FUNCTIONAL CHARACTERIZATION OF APOLIPOPROTEIN H POLYMORPHISMS AND THEIR RELATION TO SYSTEMIC LUPUS ERYTHEMATOSUS

by

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Sequence variation in gene promoters is often associated with disease risk. In this study, a 1,418 bp sequence of the 5′-flanking region of the *APOH* (encoding for β_2-glycoprotein I) has been functionally characterized by *in vitro* analysis. Associations of *APOH* promoter SNPs with systemic lupus erythematosus (SLE) risk and related phenotypes along with their effect on human plasma β_2GPI levels were examined. Transient transfections, dual-luciferase reporter gene assays were performed in COS-1 and HepG2 cells. HepG2 nuclear extracts was used for electrophoretic mobility shift assay (EMSA). A case-control design and genotyped 345 SLE women and 454 healthy control women for 12 *APOH* promoter single nucleotide polymorphisms (SNPs) (-1284C>G, -1219G>A, -1190G>C, -1076G>A, -1055T>G, -759A>G, - 700C>A, -643T>C, -627A>C, -581A>C, -363C>T -38G>A, and -32C>A). Haplotype analyses were performed using EH (Estimate Haplotype-frequencies) and Haploview programs. Deletion analysis localized the core promoter of *APOH* ~160 bp upstream of ATG codon with the presence of critical *cis*-acting elements between -166 and -65. Functional relevance for three SNPs (-1219G>A, -643T>C and -32C>A) that resulted in lower promoter activity (51%, 40% and 37%, respectively) as compared to the wild-type alleles in COS-1 cells. EMSA demonstrated HepG2 nuclear protein(s) bind to the elements located in the regions of the three SNPs. Overall haplotype distribution of the *APOH* promoter SNPs was significantly different between cases

and controls (P = 0.009). The -643C allele was found to be protective against carotid plaque formation (adjusted OR = 0.37, P = 0.013) among SLE patients. Three-site haplotype analysis revealed one haplotype carrying -32A to be significantly associated with decreased plasma β_2GPI levels (P < 0.001) and another haplotype harboring the minor allele for -1219A showed a significant albeit less pronounced association (P = 0.046). Our data indicate that *APOH* promoter variants may be involved in the etiology of SLE, especially the risk for autoimmune-mediated cardiovascular disease.

Public Health Significance: Cardiovascular disease is the leading cause of death in the U.S. and other developed nations. Understanding its pathogenesis will help in formulating newer therapeutic strategies and treatment that may decrease the adverse consequences of accelerated atherosclerosis in SLE patients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 INTRODUCTION

1.1 SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a prototypic autoimmune inflammatory disease that can affect virtually any organ system. It primarily affects premenopausal women (female:male ratio = 9:1) and is characterized by a plethora of clinical manifestations, including glomerulonephritis, arthritis, pleuritis, pericarditis and vasculitis [1]. Many theories have been advanced as explanations for the gender imbalance in SLE prevalence, including genetic factors, differential environmental and occupational exposures, and hormonal effects [2]. There is a bimodal pattern of mortality in SLE, with early deaths from active disease, that included renal insufficiency, infection, and sepsis and late deaths from cardiovascular origin [3]. Active lupus is also characterized serologically by high titers of circulating antibodies directed against native DNA (anti-DNA antibodies), and the presence of these antibodies could pre-date disease manifestations by many years [4]. Nephritis is a predictor of poor prognosis and a major cause of death in patients with active SLE [5].

1.1.1 Autoantibodies in SLE

The serological hallmark of SLE is the production of autoantibodies directed against components of the cellular nucleus. Autoantibodies are typically present several years prior to diagnosis of

SLE and serve as markers for future disease in otherwise normal individuals [6]. Antinuclear antibodies (ANA) refer to a diverse group of antibodies that target nuclear and cytoplasmic antigens and have a role in transcription or translation, in the cell cycle or as structural proteins. The ANA test is the most common screening test used in the laboratory diagnosis of SLE. Virtually all patients with SLE have ANA while most patients with ANA do not have SLE.

Antibodies to double-stranded DNA (anti-dsDNA) are of great importance in SLE and nephritis, since presence of anti-dsDNA and its titer have been correlated with SLE activity [7]. These are among the most specific autoantibodies in SLE. However, it is not particularly sensitive owing to the fact that it may be present transiently, occurring in only 50–60% of lupus patients at some point in the course of their disease [6]. Among the other autoantibodies are anti-histone and antibodies to extractable nuclear antigens (ENA) [RNP, Sm, SS-A (Ro), or SS-B (La)].

Antiphospholipid antibodies (APA) are autoantibodies associated with thrombophilic disorder named antiphospholipid syndrome (APS), defined by arterial and/or venous thrombosis, recurrent fetal loss, and neurologic disorders. APA represent a group of heterogeneous antibodies [anticardiolipin antibodies (aCL), lupus anticoagulant (LAC), and anti-β_2-glycoprotein I (anti-β_2GPI)] which are detected in a variety of conditions, including SLE. APA are found in ~ 1-10% of a general Caucasian US population *versus* (*vs.*) 30-70% in patients with SLE and APS [8-10].

1.1.2 Diagnosis of SLE

Diagnosis is not easy as there is no single set of symptoms that are uniformly specific to SLE and no laboratory tests conclusively prove that a person has or does not have SLE. For an early

diagnosis, a complete systems review, analysis of the urine for blood and protein, a complete blood count that can reveal cytopenia or anaemia, liver and kidney function, ANA testing, testing for autoantibodies to dsDNA, Ro (SS-A), La (SS-B), nRNP, Sm autoantigens, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels (a high ESR with normal CRP levels can indicate SLE) needs to be carried out. APA should be evaluated in women with thrombotic events or with a history of morbidity in pregnancy. Correct diagnosis of SLE, whether affecting an adult or a child, requires an integration of a patient's symptoms, physical examination findings and the results of diagnostic testing. The revised American College of Rheumatology (ACR) classification criteria is commonly used by rheumatologists to diagnose SLE (Table 1). In these classification criteria, a person is said to have lupus if they have 4 or more of the 11 criteria, either simultaneously or over time. The 1982 criteria were further revised in 1997 by the Diagnostic and Therapeutic Criteria Committee of the ACR to delete the lupus erythematosus (LE) cell preparation, which was not frequently used, and add changes in the immunologic disorder category.

