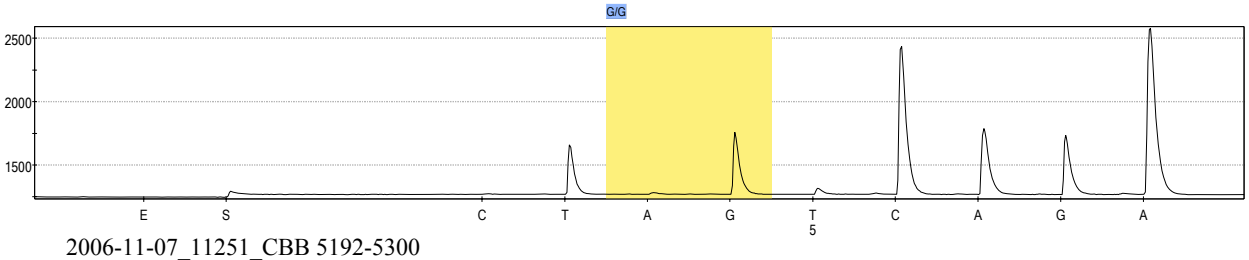


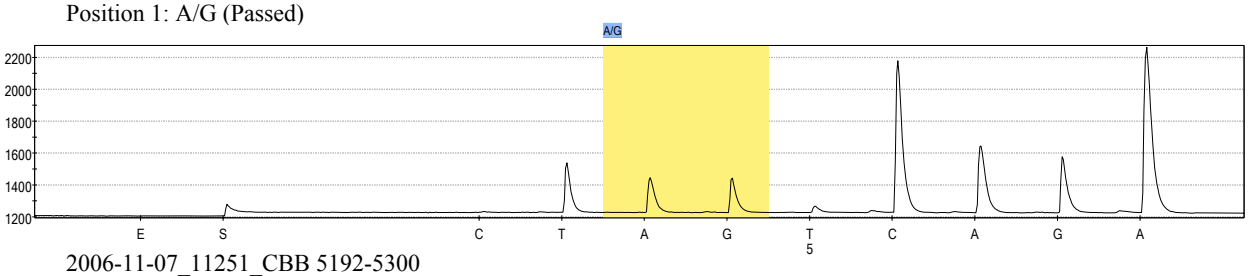
Figure 5. Theoretical outcomes of simplex entry APOH/rs8178848



2006-11-07_11251_CBB 5192-5300

Well A7 Sample: 5198

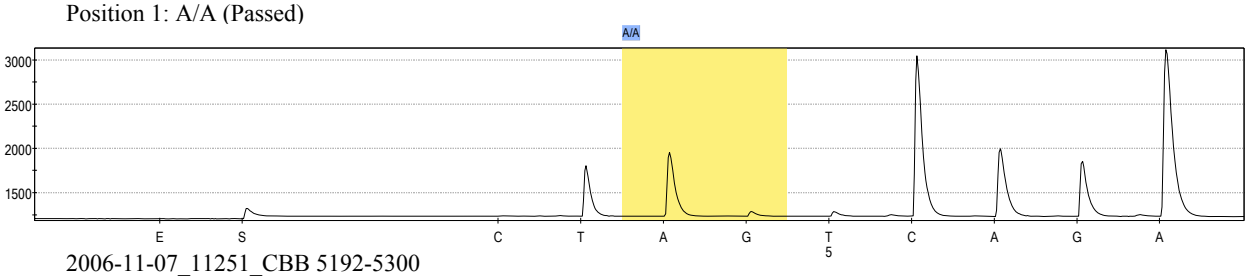
Position 1: A/G (Passed)



2006-11-07_11251_CBB 5192-5300

Well H6 Sample: 5296

Position 1: A/A (Passed)



2006-11-07_11251_CBB 5192-5300

Well H12 Sample: NTC

Position 1: A/G (Failed)