Table 1. ACR Criteria for Classification of SLE

ACR 1982 criteria	ACR 1997 revised criteria
Malar Rash	Malar Rash
Discoid Rash	Discoid Rash
Photosensitivity	Photosensitivity
Oral *or* nasal ulcerations	Oral *or* nasal ulcerations
Nonerosive Arthritis	Nonerosive Arthritis
Pleuritis *or* Pericarditis	Pleuritis *or* Pericarditis
Nephritis/Renal Disorder - Proteinuria > 0.5g/day, *or* > 3+ *or* Cellular casts	Nephritis/Renal Disorder - Proteinuria > 0.5g/day, *or* > 3+ or Cellular casts
Neurologic Disorder – Seizures *or* Psychosis	Neurologic Disorder – Seizures *or* Psychosis
Hematologic Disorder - Hemolytic anemia *or* Leukopenia *or* Lyphopenia *or* Thrombocytopenia	Hematologic Disorder - Hemolytic anemia *or* Leukopenia *or* Lyphopenia *or* Thrombocytopenia
Immunologic Disorder - Anti-DNA: antibody to native DNA *or* Anti-Sm: antibody to Sm nuclear antigen *or* Positive LE cell preparation *or* Biologically false-positive test for Syphilis	Immunologic Disorder - Anti-DNA: antibody to native DNA *or* Anti-Sm: antibody to Sm nuclear antigen *or* Positive finding of antiphospholipid antibodies on: 1. an abnormal serum level of IgG or IgM anticardiolipin antibodies, 2. a positive test results for lupus anticoagulant using a standard method, or 3. a false-positive test for at least 6 months confirmed by Treponema pallidum Immobilization or fluorescent treponemal antibody absorption test
Positive Antinuclear Antibody	Positive Antinuclear Antibody

Adapted from Tucker, 2007 [11]

1.1.3 Clinical Features of SLE

Skin disease is the second most frequent clinical manifestation of SLE [12]. The current ACR classification criteria for SLE include four somewhat overlapping dermatologic criteria, butterfly rash, discoid lupus, photosensitivity, and oral ulcers and thus patients can be classified as having

SLE with only skin manifestations (cutaneous lupus erythematosus). 50–100% of SLE patients have fatigue, weight loss or fever in the absence of infection. Musculoskeletal problems (arthritis, myositis or arthralgia) and renal problems are found in SLE patients (proteinuria, haematuria). Haematological problems include thrombocytopenia, anaemia or leucopenia. Among the other complications include reticuloendothelial anomalies (splenomegaly, hepatomegaly); neuropsychiatric problems (seizures, psychosis); and gastrointestinal problems (vomiting, nausea or abdominal pain). Cardiac abnormalities are seen in 15% of patients (endocarditis, pericarditis or myocarditis). Pulmonary problems are seen in 2–12% of the affected (pulmonary hypertension). Both clinically and scientifically, the variable organ manifestations of SLE pose a particular challenge to rheumatologists. Validated scores for disease activity of SLE include British Isles Lupus Assessment Group (BILAG) index, the European Consensus Lupus Activity Measurement (ECLAM), the Systemic Lupus Activity Measure (SLAM), the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and the Lupus Activity Index (LAI). In addition to the assessment of disease activity, the evaluation of damage using the validated SLICC/ACR damage index and health-related quality of life (MOS SF-36) is also advised for clinical research [13].

1.1.4 SLE and Cardiovascular Risk

A large body of evidence indicates that aCL are significantly associated with thrombotic events in patients with SLE [14]. Several subsequent reports have supported an increased cardiovascular risk among SLE patients. Cohort studies have shown that the increased cardiovascular risk in SLE patients cannot be fully explained by the higher prevalence of traditional risk factors such as

hypertension and dyslipidemia and that disease-specific factors play an additional role [15-17]. Several studies have recently demonstrated that risk factors for cardiovascular disease (CVD) in SLE are both traditional and nontraditional risk factors acting in concert [18]. Traditional risk factors implicated in SLE include, for example, dyslipidemia (especially high triglycerides), hypertension, and renal disease. While non-traditional risk factors such as inflammation, APA and low-density lipoprotein (LDL) oxidation are also associated with CVD in SLE. Many reports have been published that have assessed the prevalence and risk factors for the development of accelerated atherosclerosis in SLE patients [19]. Case-control studies confirmed that atherosclerosis develops prematurely, independently of traditional risk factors for CVD [20, 21]. Premature coronary heart disease (CHD) is a major cause of morbidity and mortality in SLE patients; more pronounced in younger women (35-44 years) for whom the estimated risk is more than 50 fold [22]. Although the pathogenesis of vascular disease in SLE is unknown, it is thought to be multifactorial and of atherosclerotic origin. Atherosclerosis involves both atherosis (fatty degeneration) and sclerosis (vessel stiffening). Both of these processes can be evaluated with noninvasive ultrasound imaging techniques. Atherosis in the carotid arteries is evaluated using B-mode ultrasound, which measures the degree of focal plaque and intima-media wall thickness (IMT). Vascular stiffness is evaluated using pulse-wave velocity (PWV), which measures the rate at which arterial pulse waves move along the vessel. Plaque and IMT are measures of the structural properties of the arterial wall, whereas vascular stiffness is a measure of the functional properties. Longitudinal studies have shown that all three of these subclinical measures of CVD are significant predictors of future cardiovascular events and mortality [23]. Both carotid IMT and plaque progression can be measured using B-mode ultrasound in women with SLE. Using these modalities, increased rates of carotid focal plaque (37.1% *vs.* 15.2%) [20] and coronary

calcium (30.7% *vs.* 8.7%) [24] have been reported in women with SLE compared with controls. Plaque progression appears to be greater in women with SLE than in healthy women, and monitoring its progression may be more useful than monitoring IMT in women with SLE. Rates of progression of both IMT and plaque have been related to both SLE-associated and traditional risk factors, and these may work together to increase CVD risk [23]. To date, IMT and plaque assessment have not been used in longitudinal studies of women with SLE to evaluate rates of progression and the factors that predict change in subclinical atherosclerosis. Recently, Thompson et al (2008) [25] have reported the use of carotid B-mode ultrasound as a surrogate end point in future clinical trial to examine the efficacy of CVD prevention strategies in SLE patients. The three major steps to SLE-related atherogenesis are illustrated in Figure 1: (A) Endothelial injury and leukocyte recruitment, (B) Foam cell formation and progression to the fibrous plaque, and (C) Plaque rupture and thrombosis. Endothelial injury and leukocyte recruitment that can act as autoantigens promote local inflammation, foam cell formation and development of fibrous plaque [26]. This unchecked inflammatory response can lead to increased vulnerability of the plaque to rupture and to form an acute occlusive thrombus.