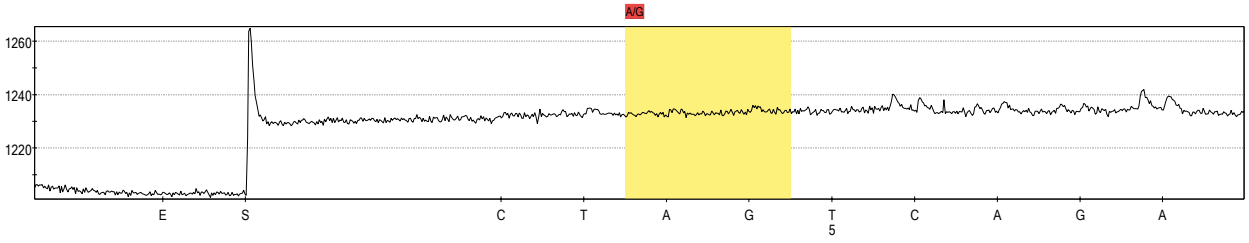


Figure 6. Representative pyrograms of simplex entry APOH/rs8178848

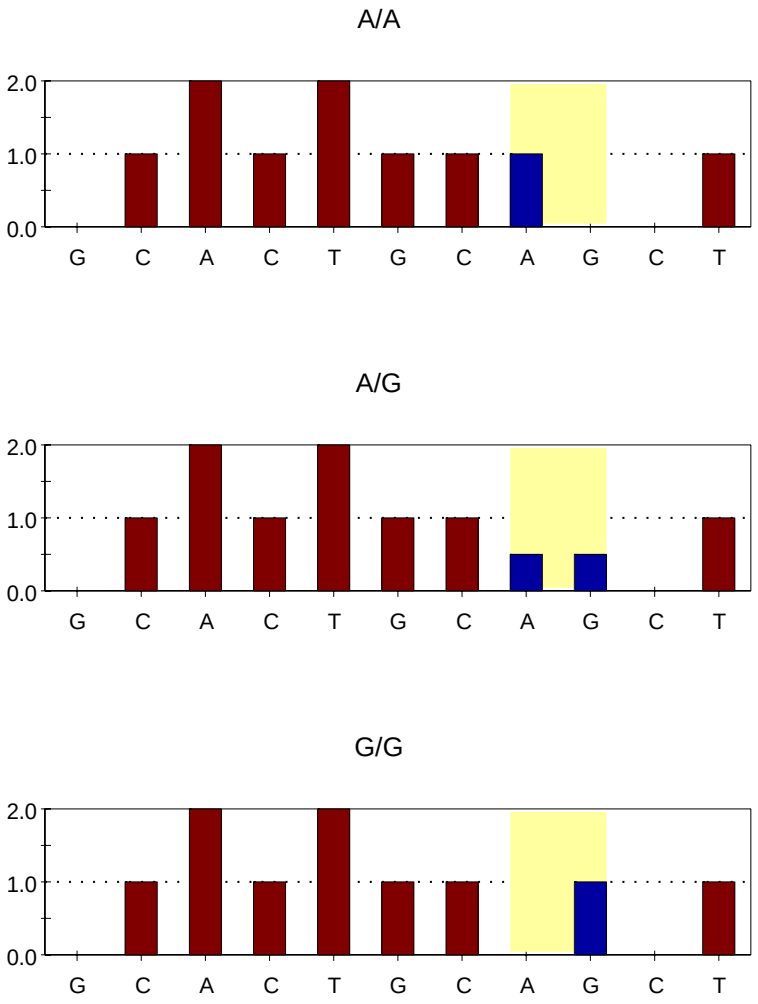


Figure 7. Theoretical outcomes of simplex entry APOH/rs8178826

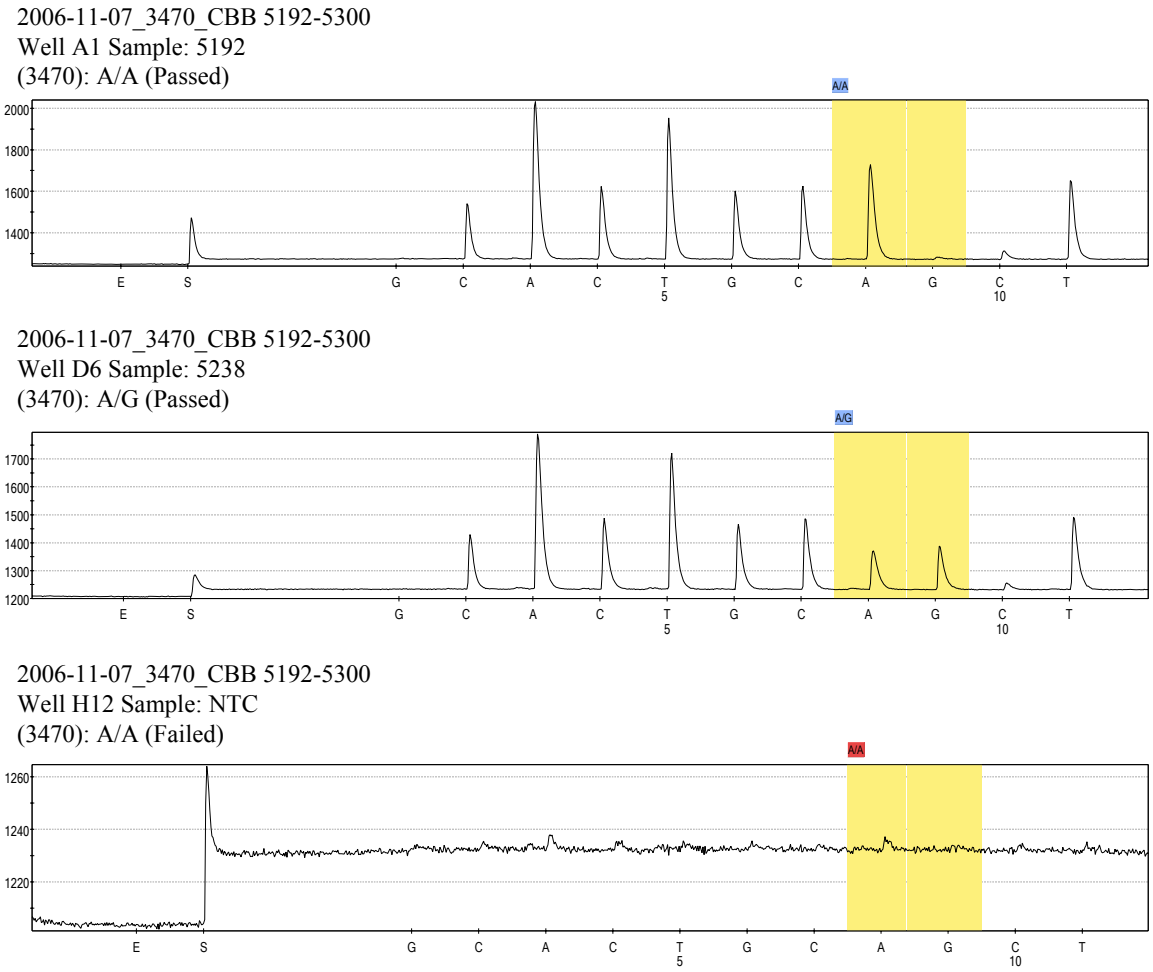


Figure 8. Representative pyrograms of simplex entry APOH/rs8178826

2.2.2 TaqMan Analysis

The genotypes of *APOH*/rs2873966, *APOH*/rs3176975, *APOH*/rs6933, *APOH*/rs7211492, *APOH*/rs8178839, and *APOH*/rs8178858 were analyzed using pre-made TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA). The assay identification numbers for each TagSNP are listed in Table 5.

Table 5. TaqMan® SNP Genotyping Assays

APOH TagSNP reference sequence number	TaqMan® Genotyping Assay
APOH/rs2873966	7567311 (functionally tested)
APOH/rs3176975*	C___2658421_1_ (validated)
APOH/rs6933	29504985 (functionally tested)
APOH/rs7211492	16090146 (functionally tested)
APOH/rs8178839	29176909 (functionally tested)
APOH/rs8178858	29992830 (functionally tested)

*This TagSNP was previously analyzed by Kamboh et al. (1999, 2004)

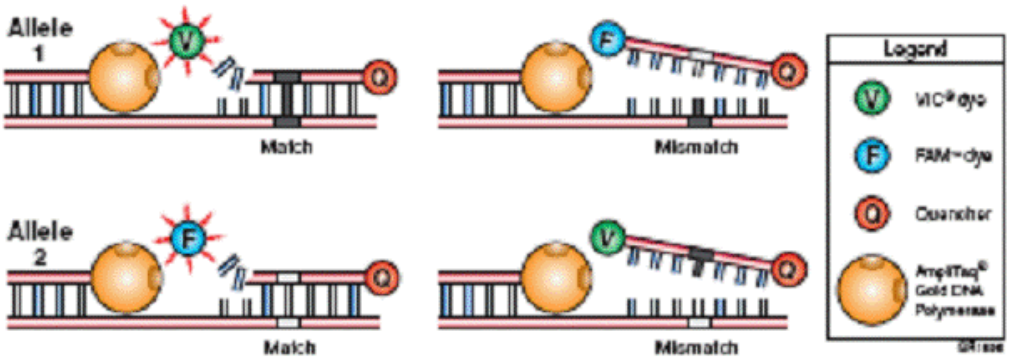
TaqMan is a two-step procedure that requires amplification of the product and a subsequent endpoint reading using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The TaqMan protocol involves filling 384-well plates with purified genomic DNA. To this, Genotyping Assay Mix is added; this mix contains sequence-specific forward and reverse primers, one TaqMan minor groove binder (MGB) probe labeled at the 5′ end with VIC dye and one TaqMan MGB probe labeled at the 5′ end with FAM dye to detect the respective alleles. A nonfluorescent quencher (NFQ) is attached to the 3′ end of the probes. PCR is performed using a PTC-200 Peltier Thermal Cycler (MJ Research) with a temperature cycle described in Table 6:

Table 6. Thermal Cycler Conditions for TaqMan®

Times and Temperatures		
Initial Step	Denaturation	Primer Annealing & Extension
HOLD	40 Cycles	
10min @ 95°C	15sec @92°C	1min @ 60°C

During the annealing step, each TaqMan MGB probe anneals to the sequence between the forward and reverse primers. AmpliTaq Gold DNA polymerase cleaves only probes that

hybridize to the target sequence; the process results in separation of the reporter dye from the quencher dye, and a consequent release of fluorescence. When probes do not hybridize to the target sequence, the reporter dye and quencher dye essentially "cancel each other out" and fluorescence is suppressed; this is termed Förster-type energy transfer (Förster, 1948). This selective annealing of TaqMan MGB probes, therefore, enables allelic discrimination. Figure 9 illustrates the fluorescent outcomes of probe matches and mismatches (Livak et al. 1995).



The table below shows the correlation between fluorescence signals and sequences in the sample.

A substantial increase in...	Indicates...
VIC® dye fluorescence only	Homozygosity for allele 1
FAM™ dye fluorescence only	Homozygosity for allele 2
Both fluorescence signals	Heterozygosity allele 1-allele 2

Figure 9. Schematic representation of TaqMan probe-target matches and mismatches

2.3 STATISTICAL METHODS

Allele frequencies for each TagSNP were calculated by the allele counting method. Observed genotype frequencies were compared to Hardy-Weinberg equilibrium (HWE), and the significance of differences was tested by the Chi-squared goodness-of-fit test. The significance of deviations between genotype frequencies and allele frequencies was tested by Fisher's exact test and standard Z-test of two binomial proportions, respectively. These tests were used for association analyses of allele and genotype distributions of 12 APOH SNPs with SLE, race, renal disease in white patients, and APA in black and white patients and controls stratified by racial group. Due to the small size of our black population, renal disease association tests were performed for white subjects only, and antibody association studies were performed on combined data for black and white subjects as well. All computations were performed using the R statistical software package (Version 2.1.1, http://www.r-project.org). Linkage disequilibrium (LD) between SNPs was estimated by both D′ and r^2 calculations using Haploview statistical software. Haplotype analysis was performed using both EH and R statistical software programs.

3.0 RESULTS

3.1 HARDY-WEINBERG EQUILIBRIUM (HWE) OF APOH SNPs

For all APOH SNPs that were genotyped, observed genotype frequencies were found to be in Hardy-Weinberg equilibrium (HWE) for our white population. HWE P-values could not be accurately estimated for the black population due to small sample size (Table 7).

Although the genotypes of several samples could not be determined by the methods used in this study, the overall failure rate was less than 10% for each of the genotyped SNPs. Failure rates for each individual SNP were as follows: APOH/rs8178848 (1.34%), APOH/rs8178826 (4.47%), APOH/rs2873966 (1.23%), APOH/rs6933 (3.91%), APOH/rs7211492 (0.67%), APOH/rs8178858 (9.50%), APOH/rs8178839 (1.12%).

All failed samples were repeated, as well as 10% of the successfully genotyped samples to check genotyping error. Genotyping error was calculated as the number of repeated samples with contradictory genotypes dived by the total number of repeated samples for each SNP. This error rate was well below 3% for most of the SNPs analyzed here, and genotyping error rates for each individual SNP were as follows: APOH/rs8178848 (0.32%), APOH/rs8178826 (0.0%), APOH/rs2873966 (0.36%), APOH/rs6933 (2.69%), APOH/rs7211492 (0.71%), APOH/rs8178858 (2.68%), APOH/rs8178839 (1.08%).

Table 7. HWE of APOH SNPs

rs8178848			**rs8178839**		
	Cases	*Controls*		*Cases*	*Controls*
HWE white	P=0.051	P=0.5991	HWE white	P=0.2467	P=0.1939
HWE black	P=0.0132	P=<0.0005	HWE black	P=0.9609	P=0.4299

rs2873966			**rs8178826**		
	Cases	*Controls*		*Cases*	*Controls*
HWE white	P=0.6692	P=0.8913	HWE white	P=0.8008	P=0.7348
HWE black	P=0.001	P=0.8788	HWE black	P= ----	P= ----

rs7211492			**rs6933**		
	Cases	*Controls*		*Cases*	*Controls*
HWE white	P=0.3013	P=0.3612	HWE white	P=0.4308	P=0.9151
HWE black	P=0.7632	P=0.8728	HWE black	P=0.121	P=0.549

rs3760292			**rs8178858**		
	Cases	*Controls*		*Cases*	*Controls*
HWE white	P=0.8351	P=0.4157	HWE white	P=0.6756	P=0.6406
HWE black	P=0.6181	P=0.4683	HWE black	P=0.3823	P= ----

rs8178847			**rs3176975**		
	Cases	*Controls*		*Cases*	*Controls*
HWE white	P=0.5633	P=0.6367	HWE white	P=0.4002	P=0.5422
HWE black	P=0.3187	P=0.7771	HWE black	P=0.5134	P=0.0573

rs1801689			**rs1801690**		
	Cases	*Controls*		*Cases*	*Controls*
HWE white	P=0.2495	P=0.3592	HWE white	P=0.9221	P=0.3378
HWE black	P= ----	P= ----	HWE black	P= ----	P=0.9370

3.2 DISTRIBUTION OF APOH SNPS IN WHITES AND BLACKS

The genotype and allele frequencies of twelve APOH SNPs were compared between white and black subjects (Table 8). A significant difference in genotype distribution between races was found for *APOH*/rs2873966 (p=0.001), *APOH*/rs6933 (p=0.003), *APOH*/rs8178858 (p=0.025), *APOH*/rs3176975 (p=<0.0001), *APOH*/rs1801689 (p=0.021), and *APOH*/rs1801690 (p=0.012).