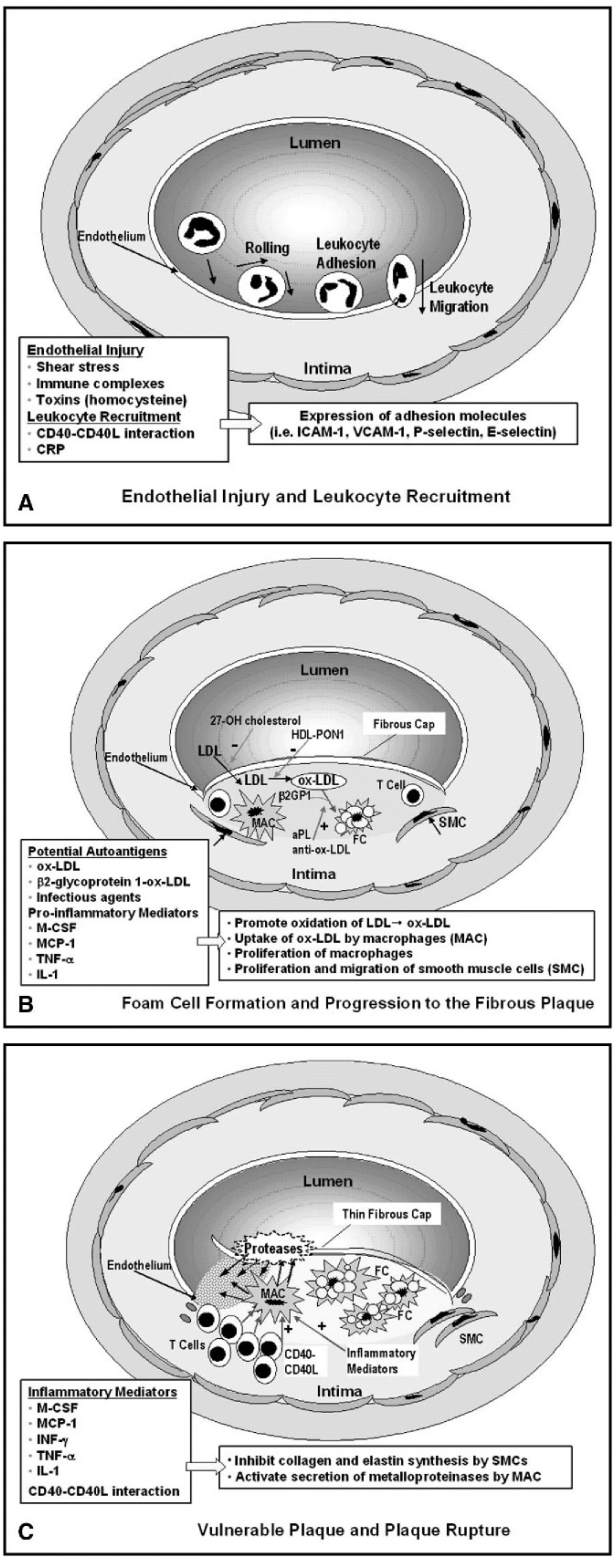


Figure 1. Three major steps involved in the vascular biology of atherogenesis seen in SLE
From Kao et al. 2003 [26]

1.1.5 SLE and Lupus Nephritis

Lupus nephritis is a serious manifestation in SLE, which affects over half of the SLE population, and could result in either acute or chronic renal failure. Cardinal features of lupus nephritis include the deposition of immunoglobulins within the glomerular and tubular basement membranes and amplification of local inflammatory responses, leading ultimately to renal fibrosis. Renal involvement in SLE is an important cause of morbidity and mortality, reaching a prevalence of 39% during the course of the disease [5] . Kidney involvement in patients with SLE is a common and serious complication that is often associated with a poor long-term prognosis. Aggressive immunosuppression is effective in controlling renal lupus flares and has improved disease outcomes, but is associated with significant morbidity (infection, malignancy, metabolic disturbances, infertility) and mortality [27]. While many different immunologic and non-immunologic factors contribute to disease expression in lupus nephritis, a large body of evidence suggests that the production of anti-DNA antibodies and the formation of glomerular immune deposits are important initial events in the pathogenesis of the disease [28]. The diversity of clinical presentations of lupus nephritis parallels the diversity of pathologic lesions seen in the kidneys of patients with SLE [29]. Renal manifestations range from asymptomatic hematuria (presence of red blood cells in urine) or proteinuria (presence of excess serum proteins in urine) to overt nephritic and nephrotic syndromes, rapidly progressive glomerulonephritis, and chronic renal failure. The criterion for diagnosis of a renal disorder includes the presence of: a) persistent proteinuria of greater than 0.5 g per day (or greater than 3+ urine dipstick reaction for albumin), or b) cellular casts, including red blood cell, hemoglobin, granular, renal tubular cell, or mixed. Urinalysis is one of the most important and effective methods to detect and monitor the activity of lupus nephritis [30]. Depression of classic complement pathway components and

high titers of anti-DNA, anti-nucleosome, or anti-C1q antibodies identify patients are increased risk of renal involvement or flares of nephritis [29]. Compelling evidence suggests that anti-DNA antibodies, in addition to being an important diagnostic marker, are also actively involved in the pathogenesis of lupus nephritis through their ability to bind to cell surface antigens or components of the glomerular basement membrane either directly (cross-reactivity) or indirectly (via chromatin material) [31].