Allele frequencies were significantly different ($p \leq 0.050$) for all SNPs except three: *APOH*/rs8178839 (p=0.558), *APOH*/rs8178848 (p=0.718), and *APOH*/rs8178847 (p=0.555).

Table 8. Distribution of APOH SNPs in whites and blacks

APOH SNP	Genotype/ Allele	Whites	Blacks	APOH SNP	Genotype/ Allele	Whites	Blacks
rs3760292	*Genotypes*	*n*(%)	*n*(%)	**rs2873966**	*Genotypes*	*n*(%)	*n*(%)
	TT	537(72.67)	66(82.50)		GG	397(50.06)	61(68.54)
	TC	184(24.90)	14(17.50)		GA	325(40.98)	27(30.34)
	CC	18(2.44)	0		AA	71(8.95)	1(1.12)
	total	739	80		total	793	89
		P=0.123				**P=0.001**	
	Alleles				*Alleles*		
	T	0.851	0.913		G	0.706	0.837
	C	0.149	0.088		A	0.294	0.163
		P=0.011				**P=<0.0001**	
rs6933	*Genotypes*	*n*(%)	*n*(%)	**rs7211492**	*Genotypes*	*n*(%)	*n*(%)
	AA	233(30.26)	43(48.86)		AA	678(84.96)	83(93.26)
	AG	375(48.70)	32(36.36)		AG	112(14.04)	6(6.74)
	GG	162(21.04)	13(14.77)		GG	8(1.00)	0
	total	770	88		total	798	89
		P=0.003				**P=0.113**	
	Alleles				*Alleles*		
	A	0.546	0.670		A	0.920	0.966
	G	0.454	0.330		G	0.080	0.034
		P=0.001				**P=0.002**	
rs8178826	*Genotypes*	*n*(%)	*n*(%)	**rs8178839**	*Genotypes*	*n*(%)	*n*(%)
	AA	745(97.01)	85(100.00)		TT	622(78.24)	66(75.00)
	AG	23(2.99)	0		TG	163(20.50)	21(23.86)
	GG	0	0		GG	10(1.26)	1(1.14)
	total	768	85		total	795	88
		P=0.156				**P=0.703**	
	Alleles				*Alleles*		
	T	0.985	1.000		T	0.885	0.869
	C	0.015	0.000		G	0.115	0.131
		P=<0.0001				**P=0.558**	

Table 8 continued. Distribution of APOH SNPs in whites and blacks

APOH SNP	Genotype/ Allele	Whites	Blacks	APOH SNP	Genotype/ Allele	Whites	Blacks
rs8178848	*Genotypes*	*n*(%)	*n*(%)	**rs8178858**	*Genotypes*	*n*(%)	*n*(%)
	GG	697(87.78)	78(89.66)		GG	461(63.67)	66(78.57)
	GA	92(11.59)	8(9.20)		GA	230(31.77)	16(19.05)
	AA	5(0.63)	1(1.15)		AA	33(4.56)	2(2.38)
	total	794	87		total	724	84
		P=0.475				**P=0.025**	
	Alleles				*Alleles*		
	G	0.936	0.943		G	0.796	0.881
	A	0.064	0.057		A	0.204	0.119
		P=0.718				**P=0.002**	
rs8178847	*Genotypes*	*n*(%)	*n*(%)	**rs3176975**	*Genotypes*	*n*(%)	*n*(%)
	GG	593(87.33)	59(85.51)		GG	448(60.79)	23(30.67)
	GA	83(12.22)	9(13.04)		GT	247(33.51)	34(45.33
	AA	3(0.44)	1(1.45)		TT	42(5.70)	18(24.00)
	total	679	69		total	737	75
		P=0.361				**P=<0.0001**	
	Alleles				*Alleles*		
	G	0.934	0.920		G	0.775	0.533
	A	0.066	0.080		T	0.225	0.467
		P=0.555				**P=<0.0001**	
rs1801689	*Genotypes*	*n*(%)	*n*(%)	**rs1801690**	*Genotypes*	*n*(%)	*n*(%)
	TT	613(92.04)	67(100.00)		GG	715(90.74)	84(98.82)
	TG	52(7.81)	0		GC	72(9.14)	1(1.18)
	GG	1(0.15)	0		CC	1(0.13)	0
	total	666	67		total	788	85
		P=0.021				**P=0.012**	
	Alleles				*Alleles*		
	T	0.959	1.000		G	0.953	0.994
	G	0.041	0.000		C	0.047	0.006
		P=<0.0001				**P=<0.0001**	

3.3 LINKAGE DISEQUILIBRIUM ANALYSIS OF APOH SNPs

Pairwise linkage disequilibrium (LD) analysis was performed using Haploview statistical software for all 12 SNPs among white cases (Figure 10), white controls (Figure 11), and all white subjects combined (Figure 12). LD was not examined for our black population due to small sample size.

Both D' and r^2 values are presented here to demonstrate the extent of LD between SNPs. The SNPs are aligned from left to right in the order in which they are located in the APOH gene (from promoter to 3' region). Coding SNPs are marked with an '*'. The highest r^2 values generated from this analysis were observed in white cases only and included LD between *APOH*/8178847 and *APOH*/8178848 (r^2=0.76), between *APOH*/8178847 and *APOH*/1801690 (r^2=0.60), and between *APOH*/8178848 and *APOH*/1801690 (r^2=0.58). Similar r^2 values were observed for white cases, controls, and all whites combined between *APOH*/8178839 and *APOH*/8178847 (r^2=0.51) and between *APOH*/3760292 and *APOH*/3176975 (r^2=0.53).

There were several blocks of D' values equal to 1.0 in this analysis, more so in white cases than controls. This is an example of "complete LD" without "perfect LD" – a concept that will be addressed in further detail in Section 4.1.2 of this paper.

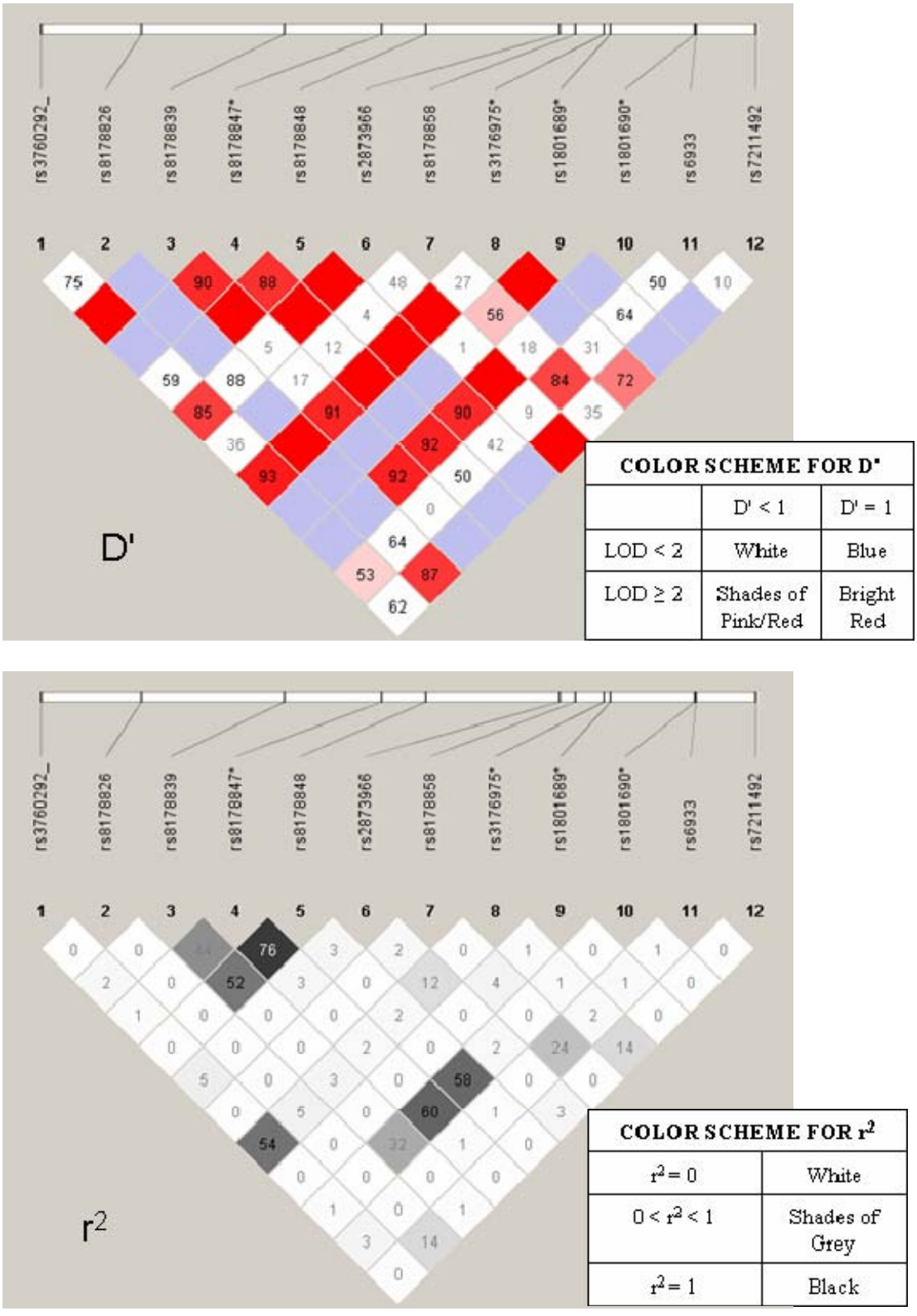


Figure 10. LD analysis of white cases

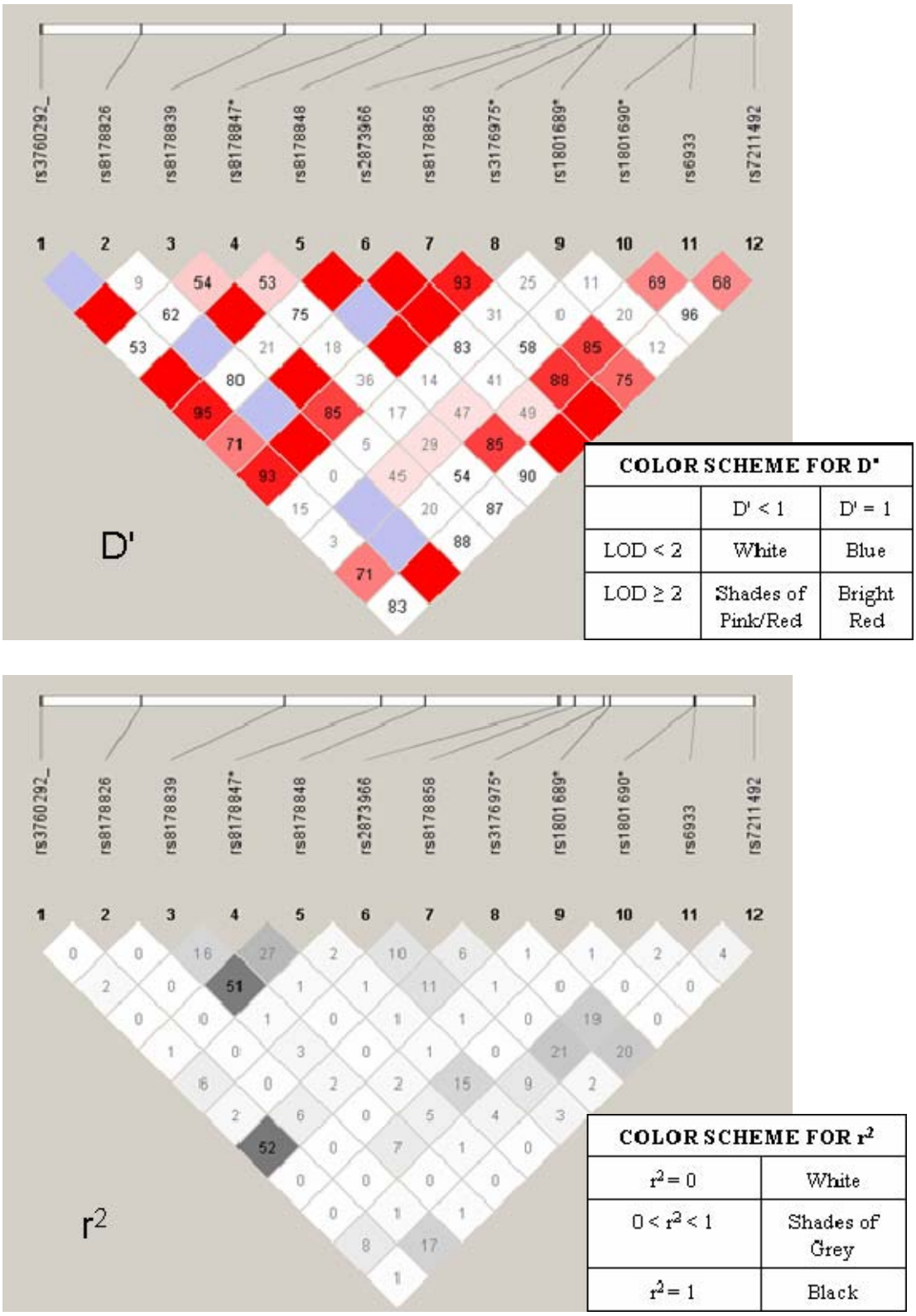


Figure 11. LD analysis of white controls

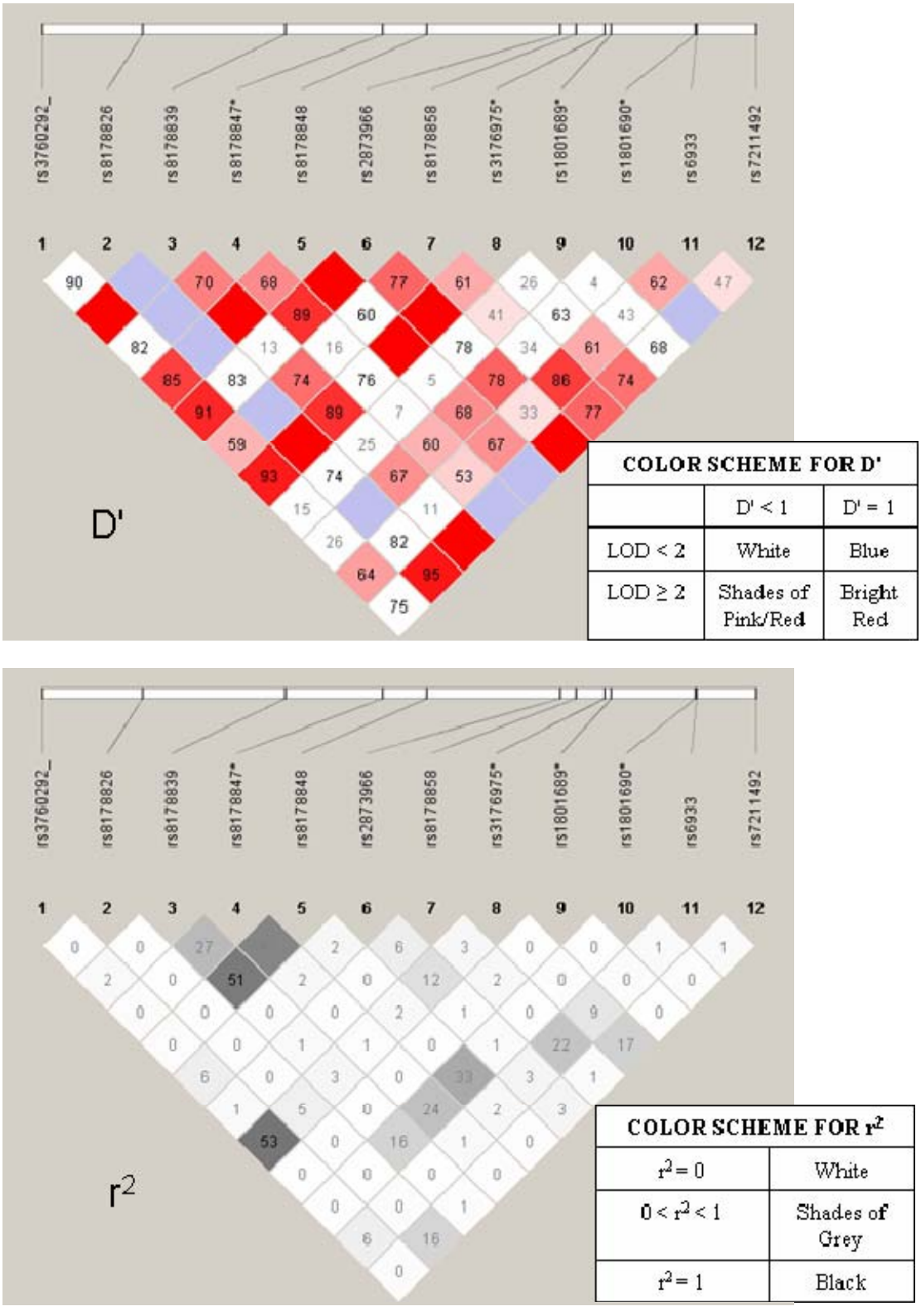


Figure 12. LD analysis of all white subjects

3.4 ASSOCIATION OF APOH POLYMORPHISMS WITH SLE

3.4.1 Association of APOH SNPs with SLE in whites and blacks

The genotype and allele frequencies of twelve APOH SNPs were compared between cases and controls within white and black cohorts (Table 9). Using a cut-off of P=0.05, no significant difference in genotype or allele distribution between patients with SLE and healthy controls was found for any of these twelve SNPs.