1.1.6 Epidemiology of SLE

SLE is a heterogeneous disease with a multifactorial aetiology. There is now enough evidence to support ethnic and geographical variations in the manifestations of SLE including disease frequency, serological changes, organ involvement and overall prognosis. There are several studies of SLE prevalence and incidence in different areas of the world. Danchenko et al. (2006) [32] have summarized the results of over 60 such studies. A number of studies carried out in North America have shown Hispanics and American Blacks to have an excess morbidity from SLE and a younger age of onset than Caucasians [33-35]. Similar studies have also shown evidence for an excess in the prevalence of SLE among Orientals as compared to Caucasians [33]. The lowest incidences of SLE were seen among Caucasian Americans, Canadians and Spaniards with incidences of 1.4, 1.6 and 2.2 cases per 100,000 people respectively. Throughout Europe, the highest incidences were found in France (5 cases/100,000), Sweden (4.7 cases/100,000), and in Asian (10 cases/100,000) and Afro-Caribbean (21.9 cases/100,000) residents of the UK. The prevalence patterns summarized by Danchenko et al. (2006) [32] were similar to those of incidence, but no clear north–south or east–west pattern emerged. A higher

prevalence of SLE among people of sub-Saharan African descent living in North America, the Caribbean and Europe, compared with residents of sub-Saharan Africa, was summarized by Bae et al. (1998) [36]. Whether these variations are secondary to genetic differences or environmental changes such as socioeconomic status, availability of healthcare facilities, certain toxins, and dietary habits is not fully understood. However, it is likely to be due to interplay of these known, and other yet unrecognized factors.

1.1.7 Childhood SLE

SLE is a relatively rare disease in childhood, with estimated incidence ranging from 10 to 20 per 100,000 children, depending on the ethnic population [11]. Approximately 15% of individuals with SLE will have the onset of their disease prior to the age of 18 years; children and adolescents generally have a more severe disease presentation, develop disease damage more quickly than adults with SLE and have a higher overall burden of disease over their lifetimes [37]. The 1997 ACR classification criteria for SLE, which are, in general, used as diagnostic criteria among adult rheumatologists, are appropriate for use in children and adolescents. The pattern of clinical and serologic presenting features in children and adolescents with SLE is different in general from the adult SLE population, with a more severe disease onset in general. Early and accurate diagnosis of SLE in children and adolescents is crucial for initiating treatment to prevent damage.

1.1.8 Genetic Model of SLE

Genetic factors play an important role in SLE susceptibility, as reflected by heritability of up to 66% [38] and a 20-80 times greater risk for family members [39]. Concordance rates in twin studies range from 2% to 5% for dizygotic twins and 24–60% for monozygotic twins [40-42]. The current model for the development of autoimmune disease such as SLE involves multiple different susceptibility genes interacting with a variety of potential environmental exposures (Figure 2). Several environmental (or non-genetic) factors have been discussed in SLE susceptibility, including viruses, hormones, smoking, alcohol intake, exposure to aromatic amines, pesticides, silica, organic solvents, heavy metals and ultraviolet light, as well as dietary factors such as alfalfa sprouts and saturated fats [43]. Environmental exposures may lead to the production of autoreactive T cells and autoantibodies, the stimulation of pro- and anti-inflammatory cytokines, and target end-organ damage, but are not very convincing as agents causing SLE. Hormones have multiple influences on the regulation of the immune system and profound effects in murine models of lupus [44]. Menopausal factors such as menopausal status, age at menopause and postmenopausal hormone (PMH) therapy also have been evaluated as risk factors for SLE. The association of hair dyes with the risk of lupus has been examined in several other case-control studies in a variety of settings [45] and none or only a very weak association was found. Ultraviolet light is a well-known factor capable of eliciting a flare in SLE patients [46]. This is thought to be dependent on the capacity of particularly ultraviolet B (UVB) to induce apoptosis in keratinocytes due to formation of reactive oxygen species (ROS) and by direct DNA damage, but may also be related to altered cytokine expression patterns or effects of vitamin D production [47]. The anti-SS-A/Ro antibody is the most closely associated with photosensitivity in SLE patients. Studies exploring the role of socioeconomic factors as risk

factors for SLE are limited, although poorer outcomes and higher disease activity have been associated with measures of socioeconomic status [48]. In SLE, epigenetic mechanisms has been proposed as important, possibly being the target for drugs and environmental factors thus mediating the interaction between genes and the environment [49].

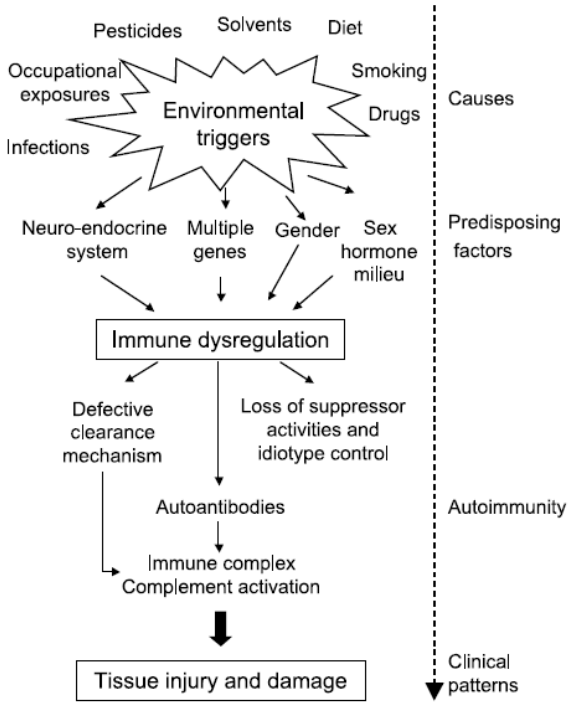


Figure 2. Role of Environment in the pathogenesis of SLE
From Sarzi-Puttini et al. 2005 [46]

1.1.9 Genetics of SLE

To date, two high-density case-control genome-wide association analyses have been published for SLE that have provided further support for several existing SLE susceptibility genes and have also helped to identify novel candidate genes [50, 51]. One study by The International

Consortium for Systemic Lupus Erythematosus Genetics (SLEGEN) used a cohort of 720 SLE cases and 2337 controls. Genotyping was performed using the Illumina HumanHap 300 BeadChip, resulting in the successful genotyping of over 300 000 SNPs [50]. The second study used a cohort of 1311 cases and 1783 controls [51]. This study used the Illumina HumanHap550 bead chip, resulting in over 500 000 SNPs successfully genotyped. Both studies confirmed strong genome-wide significance for the established susceptibility genes, the major histocompatibility complex (MHC) locus, interferon regulatory factor 5 (*IRF5)* and signal transducer and activator of transcription 4 (*STAT4*). Additional risk loci were identified for SLE susceptibility including B cell receptor signaling (B-lymphoid tyrosine kinase), *BLK–C8orf13* on chromosome 8 and integrin, alpha M complement component 3 receptor 3 subunit (*ITGAM)* and integrin, alpha X complement component 3 receptor 4 subunit (*ITGAX)* on chromosome 16.