Table 9. Distribution of APOH SNPs between cases and controls in whites and blacks

APOH SNP	Genotype/Allele	Whites		Blacks	
rs3760292	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	TT	219(71.10)	318(73.78)	36(85.71)	30(78.95)
	TC	82(26.62)	102(23.67)	6(14.29)	8(21.05)
	CC	7(2.27)	11(2.55)	0	0
	total	308	431	42	38
		P=0.646		**P=0.558**	
	Alleles				
	T	0.844	0.856	0.929	0.895
	C	0.156	0.144	0.071	0.105
		P=0.525		**P=0.453**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs6933	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	AA	113(33.73)	120(27.59)	21(44.68)	22(53.66)
	AG	157(46.87)	218(50.11)	17(36.17)	15(36.59)
	GG	65(19.40)	97(22.30)	9(19.15)	4(9.76)
	total	335	435	47	41
		P=0.175		**P=0.459**	
	Alleles				
	T	0.572	0.526	0.628	0.720
	C	0.428	0.474	0.372	0.280
		P=0.077		**P=0.192**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178826	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	AA	318(97.25)	427(96.83)	45(100.00)	40(100.00)
	AG	9(2.75)	14(3.17)	0	0
	GG	0	(0.00)	0	0
	total	327	441	45	40
		P=0.832		**P=1.000**	
	Alleles				
	A	0.986	0.984	1.000	1.000
	G	0.014	0.016	0.000	0.000
		P=0.734		**P= ----**	

Table 9 continued. Distribution of APOH SNPs between cases and controls in whites and blacks

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178848	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	GG	303(87.32)	394(88.14)	42(91.30)	36(87.80)
	GA	40(11.53)	52(11.63)	3(6.52)	5(12.20)
	AA	4(1.15)	1(0.22)	1(2.17)	0
	total	347	447	46	41
		P=0.301		**P=0.467**	
	Alleles				
	G	0.931	0.940	0.946	0.939
	A	0.069	0.060	0.054	0.061
		P=0.483		**P=0.852**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178847	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	GG	254(86.99)	339(87.60)	31(81.58)	28(90.32)
	GA	36(12.33)	47(12.14)	6(15.79)	3(9.68)
	AA	2(0.68)	1(0.26)	1(2.63)	0
	total	292	387	38	31
		P=0.781		**P=0.596**	
	Alleles				
	G	0.932	0.937	0.895	0.952
	A	0.068	0.063	0.105	0.048
		P=0.704		**P=0.201**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs1801689	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	TT	270(93.43)	343(90.98)	37(100.00)	32(100.00)
	TG	18(6.23)	34(9.02)	0	0
	GG	1(0.35)	(0.00)	0	0
	total	289	377	37	32
		P=0.188		**P=1.000**	
	Alleles				
	T	0.965	0.955	1.000	1.000
	G	0.035	0.045	0.000	0.000
		P=0.328		**P= ----**	

Table 9 continued. Distribution of APOH SNPs between cases and controls in whites and blacks

APOH SNP	Genotype/Allele	Whites		Blacks	
rs2873966	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	GG	174(50.58)	223(49.67)	31(64.58)	30(73.17))
	GA	139(40.41)	186(41.43)	17(35.42)	10(24.39)
	AA	31(9.01)	40(8.91)	0	1(2.44)
	total	344	449	48	41
		P=0.965		**P=0.302**	
	Alleles				
	G	0.708	0.704	0.823	0.854
	A	0.292	0.296	0.177	0.146
		P=0.860		**P=0.577**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs7211492	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	AA	300(86.71)	378(83.63)	44(91.67)	39(95.12)
	AG	43(12.43)	69(15.27)	4(8.33)	2(4.88)
	GG	3(0.87)	5(1.11)	0	0
	total	346	452	48	41
		P=0.471		**P=0.683**	
	Alleles				
	A	0.929	0.913	0.958	0.976
	G	0.071	0.087	0.042	0.024
		P=0.221		**P=0.516**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178839	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	TT	270(78.49)	352(78.05)	34(72.34)	32(78.05)
	TG	67(19.48)	96(21.29)	12(25.53)	9(21.95)
	GG	7(2.03)	3(0.67)	1(2.13)	0
	total	344	451	47	41
		P=0.201		**P=0.895**	
	Alleles				
	T	0.882	0.887	0.851	0.890
	G	0.118	0.113	0.149	0.110
		P=0.774		**P=0.437**	

Table 9 continued. Distribution of APOH SNPs between cases and controls in whites and blacks

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178858	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	GG	215(63.99)	246(63.40)	34(72.34)	32(86.49)
	GA	106(31.55)	124(31.96)	11(23.40)	5(13.51)
	AA	15(4.46)	18(4.64)	2(4.26)	0
	total	336	388	47	37
		P=0.991		**P=0.240**	
	Alleles				
	G	0.798	0.794	0.840	0.932
	A	0.202	0.206	0.160	0.068
		P=0.858		**P=0.054**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs3176975	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	GG	195(60.00)	253(61.41)	11(26.19)	12(36.36)
	GT	110(33.85)	137(33.25)	23(54.76)	11(33.33)
	TT	20(6.15)	22(5.34)	8(19.05)	10(30.30)
	total	325	412	42	33
		P=0.844		**P=0.187**	
	Alleles				
	G	0.769	0.780	0.536	0.530
	T	0.231	0.220	0.464	0.470
		P=0.613		**P=0.947**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs1801690	*Genotypes*	*Cases*(%)	*Controls*(%)	*Cases*(%)	*Controls*(%)
	GG	302(89.88)	413(91.37)	44(100.00)	40(97.56)
	GC	33(9.82)	39(8.63)	0	1(2.44)
	CC	1(0.30)	(0.00)	0	0
	total	336	452	44	41
		P=0.446		**P=0.482**	
	Alleles				
	G	0.948	0.957	1.000	0.988
	C	0.052	0.043	0.000	0.012
		P=0.413		**P=0.314**	

3.4.2 Haplotype associations with SLE

The maximum number of SNPs that could be efficiently handled by our haplotype software is nine; therefore, the three coding SNPs that are not TagSNPs were excluded (*APOH*/8178847, *APOH*/1801689, and *APOH*/1801690).

Nine-site haplotype analysis was performed using R software to determine the association of APOH TagSNPs with SLE in the entire white cohort, including 454 controls and 349 cases. Haplotype analysis was also performed using both EH and R software excluding all samples that had missing genotype information for any of the nine TagSNPs in the white cohort, including 320 controls and 256 cases. R program results including all samples were largely consistent with the R program results excluding samples with missing data. The EH and R program outputs that excluded samples calculated virtually identical haplotype frequencies; R program results are presented here, since this program is able to calculate P-values while EH is not. Haplotype analysis was not carried out for the black population due to lack of a larger sample size.

The parameters of the analysis included a minimum MAF of 0.01 and 18 degrees of freedom. In total, 42 haplotypes were observed; the 11 most frequent haplotypes (pool frequency ≥ 0.01) are summarized in Table 10. The alleles are aligned from left to right in 5' to 3' orientation in the APOH gene (as listed below Table 10). Major alleles are marked in bold type, while minor alleles are in regular type. The frequencies of each haplotype in white cases, controls, and all whites are listed along with corresponding P-values. Six of these haplotypes were significantly different between cases and controls (#'s 1, 2, 3, 5, 7, and 9, which are highlighted in Table 10). Haplotypes that were more frequent in controls are marked in blue font, while haplotypes that were more frequent in cases are marked in red font. There were four

common haplotypes that are present in greater than 10% of the population. The overall difference in haplotypes of cases and controls was calculated by R software and was found to be significant (p<.0001).

Table 10. Haplotype analysis of 9 TagSNPs in white cases and controls

Haplotypes	Case freq (n=256)	Control freq (n=320)	Pool freq	P-values
TATGAGGAA	0.144	0.228	0.199	0.031
TATGGAGGA	0.121	0.196	0.163	0.006
TATGGGGGA	0.103	0.191	0.159	0.002
CATGGGTAA	0.114	0.129	0.123	0.587
TATGGGGAA	0.162	0.006	0.064	<0.001
TAGAGGGAA	0.055	0.053	0.055	0.794
TATGGGTAG	0.021	0.055	0.042	0.017
TAGGAGGGA	0.032	0.047	0.041	0.235
TATGAGGGA	0.073	0.016	0.036	<0.001
CATGGGTGA	0.017	0.007	0.012	0.210
TGTGGGTAG	0.011	0.012	0.012	0.809

Overall P-Value<0.0001

TagSNPs: rs3760292, rs8178826, rs8178839, rs8178848, rs2873966, rs8178858, rs3176975, rs6933, rs7211492

3.5 ASSOCIATION OF APOH POLYMORPHISMS WITH RENAL DISEASE IN WHITE PATIENTS WITH SLE

The genotype and allele frequencies of twelve APOH SNPs were compared between white cases with and without renal disease (Table 11). This comparison was also performed for black cases with and without renal disease; however, due to small sample size and inability to interpret P-values these data are not shown. Using a cut-off of P=0.05, only one significant difference in genotype or allele distribution between patients with SLE and healthy controls was found for these twelve SNPs. The minor allele frequency of *APOH*/2873966 was significantly less for those with renal disease than those with no renal disease (p=0.023).