1.1.10 Current SLE Candidate Genes

Linkage has proven a rather poor way to actually identify candidate genes in lupus. True candidates have been identified by a variety of other methods including association studies, mouse work, expression data and studies in other diseases. The human MHC on chromosome 6p11-21 has shown the most consistent and significant linkage and association of all the lupus susceptibility genes [52]. The SLEGEN genome wide association analysis has now provided evidence of independent signals arising from both human leukocyte antigen (*HLA)* Class II and Class III regions [50]. Even before the genome-wide association studies, there was growing evidence to implicate *IRF5* as an important SLE susceptibility gene. The type I interferon (IFN-*α* and -*ß*) system has been proposed as having a pivotal role in the development and maintenance

of the disease process in SLE [53]. Three functional polymorphisms have been identified in *IRF5* that are associated with SLE susceptibility; SNP rs2004640 where the associated T allele creates a donor splice site allowing expression of several unique *IRF5* isoforms [54-56], a second mutation rs10954213 located in the 3´untranslated region (3´UTR) that creates a functional polyadenylation site and hence a shorter and more stable gene transcript [55], and an insertion/deletion in the 6th exon that defines the isoforms of *IRF5* that are translated into protein [57].The best current genetic model for *IRF5* is a SLE risk haplotype carrying functional mutations that is associated with higher serum IFNα activity in SLE patients positive for either anti-RNA binding protein or anti-dsDNA autoantibodies [58]. The broad range of immunological function of candidate genes highlights the complexity of lupus pathogenesis. To some extent they may be grouped into functional categories. Complement component *C1q*, mannose binding lectin *(MBL)* and C- reactive protein (*CRP)* may affect availability of nuclear antigens [59]; tyrosine kinase *(TYK2)* [54] and toll-like receptors (*Tlr7)* (mouse) [60] are involved in the activation of innate immune mechanisms; cytotoxic T lymphocyte antigen-4 *(CTLA4)* [61], programmed cell death 1 (*PDCD1)* [62, 63], protein tyrosine phosphatase nonreceptor 22 (*PTPN22)* [64] and the signaling lymphocyte activation molecule (*SLAM)* genes (mouse) [65] affect the activity and survival of potentially autoreactive B- and T-cells; while the immunoglobulin Fc γ receptor genes [66] play an important role in the regulation of the humoral induced-immune response. Among the other candidate genes included are *ITGAM* [67], *STAT4* [68], *BLK* [51], B-cell scaffold protein with ankyrin repeats (*BANK1*) [69], and tumor necrosis factor superfamily 4 (*TNFSF4*) [70]. Recent reports have identified SNPs in the tumor necrosis factor alpha–induced protein 3 (*TNFAIP3*) region to be associated in the etiology of SLE among individuals of European ancestry [71]. The *TNFAIP3* encodes an ubiquitin editing enzyme, A20

that restricts NF-$_K$B–dependent signaling and prevents inflammation. Recently, a 'genetic hypothesis' was postulated elucidating the role of key SLE candidate genes in the immunological mechanisms associated with SLE disease pathogenesis (Figure 3) [72].

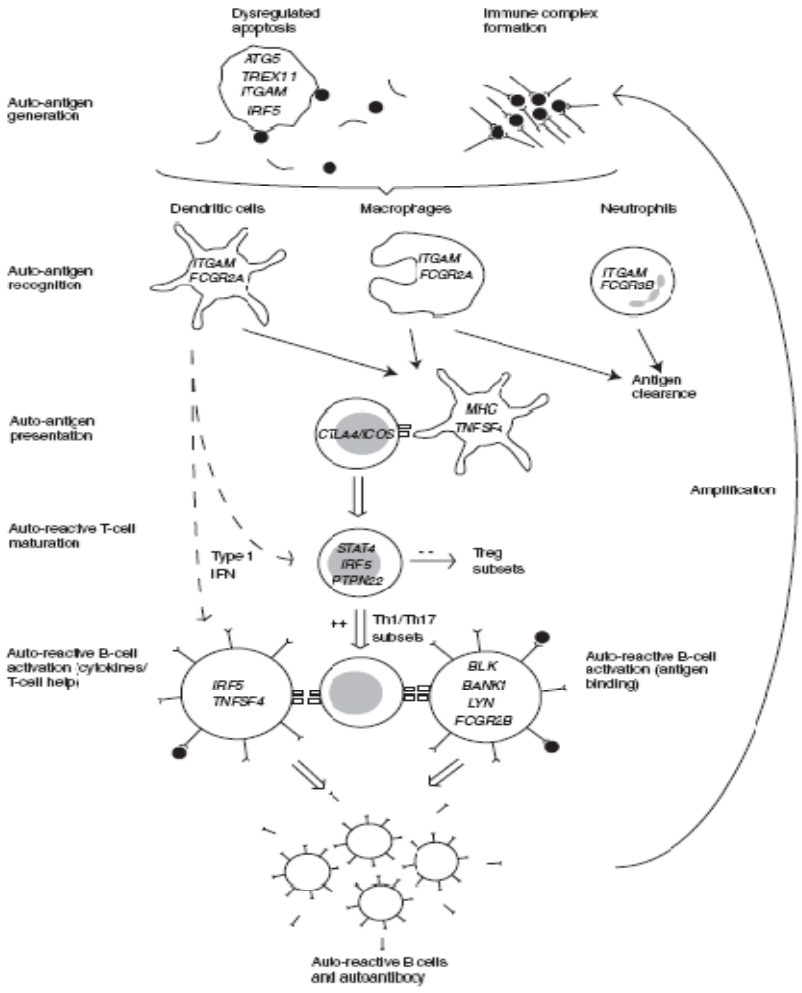


Figure 3. Proposed mechanism for the role of key candidate genes in the SLE pathogenesis
From Rhodes and Vyes, 2008 [72]. Genes are italicized within cells that are sites of their primary expression

1.1.11 Prognosis and Preventive strategies in SLE

The prognosis of patients with SLE has improved tremendously in the past few decades. While the 5-year survival of SLE was below 50% in the 1950s, recent studies report survival of 85% at 10 years and 75% at 20 years [73]. The main reasons for this improvement are the efficacy of current treatment protocol and the development of laboratory tests to detect ANA, which has made the early diagnosis of SLE easier in a great amount of cases. Despite the improvement of SLE short- and medium term survival observed in the last decades, the long-term prognosis of these patients remains poor, mainly due to complications of the disease and/or of its treatment. Therefore, long-term prognosis in SLE can be improved by adopting strategies early in the disease course that can contribute to reducing long-term complications, including screening for and prophylaxis against infections, control of risk factors for atherosclerosis, and cancer surveillance [74]. Infections are an important cause of hospitalization and death in SLE. All types of infections have been reported: bacterial, viral and opportunistic [46]. Among the main risks factors for infections in SLE, there are severe disease manifestations, particularly glomerulonephritis, and corticosteroid and immunosuppressant use. All patients should be screened for *Mycobacterium tuberculosis* and for viral infections, including human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV), before starting treatment with corticosteroids and immunosuppressants. Regular surveillance and control of traditional risk factors is mandatory, and so is the appropriate use of the statins, anti-malarial agents, anti-platelet agents, and anti-coagulation for primary and secondary prevention of arterial thromboembolism in SLE patients [75]. Control of disease activity and/or severity using the lowest effective dosage of corticosteroids is also recommended. For prevention and/or early diagnosis of malignancies, it is important that SLE patients regularly undergo the age-

related specific cancer screening recommended for the general population. Moreover, strategies directed at preventing clinical manifestations in asymptomatic ANA–positive individuals or in APA–positive SLE patients, as well as at preventing severe manifestations in patients with mild SLE at the time of the diagnosis should be considered [74]. To date; there have been only few high quality randomized controlled trails (RCTs) in SLE, particularly for manifestations other than nephritis and thus, several important issues have not been adequately addressed. Importantly, end-points currently used in SLE trials have not been validated in clinical trials. These findings underscore the need to establish international networks to facilitate clinical trials addressing these issues and testing new therapies. Recently, the European League Against Rheumatism (EULAR), have developed recommendations for the management of SLE and for points to consider in the design of SLE trials [76].

1.2 APOLIPOPROTEIN H

Human apolipoprotein H (APOH), also known as β_2-glycoprotein I (β_2GPI), is a plasma glycoprotein first discovered in 1961 [77] (in this study, we will use *APOH* to refer to the gene as used in human genome databases and β_2GPI to refer to the protein as commonly used in the rheumatology literature). β_2GPI is a major autoantigen recognized by predominant APA found in sera of many autoimmune diseases such as primary antiphospholipid syndrome (PAPS) and SLE. The plasma concentration of this glycoprotein is approximately 20 mg/dL of which 40% is bound to very low-density lipoprotein (VLDL), high-density lipoprotein (HDL), and chylomicrons [78]. β_2GPI is expressed primarily in the liver and sporadically in intestinal cell lines and tissues [79]. β_2GPI is a major urinary protein excreted in patients with renal tubular

diseases such as adult Fanconi syndrome [80]. The level of *APOH* mRNA expressed by HepG2 cells is downregulated when incubated with inflammatory mediators, implying that β_2GPI is a negative acute-phase protein [81].

1.2.1 Structural Characterization of Apolipoprotein H

1.2.1.1 5'-Upstream Region

Primer extensions determined alternate transcription start sites (TSSs) at 31 base pairs (bp) and 21 bp upstream of the translation start codon [82]. TSS 31 bp upstream agreed completely with the consensus for an initiator element (*Inr*) known to sustain transcription initiation. Wang and Chiang (2004) [83] have initially characterized the 5′-flanking region and proximal promoter of *APOH* using transient transfection assays in hepatoma cell lines. Serial deletion analysis of *APOH* 5′-flanking sequence revealed strong promoter activity in the regions between -197 to +7 in hepatoma cells but not in HeLa cells. Several consensus sequence elements relevant for regulation of transcription in the liver were determined in the 5′ upstream region of *APOH*. Gene expression of *APOH* is governed by distinct liver-specific transcriptional regulation involving an atypical TATA box (TATTTA), located between positions -97 and -92 and a hepatic nuclear factor-1α (HNF-1α) element binding between regions -65 to -51, that play an essential role in the transcriptional initiation of the *APOH* promoter. Co-transfection of *APOH* promoter-luciferase vector with HNF-1α expression vector in Huh7 and HNF-1-deficient HeLa cells demonstrated the transactivation effect of HNF-1α on *APOH* promoter activity. In addition, overexpression of HNF-1α also enhanced the endogenous β_2GPI expression. These results indicated that both the

atypical TATA box and HNF-1α *cis*-element are critical for *APOH* transcription and the HNF-1α maybe mediating cell type-specific transcriptional regulation leading to differential expression of *APOH* in humans.

1.2.1.2 Gene Structure of Apolipoprotein H

The human *APOH* locus has been mapped to chromosome 17q23-qter using somatic cell hybrids [84] and was localized to chromosome 17q23–24 using fluorescent in situ hybridization [82]. *APOH* spans 18 kilobases (kb) on chromosome 17q23-24 and is comprised of eight exons that encode for a mature protein of 345 amino acid (aa) residues, including 19 aa in the signal peptide which is cleaved in the mature protein [81, 84, 85]. The 326 amino acid sequence was originally determined by sequencing the purified protein and was found to be one of the most proline-rich eukaryotic proteins with a frequent occurrence of cysteine-proline linkages at regular intervals [82]. *APOH* cDNA has been cloned and sequenced from human hepatic cells and the hepatoma cell line HepG2 that are major sites of biosynthesis for β_2GPI [81, 84]. β_2GPI is a single chain glycoprotein of 326 amino acid residues containing 22 cysteine (Cys) residues that form 11 disulfide bridges and four N-linked glycosylation sites [84, 85] The positions of all 11 disulfide bonds in human β_2GPI are identical to those seen in bovine β_2GPI [86]. The amino acid sequence can be divided into four contiguous homologous regions of about 60 aa residues known as short consensus repeats (SCRs) and an additional fifth C-terminal domain. The SCRs, also known as GP–1 domains, sushi domains, and complement control protein (CCP) repeats are widely distributed in the proteins of the complement system and also in some non-complement proteins such as the interleukin-2 receptor and the selectin family of endothelial leukocyte adhesion

molecules [84]. Each β_2GPI SCRs contain four invariant Cys residues that form two disulfide bridges in a conserved pattern of Cys-1 bound to Cys-3 and Cys-2 to Cys-4 [82]. In contrast to the first four SCR domains, the fifth and most C-terminal domain has six Cys residues resulting in three disulfide bonds that have been mapped with the disulfide linkage pattern shown to be as Cys 1-4, Cys 2-5, and Cys 3-6 [84].The cDNA consists of eight exons (~ 1.2 kb), separated by large introns (~ 16.2 kb) [82]. Exon 1 codes for the 5′-UTR and the signal peptide, SCRs 1, 3, and 4 are encoded by a single exon while SCRs 2 and 5 are encoded by two exons and exon eight also codes for the C-terminus and 3′-UTR (Figure 4).