Table 11. Distribution of APOH SNPs among white cases with and without renal involvement

APOH SNP	Genotype/ Allele	No Renal Disease	Renal Disease	APOH SNP	Genotype/ Allele	No Renal Disease	Renal Disease
rs3760292	*Genotypes*	*n*(%)	*n*(%)	**rs2873966**	*Genotypes*	*n*(%)	*n*(%)
	TT	164(70.39)	55(73.33)		GG	116(46.96)	58(59.79)
	TC	63(27.04)	19(25.33)		GA	106(42.91)	33(34.02)
	CC	6(2.58)	1(1.33)		AA	25(10.12)	6(6.19)
	total	233	75		total	247	97
		P=0.888				**P=0.098**	
	Alleles				*Alleles*		
	T	0.839	0.860		G	0.684	0.768
	C	0.161	0.140		A	0.316	0.232
		P=0.526				**P=0.023**	
rs6933	*Genotypes*	*n*(%)	*n*(%)	**rs7211492**	*Genotypes*	*n*(%)	*n*(%)
	AA	83(34.30)	30(32.26)		AA	214(85.94)	86(88.66)
	AG	114(47.11)	43(46.24)		AG	32(12.85)	11(11.34)
	GG	45(18.60)	20(21.51)		GG	3(1.20)	0
	total	242	93		total	249	97
		P=0.829				**P=0.716**	
	Alleles				*Alleles*		
	A	0.579	0.554		A	0.924	0.943
	G	0.421	0.446		G	0.076	0.057
		P=0.563				**P=0.337**	
rs8178826	*Genotypes*	*n*(%)	*n*(%)	**rs8178839**	*Genotypes*	*n*(%)	*n*(%)
	AA	227(97.01)	91(97.85)		TT	194(78.23)	76(79.17)
	AG	7(2.99)	2(2.15)		TG	51(20.56)	16(16.67)
	GG	0	0		GG	3(1.21)	4(4.17)
	total	234	93		total	248	96
		P=1.000				**P=0.165**	
	Alleles				*Alleles*		
	T	0.985	0.989		T	0.885	0.875
	C	0.015	0.011		G	0.115	0.125
		P=0.655				**P=0.717**	

Table 11 continued. Distribution of APOH SNPs among white cases with and without renal involvement

APOH SNP	Genotype/ Allele	No Renal Disease	Renal Disease	APOH SNP	Genotype/ Allele	No Renal Disease	Renal Disease
rs8178848	*Genotypes*	*n*(%)	*n*(%)	**rs8178858**	*Genotypes*	*n*(%)	*n*(%)
	GG	217(86.80)	86(88.66)		GG	158(65.02)	57(61.29)
	GA	30(12.00)	10(10.31)		GA	73(30.04)	33(35.48)
	AA	3(1.20)	1(1.03)		AA	12(4.94)	3(3.23)
	total	250	97		total	243	93
		P=0.879				**P=0.595**	
	Alleles				*Alleles*		
	G	0.928	0.938		G	0.800	0.790
	A	0.072	0.062		A	0.200	0.210
		P=0.626				**P=0.773**	
rs8178847	*Genotypes*	*n*(%)	*n*(%)	**rs3176975**	*Genotypes*	*n*(%)	*n*(%)
	GG	193(86.94)	61(87.14)		GG	140(59.32)	55(61.80)
	GA	28(12.61)	8(11.43)		GT	79(33.47)	31(34.83)
	AA	1(0.45)	1(1.43)		TT	17(7.20)	3(3.37)
	total	222	70		total	236	89
		P=0.656				**P=0.497**	
	Alleles				*Alleles*		
	G	0.932	0.929		G	0.761	0.792
	A	0.068	0.071		T	0.239	0.208
		P=0.876				**P=0.384**	
rs1801689	*Genotypes*	*n*(%)	*n*(%)	**rs1801690**	*Genotypes*	*n*(%)	*n*(%)
	TT	205(93.18)	65(94.20)		GG	224(89.96)	78(89.66)
	TG	14(6.36)	4(5.80)		GC	24(9.64)	9(10.34)
	GG	1(0.45)	0		CC	1(0.40)	0
	total	220	69		total	249	87
		P=1.000				**P=0.879**	
	Alleles				*Alleles*		
	T	0.964	0.971		G	0.948	0.948
	G	0.036	0.029		C	0.052	0.052
		P=0.661				**P=0.980**	

3.6 ASSOCIATION OF APOH POLYMORPHISMS WITH ANTIPHOSPOHLIPID ANTIBODIES (APA) IN WHITES AND BLACKS

The genotype and allele frequencies of twelve APOH SNPs were compared between APA negative and APA positive groups stratified by race (Table 12). Subjects that were "APA positive" included those that were positive for any of the three antipohospohlipid antibodies (aCL, LAC, and anti-apoH). APA negative individuals included those who were not positive for any of the APA. Only one significant difference was observed, and this was between allele frequencies of black cases and controls for *APOH*/8178848 (p=0.041).

Each of the twelve APOH SNPs was also analyzed for association with aCL, LAC, and anti-apoH separately in all subjects stratified by race. No significant differences were observed in these comparisons (data not shown).

In addition, Each of the twelve APOH SNPs was analyzed for association with aCL, LAC, anti-apoH, and any APA separately in cases and controls stratified by race. Once again, no significant difference was observed for these comparisons (data not shown).

Table 12. Distribution of APOH SNPs among whites and blacks with and without APA

APOH SNP	Genotype/Allele	Whites		Blacks	
rs3760292	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	TT	231(73.57)	253(71.67)	22(81.48)	38(82.61)
	TC	77(24.52)	89(25.21)	5(18.52)	8(17.39)
	CC	6(1.91)	11(3.12)	0	0
	total	314	353	27	46
		P=0.616		**P=1.000**	
	Alleles				
	T	0.858	0.843	0.907	0.913
	C	0.142	0.157	0.093	0.087
		P=0.427		**P=0.909**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs6933	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	AA	85(27.33)	117(32.87)	12(42.86)	21(45.65)
	AG	156(50.16)	167(46.91)	13(46.43)	17(36.96)
	GG	70(22.51)	72(20.22)	3(10.71)	8(17.39)
	total	311	356	28	46
		P=0.294		**P=0.612**	
	Alleles				
	T	0.524	0.563	0.661	0.641
	C	0.476	0.437	0.339	0.359
		P=0.153		**P=0.810**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178826	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	AA	301(95.86)	349(97.21)	27(100.00)	45(100.00)
	AG	13(4.14)	10(2.79)	0	0
	GG	0	(0.00)	0	0
	total	314	349	27	45
		P=0.397		**P=1.000**	
	Alleles				
	A	0.979	0.986	1.000	1.000
	G	0.021	0.014	0.000	0.000
		P=0.345		**P= ----**	

Table 12 continued. Distribution of APOH SNPs among whites and blacks with and without APA

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178848	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	GG	284(87.93)	324(87.57)	27(96.43)	38(84.44)
	GA	37(11.46)	43(11.62)	1(3.57)	6(13.33)
	AA	2(0.62)	3(0.81)	0	1(2.22)
	total	323	370	28	45
		P=1.000		**P=0.304**	
	Alleles				
	G	0.937	0.934	0.982	0.911
	A	0.063	0.066	0.018	0.089
		P=0.836		**P=0.041**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178847	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	GG	262(86.75)	281(87.27)	23(92.00)	33(80.49)
	GA	38(12.58)	40(12.42)	2(8.00)	7(17.07)
	AA	2(0.66)	1(0.31)	0	1(2.44)
	total	302	322	25	41
		P=0.890		**P=0.667**	
	Alleles				
	G	0.930	0.935	0.960	0.890
	A	0.070	0.065	0.040	0.110
		P=0.761		**P=0.115**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs1801689	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	TT	273(91.30)	293(92.43)	25(100.00)	39(100.00)
	TG	25(8.36)	24(7.57)	0	0
	GG	1(0.33)	(0.00)	0	0
	total	299	317	25	39
		P=0.710		**P=1.000**	
	Alleles				
	T	0.955	0.962	1.000	1.000
	G	0.045	0.038	0.000	0.000
		P=0.522		**P= ----**	

Table 12 continued. Distribution of APOH SNPs among whites and blacks with and without APA

APOH SNP	Genotype/Allele	Whites		Blacks	
rs2873966	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	GG	156(48.60)	190(51.49)	19(67.86)	34(72.34)
	GA	139(43.30)	145(39.30)	9(32.14)	13(27.66)
	AA	26(8.10)	34(9.21)	0	0
	total	321	369	28	47
		P=0.557		**P=0.794**	
	Alleles				
	G	0.702	0.711	0.839	0.862
	A	0.298	0.289	0.161	0.138
		P=0.718		**P=0.712**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs7211492	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	AA	272(83.95)	314(84.86)	25(89.29)	45(95.74)
	AG	49(15.12)	52(14.05)	3(10.71)	2(4.26)
	GG	3(0.93)	4(1.08)	0	0
	total	324	370	28	47
		P=0.927		**P=0.356**	
	Alleles				
	A	0.915	0.919	0.946	0.979
	G	0.085	0.081	0.054	0.021
		P=0.798		**P=0.336**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178839	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	TT	251(78.19)	286(77.30)	23(82.14)	31(67.39)
	TG	65(20.25)	80(21.62)	5(17.86	14(30.43)
	GG	5(1.56)	4(1.08)	0	1(2.17)
	total	321	370	28	46
		P=0.808		**P=0.326**	
	Alleles				
	T	0.883	0.881	0.911	0.826
	G	0.117	0.119	0.089	0.174
		P=0.904		**P=0.123**	

Table 12 continued. Distribution of APOH SNPs among whites and blacks with and without APA

APOH SNP	Genotype/Allele	Whites		Blacks	
rs8178858	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	GG	175(60.98)	216(63.53)	20(76.92)	37(84.09)
	GA	97(33.80)	108(31.76)	6(23.08)	7(15.91)
	AA	15(5.23)	16(4.71)	0	0
	total	287	340	26	44
		P=0.825		**P=0.531**	
	Alleles				
	G	0.779	0.794	0.885	0.920
	A	0.221	0.206	0.115	0.080
		P=0.509		**P=0.498**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs3176975	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	GG	177(60.20)	210(60.00)	9(34.62)	11(29.73)
	GT	99(33.67)	120(34.29)	12(46.15)	15(40.54)
	TT	18(6.12)	20(5.71)	5(19.23)	11(29.73)
	total	294	350	26	37
		P=0.965		**P=0.686**	
	Alleles				
	G	0.770	0.771	0.577	0.500
	T	0.230	0.229	0.423	0.500
		P=0.965		**P=0.392**	