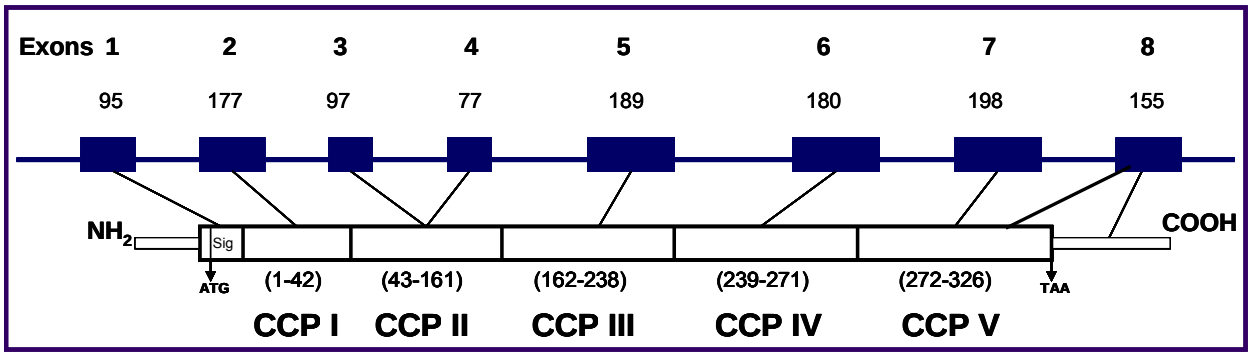


Figure 4. Schematic representation of human *APOH* showing the intron-exon organization

The positions of exons are shown as blue boxes, and introns are shown as lines connecting the exons. CCP repeat modules are shown below as connected rectangles. Position of translation initiation codon (ATG) and termination codon (TAA) are also shown.

1.2.1.3 Crystal Structure of β_2-glycoprotein I

Crystal model of β_2GPI containing 326 aa and four glycans revealed four CCP modules and a distinctly folded fifth C-terminal domain arranged like beads on a string to form an elongated J-

shaped molecule [87, 88]. The four N-terminal CCP modules are arranged along their long axis in a slight right-handed spiral to join the distinct fifth domain (Figure 5). The secondary structure of each module is comprised of several short, anti-parallel β-strands wrapped around a well defined hydrophobic core. The structure is stabilized by two disulfide bonds that are N- and C-terminally disposed at opposite poles of the module. The β-strands run approximately parallel and anti-parallel to the long axis, with the first and third turns located close to the C-terminus and second and fourth turns near the N-terminus. The fifth domain folds into a central β-spiral composed of four anti-parallel β –sheets and one short α-helix that are held together by three disulfide bridges in a 1–4, 2–5, 3–6 pattern.

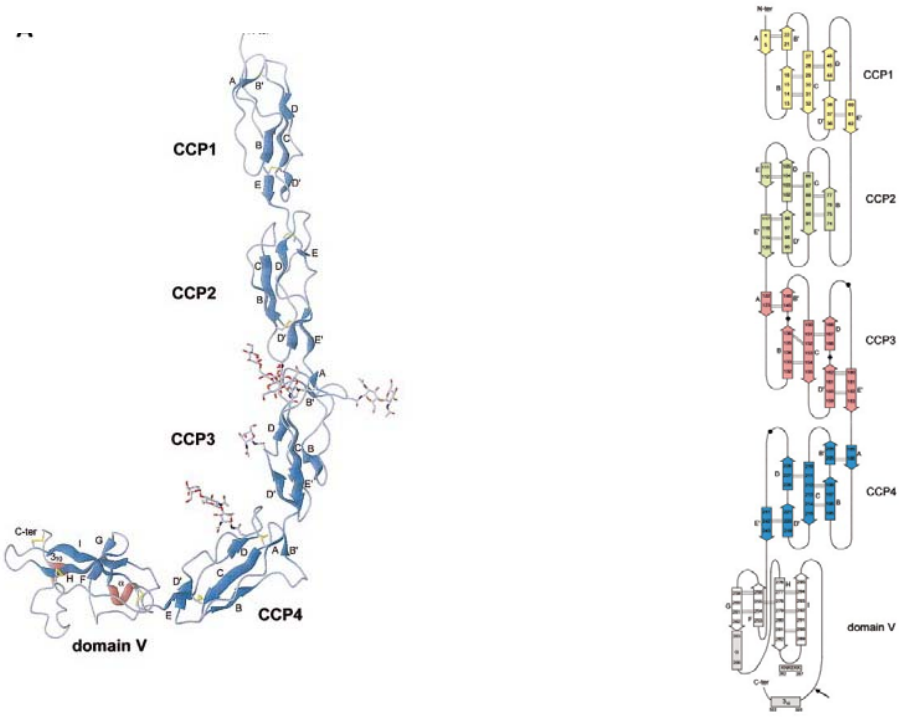


Figure 5. Crystal (secondary) structures of β_2GPI
From Schwarzenbacher et al. 1999 [88]

1.2.1.4 Fifth Domain of β_2-glycoprotein I

The first four GP-I domains (~60 aa each) of β_2GPI are all structurally related, while the fifth domain (84 aa) is the most variable and includes a cluster of lysine residues (282–287), four highly conserved hydrophobic amino acids (313–316), and three disulfide bonds instead of two, as present in each of the preceding four domains. This variable configuration of the fifth domain of β_2GPI is essential for the binding of β_2GPI to anionic phospholipids (Figure 6) [89].