APOH SNP	Genotype/Allele	Whites		Blacks	
rs1801690	*Genotypes*	APA -(%)	APA+(%)	APA -(%)	APA+(%)
	GG	292(90.40)	340(90.67)	28(100.00)	47(97.92)
	GC	31(9.60)	34(9.07)	0	1(2.08)
	CC	0	1(0.27)	0	0
	total	323	375	28	48
		P=0.944		**P=1.000**	
	Alleles				
	G	0.952	0.952	1.000	0.990
	C	0.048	0.048	0.000	0.010
		P=0.999		**P=0.315**	

4.0 DISCUSSION

Systemic lupus erythematosus (SLE) is an autoimmune disease of great public health import, the etiology of which is as yet poorly understood. One glycoprotein in particular, apoH, is known to complex with phospholipids and form an autoantigen that is recognized by antibodies present in autoimmune diseases such as SLE. These antiphospholipid antibodies (aCL, LAC, and anti-apoH) are of great clinical relevance due to their association with antiphospholipid antibody syndrome (APS), thrombosis, and SLE.

While some studies have identified functional polymorphisms in the coding regions of the APOH gene, intronic polymorphisms have not yet been analyzed. Further, TagSNPs have not previously been used in an association study with APOH and lupus. Since it is nearly impossible to analyze all of the polymorphisms in the APOH gene individually, TagSNPs are particularly useful to work with as any associations that are found to exist between a TagSNP and disease factors may indicate that one or more SNPs that are in linkage disequilibrium with the TagSNP could be involved in the association.

4.1 ASSOCIATION OF APOH POLYMORPHISMS WITH RACE

4.1.1 Distribution of APOH SNPs in whites and blacks

The frequencies of the APOH SNPs that were analyzed in this study have been reported for sample groups of African and Caucasian descent by both the Seattle SNP database and the HapMap project; however, these samples were small (n=23 for whites, n=24 for blacks in the Seattle SNP database, and n=60 for both subgroups in HapMap). In our larger Pittsburgh cohorts, significant allelic distribution differences between whites and blacks were found for nine of the twelve SNPs, while significant genotype differences were found for six SNPs.

For one TagSNP (*APOH*/rs3176975), the minor allele frequency (MAF) was significantly less for white subjects (0.225) compared to black subjects (0.467). A similar trend was reported by the Seattle SNP database (Table 13). The genotype distribution for this coding TagSNP was also significantly different between whites and blacks.

All of the other significant differences that were observed indicated a lower MAF in black subjects compared to white subjects. For *APOH*/rs8178858, the frequency of the minor allele (A) was 0.119 and 0.204 in blacks and whites, respectively. This MAF in blacks was more than twice the frequency reported by the Seattle SNP database (0.05), while the white MAF was similar (Table 13). The genotype distribution for this intronic TagSNP was also significantly different between whites and blacks.

For *APOH*/rs2873966, the frequency of the minor allele (A) was 0.163 and 0.294 in blacks and whites, respectively. A similar trend was reported by the Seattle SNP database (Table

13). The genotype distribution for this intronic TagSNP was also significantly different between whites and blacks.

For *APOH*/rs6933, the frequency of the minor allele (G) was 0.330 and 0.454 in blacks and whites, respectively. This MAF in blacks was almost twice the frequency reported by the Seattle group (0.18), while the white MAF was similar (Table 13). The genotype distribution for this TagSNP located in the 3′UTR was also significantly different between whites and blacks.

For *APOH*/rs1801689, the frequency of the minor allele (G) was 0.041 in whites and it was not present in blacks. HapMap reported the MAF to be 0.025 in whites and similarly absent in blacks (Table 13). The genotype distribution for this coding SNP was also significantly different between whites and blacks.

For *APOH*/rs1801690, the frequency of the minor allele (C) was 0.006 and 0.047 in blacks and whites, respectively. This MAF in whites was twice the frequency reported by the Seattle SNP database (0.02), while the allele was absent in blacks (Table 13). The genotype distribution for this coding SNP was also significantly different between whites and blacks. The single individual with the GC genotype in our Pittsburgh cohort may suggest a very low MAF in the African population, Caucasian admixture in this individual's ancestry, or a new mutation.

For *APOH*/rs3760292, the frequency of the minor allele (C) was 0.088 and 0.149 in blacks and whites, respectively. This MAF in blacks was about half as frequent as reported by the Seattle SNP database (0.17), while the white MAF was similar (Table 13). The genotype distribution for this promoter TagSNP was not significantly different between whites and blacks.

For *APOH*/rs7211492, the frequency of the minor allele (G) was 0.034 and 0.080 in blacks and whites, respectively. A similar trend was reported by the Seattle SNP database (Table 13). The genotype distribution for this TagSNP located in the 3′ flanking region was not significantly different between whites and blacks.

For *APOH*/rs8178826, the frequency of the minor allele (G) was 0.015 in whites and it was not present in blacks. The Seattle SNP database found the MAF to be 0.05 in whites and similarly absent in blacks (Table 13). The genotype distribution for this intronic TagSNP was not significantly different between whites and blacks.

These associations must all be interpreted under the caveat that the black sample size was 9 to 10 times smaller than the white sample size, with black sample sizes ranging from 67 to 89 individuals (depending on the SNP) (Table 8). Therefore, minor alleles with a very low frequency (especially less than 10%) might have been mis-represented due to sampling error, causing misleading p-values to be generated. All studies to date that have reported the frequencies of these SNPs in individuals of African descent have had small sample sizes, thus it is difficult to infer that our observed frequencies truly reflect the general population. All caveats aside, significant differences in the distribution of several APOH SNPs between whites and blacks were observed in this study; therefore, other analyses were performed in races separately.

Table 13. Minor allele frequencies (MAF) of APOH SNPs

Db SNP ID	MAF in Whites		MAF in Blacks	
	Seattle SNP *HapMap	Pittsburgh	Seattle SNP *HapMap	Pittsburgh
rs3760292	.17	.149	.17	.088
rs8178826	.05	.015	.00	.00
rs8178839	.07	.115	.17	.131
rs8178847	.07	.066	.05	.080
rs8178848	.05	.064	.03	.057
rs2873966	.22	.294	.19	.163
rs8178858	.24	.204	.05	.119
rs3176975	.26	.225	.50	.467
rs1801689	.025*	.041	.00*	.00
rs1801690	.02	.047	.00	.006
rs6933	.48	.454	.18	.330
rs7211492	.12	.080	.03	.034

4.1.2 Linkage disequilibrium analysis of APOH SNPs

Hardy-Weinberg equilibrium tells us how genotype frequencies at a single locus behave under a set of assumptions; however, to gain information about how genotype frequencies over two or more loci behave (ie, independently or dependently), linkage disequilibrium (LD) analysis is useful.

It is not yet possible to predict whether most noncoding polymorphisms have functional consequences. Testing candidate SNPs directly is a useful strategy because information gained about the disease association of one SNP has implications for other SNPs. For example, a lack of association found in one SNP would rule out functionally important changes at SNPs that are in tight LD with the candidate SNP (Carlson et al. 2004). Further, when a set of densely spaced

TagSNPs within one gene are directly tested, information can be indirectly gained about those unassayed SNPs that are in the same bin.

In this study, pairwise linkage disequilibrium (LD) analysis was performed for twelve APOH SNPs among white cases, white controls, and all white subjects combined. LD for the black population was not analyzed due to small sample size. One way LD is quantified is by D – a measure of how far off gamete frequencies are from what you'd expect (H_0) if no LD were present. D' (D/D_{max}) is a measure of how "scientifically significant" an observed correlation may be.

The results of this analysis revealed several D' values of 1.0, indicating that the allelic association is as strong as possible, given the allele frequencies at two sites. This is referred to as "complete LD." This is not surprising since these APOH SNPs are linked (located in close proximity). SNPs can be linked without being in linkage disequilibrium, since LD only exists if there is an association with a population. Further, pairwise LD analysis can yield only three haplotypes, not only if there is true correlation, but also if the minor allele frequencies of SNPs are so low that the fourth haplotype (with both minor alleles) is not seen. In our analysis, this may have been the case since we were dealing with several SNPs with low minor allele frequencies.

A problem with D' is that a D' of 1.0 can be observed even if there is low correlation. For low allele frequencies, r^2 has more reliable sample properties than D'. Only when genotypes are perfectly correlated does r^2 equal 1.0 ("perfect LD"), and this can only occur if the MAFs are equal (Carlson et al. 2004). The highest r^2 value observed between these twelve SNPs was 0.76 between *APOH*/rs8178847 and *APOH*/rs8178848 for the white cases only. For the majority of blocks, the r^2 was relatively low. Since this analysis was performed with nine TagSNPs, these

results are understandable using the rational that the TagSNPs are known to be in LD with other SNPs (not analyzed in this study), but not necessarily each other.

4.2 ASSOCIATION OF APOH POLYMORPHISMS WITH SLE

4.2.1 Association of APOH SNPs with SLE in whites and blacks

The apoH protein has been shown to have anti-atherogenic properties in addition to playing a role as a major target antigen for APA present in patients with APS and secondary APS, which may exist in concert with autoimmune diseases such as SLE. For these reasons, researchers have been interested in the relationship that APOH may have with SLE. Studies have found instances of functional polymorphisms in APOH, and association analyses have been performed to investigate the impact of these polymorphisms on disease risk (Steinkasserer et al. 1993; Sanghera et al. 1997b; Kamboh et al. 2004). In theory, when the function of a gene is altered by SNPs, there may be phenotypic consequence. Therefore, association analyses of individual APOH SNPs with SLE were performed for our study.

Association of each SNP with SLE was analyzed in whites and blacks separately by comparing genotype and allele frequencies in cases and controls. Results revealed no clear-cut associations of APOH SNPs with SLE risk. The lowest P-value observed was between allele frequencies of black cases and controls in *APOH*/rs8178858 (p=0.054), indicating borderline significance (our sample size was small, however, so this result must be interpreted with caution).

Most of the observed P-values for this analysis were large, meaning there was not enough evidence to reject the null hypothesis. This could be due to either a true lack of association or to our relatively small sample sizes (especially in the black cohort), and several SNPs with low MAFs. Therefore, our results warrant further investigation of the association of these APOH SNPs with SLE.

4.2.2 Haplotype associations with SLE

The maximum number of SNPs that could be efficiently handled by our haplotype software is nine; therefore, the three coding SNPs that were not TagSNPs (located in codons 136, 306, and 316) were excluded from haplotype analysis. EH and R statistical programs were used to analyze white cases and controls (excluding all samples with missing genotypes for any of the TagSNPs). These two programs yielded virtually identical haplotype frequencies. EH, however, is not able to generate meaningful P-values for an analysis of nine SNPs, given the high degree of freedom (df=511). R program sets a lower degree of freedom (df=18); therefore, P-values could be calculated by this program (R program output is reported in this document).

Haplotype analysis of 9 APOH TagSNPs in the white population revealed an overall significant difference between the distribution of haplotypes between cases and controls ($p<0.0001$), indicating the greater informative value of haplotype analysis compared to individual SNP analysis. There were four common haplotypes that are present in greater than 10% of the population (Haplotype #'s 1-4). Of the 11 most frequent haplotypes (pool frequency ≥ 0.01), six differed significantly between cases and controls (Haplotype #'s 1, 2, 3, 5, 7, and 9). Haplotype #'s 1, 2, 3, and 7 were significantly more frequent in the white control population

compared to white cases, suggesting a protective effect. In contrast, haplotype #'s 5 and 9 were more frequent in cases compared to healthy controls, indicating a potential risk factor for SLE.

The haplotypes with the most statistical significance (p=<0.001) were #'s 5 and 9. Of particular interest is haplotype #5, which is present in 16.2% of cases vs. 0.6% of controls (about 27 times more frequent in cases). This haplotype also contains the major allele from each TagSNP. Such a finding is interesting, since one would expect the most common haplotype to contain all of the major alleles; however in this study, it appears that the most common haplotype, #1, which exists in 19.9% of the total population (14.4% and 22.8% of cases and controls, respectively), contains the minor allele for *APOH*/rs2873966. This is the second most common TagSNP, with a MAF of 29.4% in our population. The next three most frequent haplotypes account for 44.5% of our population collectively. Given the fact that most of the TagSNPs used in this study are relatively rare in the general population, it is feasible that there is no one very frequent haploytpe.

It is possible that the reason why more significance was found by haplotype analysis than single site (LD) analysis is that several small single site effects could be additive when evaluated in sum. However, the most likely explanation is that untested and potentially functional variants are present on those haplotypes. Sequencing of these haplotypes in future studies may identify the functional variants.

4.3 ASSOCIATION OF APOH POLYMORPHISMS WITH RENAL DISEASE IN WHITE PATIENTS WITH SLE

As previously mentioned, lupus nephritis is one of the most common clinical manifestations of SLE, and polymorphisms in several genes have been shown to be associated with its occurrence. For example, an allele in PDCD1 (PD-1.3A) has been shown to be associated with renal manifestations in SLE patients from northern Sweden (Johansson et al. 2005). In addition, Tripi et al. (2006) identified three promoter SNPs in PON1 that were significantly associated with lupus nephritis. The underlying mechanism of this association is not yet determined; however, it is believed that increased susceptibility of LDL to oxidation in renal disease may be one explanation (Tripi et al. 2006).

To determine a possible association of APOH SNPs with lupus nephritis, the genotype and allele frequencies of twelve APOH SNPs were compared between white cases with and without renal disease. No associations between genotype distributions of the twelve APOH SNPs and renal disease in white patients with SLE were observed. Only one significant difference in allele distribution was found. The minor allele frequency of *APOH*/2873966 was significantly less for those with no renal disease than those with renal disease (P=0.023, MAF=0.232 vs. MAF=0.316). There were 97 patients in the group with no renal disease, which is a relatively small population; therefore, although this finding may warrant further investigation, it must be interpreted with the caveat of low statistical power. Further investigation could involve repeating the analysis with a larger patient sample or investigating associations between SNPs in the same bin as the *APOH*/2873966 TagSNP with renal disease in SLE patients.

4.4 ASSOCIATION OF APOH POLYMORPHISMS WITH ANTIPHOSPOHLIPID ANTIBODIES (APA) IN WHITES AND BLACKS

Antiphospholipid antibodies (APA) are known to be associated with several clinical manifestations of SLE, and apoH is the main target antigen for APA. The prevalence of anti-apoH antibodies has been shown to be greater than 30% in SLE patients of white ancestry (Kamboh et al. 1999) and 51% in Nigerian blacks (Kamboh et al. 2004). Several other studies have found the prevalence of anti-apoH antibodies to be 49-77% of patients with antiphospholipid syndrome (APS) of various ancestries, including white, African American, Asian, and Hispanic (Hirose et al. 1999; Atsumi et al. 1999; Prieto et al. 2003).

Association studies of several APOH polymorphisms with APA have been published. For example, the Val247Leu polymorphism has been shown to be associated with anti-apoH in patients with APS (Hirose et al. 1999; Atsumi et al. 1999; Prieto et al. 2003), but not in patients with SLE (Kamboh et al. 1999), secondary APS (Atsumi et al. 1999), or a general African black sample (Kamboh et al. 2004). Two functional polymorphisms in the fifth domain of apoH (Cys306Gly and Trp316Ser) have been shown to disrupt the normal binding of anionic phospholipids to apoH (Sanghera et al. 1997b; Mehdi et al. 2000). Three missense mutations in the third domain of apoH (Ile122Thr, Arg135His, and Ala141Asp) have been shown to affect the binding of cardiolipin to apoH (Kamboh et al. 2004).

In order to ascertain any association of twelve APOH SNPs with APA in patients with SLE, the genotype and allele frequencies of these SNPs were compared between white and black SLE cohorts that were APA negative or APA positive. Subjects that were "APA positive" included those that were positive for any of three antipohospohlipid antibodies: aCL, LAC, and β_2-GPI. No associations between genotype distributions of each of the twelve APOH SNPs and

whites and blacks that were APA negative or APA positive were observed. Only one significant difference was observed between allele frequencies of black cases and controls for one intronic TagSNP, *APOH*/8178848 (p=0.041). The black sample size was small, so future studies with a larger sample size may be needed to further investigate this preliminary association. Other future directions could involve investigating associations between SNPs in the same bin as the *APOH*/8178848 TagSNP with APA in SLE patients.

Each of the twelve APOH SNPs were also analyzed for association with aCL, LAC, and anti-apoH separately in SLE subjects stratified by race. No significant differences were observed in these comparisons. In addition, SNP genotype and allele associations with APA were analyzed in black and white cases and controls; once again, no significant differences were observed for this comparison.

The lack of significant association between these APOH SNPs and APA could be explained by a small black cohort. It is interesting that studies in the past found associations with the nonsynonymous coding SNPs ; however, our sample is larger than some of these past studies so the power of the statistical analysis of this study may be greater.

4.5 CONCLUSIONS

In this study, twelve APOH SNPs, including 5 intronic TagSNPs, one TagSNP in the 3′UTR, one TagSNP in the 3′ flanking region, one promoter TagSNP, one coding TagSNP, and three coding SNPs, were genotyped in SLE patients and healthy controls. Association tests were conducted comparing allele and genotype distributions of these SNPs with SLE, race, renal disease, and APA in patients with SLE. In addition, linkage disequilibrium (LD) analysis between all twelve APOH SNPs in white cases and controls and 9-site haplotype analysis including TagSNPs in all white subjects were performed. The results of these analyses are summarized here:

1) The distribution of allele frequencies was significantly different between blacks and whites for 9 of the 12 APOH SNPs analyzed
2) Pairwise LD analysis revealed complete LD (D'=1.0) between several APOH SNPs; however there were no r^2 values over 0.80, indicating a lack of high correlation.
3) The haplotype pattern of nine TagSNPs in the white samples was significantly different between cases and controls ($p<0.0001$). Two haplotypes had significantly higher frequency in cases (indicating potential disease risk), while four haplotypes had significantly higher frequency in controls (indicating potential disease protection).
4) No associations between genotype or allele distributions in any of the twelve APOH SNPs and SLE in whites or blacks were observed.
5) No associations between genotype distributions were observed in any of the twelve APOH SNPs and renal disease in whites with SLE.
6) No associations between genotype distributions in any of the twelve APOH SNPs and whites and blacks that were APA negative or APA positive were observed.

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