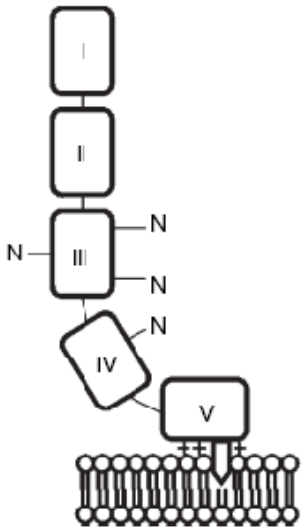


Figure 6. Schematic diagram of the domains of β_2GPI
From de Groot and Derksen, 2005 [90]. Domain V is involved in the binding to anionic phospholipids while domain I contains the binding epitope for anti- β_2GPI antibodies.

Two common structural mutations at codon 306 and codon 316 present in the fifth domain directly affect the binding of β_2GPI to anionic phospholipids [86, 91]. Missense mutation at codon 316 affects the integrity of a potential protein-lipid interaction site defined by the highly conserved (evolutionarily conserved in bovine, rat, mouse, dog, and human β_2GPI) hydrophobic

loop at positions 313-316 that is composed of residues leucine-alanine-phenylalanine-tryptophan, respectively [89]. The missense mutation at codon 306 disrupts the disulfide bond between Cys 306 and Cys 281, which plays a key role in clustering the positively charged amino acid sequence motif (position 281-288) in the fifth domain. Homozygosity of 316 mutations and compound heterozygozity of codons 306 and 316 abolished complete binding capacity of β_2GPI to phosphatidylserine [86]. Crystal structure of human β_2GPI has confirmed that the positive charge cluster (281-288), the hydrophobic loop (313-316), and integrity of disulfide bonds of the fifth domain are all essential for the lipid-binding of β_2GPI [88]. In domain V of the β_2GPI, the substitution at position 247 of Leu to Val is correlated with anti- β_2GPI antibody production in patients with primary APS. The Val^{247} allele of the Val247Leu polymorphism was significantly more frequent in primary APS patients with anti- β_2GPI antibodies than in controls or in primary APS patients without anti- β_2GPI antibodies in a British APS cohort [8]. Val^{247} allele was found to be associated with higher frequency as well as stronger reactivity with anti- β_2GPI antibodies, when compared with the Leu^{247} allele in Asian APS patients indicating it to be a major risk factor for APS in Asians [9]. Val^{247} allele was shown to be a genetic risk factor for development of APS and important in the formation of β_2GPI antigenicity [92]. Similarly, in a pediatric cohort study an association has been demonstrated for the Val247Leu substitution and both the development of APA, and APA-associated clinical manifestations in patients [93]. The significance of the position of Val247Leu polymorphism that is located between the phospholipid binding site in domain V and the potential epitopes of anti- β_2GPI antibodies in domain IV was analyzed by conformational optimization [92]. Results showed that the replacement of Leu^{247} by Val^{247} led to a significant alteration in the tertiary structure of domain V and/or the domain IV-V interaction. The VV genotype at position 247 in Mexican subjects

was found to be significantly higher frequencies in anti- β_2GPI-positive patients and in patients with arterial thrombosis than in anti- β_2GPI-negative patients or donors [10]. Recent reports have shown that the Val247Leu and Trp316Ser polymorphisms in exons 7 and 8, respectively, were not related to the presence of anti- β_2GPI antibodies in unselected Chilean patients with venous and arterial thrombosis, but they were significantly associated with venous and arterial thrombosis [94]. β_2GPI could have a potential role in the pathogenesis of HBV infection in humans. Indeed, the binding of β_2GPI to the HBV surface antigen has been reported [95, 96]. It was demonstrated that the β_2GPI cDNA transfected COS-1 cells expressed and secreted full-length recombinant β_2GPI (rβ_2GPI) into its culture medium that exhibits normal binding to recombinant hepatitis B surface antigen (rHBsAg) [95]. The authors speculated that the mode of entry for HBV into the human hepatocytes might take place when infectious HBV binds to β_2GPI complexed with chylomicrons or HDL followed by uptake through LDL receptor or the α2-macroglobulin receptor. The binding of β_2GPI with rHBsAg was disrupted by reducing the intramolecular disulfide bonds of β_2GPI and chemical modification of as few as three positively charged lysine residues of β_2GPI located in the fifth domain [96]. It was therefore, recently predicted that the binding region for rHBsAg on β_2GPI is located in the fifth domain of β_2GPI and is the same as the phospholipid binding region on β_2GPI [97].

1.2.2 Phylogenetic Analysis of Mammalian β_2-glycoprotein I Sequences

β_2GPI has been extensively characterized both at the protein and nucleotide levels from a number of other species including rat, bovine, canine, mouse and chimpanzee. Significant structural homology of β_2GPI across these several mammalian species indicates conserved regions in the protein with natural evolution that may be critical for its function. Studies have observed a high degree of similarity of human β_2GPI sequence with other mammalian species; rat - 83% [98], bovine - 83% [99, 100] mouse - 73.6% [101-103], canine - 82.5% [104], and chimpanzee - 99.4% [105]. The mature protein is composed entirely of 5 contiguous SCR domains among all species. SCRs 1-4 are approximately 60 aa residues in length and contain four conserved Cys residues, whereas SCR 5 (C-terminal) contains six Cys residues, two of which are not conserved. Interestingly, the position of Cys residues and the 11-disulphide bonds were homologous among all species. Phylogenetic analysis (Figure. 7) established the mouse β_2GPI sequence to be the most divergent species overall, reported to date. It is most similar to human (73.6%) and least similar to bovine sequence (72%) indicating that mouse β_2GPI has accumulated more mutations and hence is a faster evolving species than others. Chromosomal location of mouse β_2GPI was mapped to chromosome 11 and the TSS was identified by primer extension 44 nucleotides upstream of the initiator AUG codon. In contrast to human and canine β_2GPI, the mouse β_2GPI is not an acute phase reactant, since there was no down regulation of β_2GPI mRNA levels in response to pro-inflammatory cytokines. The nucleotide sequences between human and chimpanzee *APOH* were 99.6% identical between the two species, whereas at the protein level there was 99.4% identity between human and chimpanzee β_2GPI. The prevalence of anti- β_2GPI antibodies in chimpanzees (64%) was found to be unusually high as compared to humans.

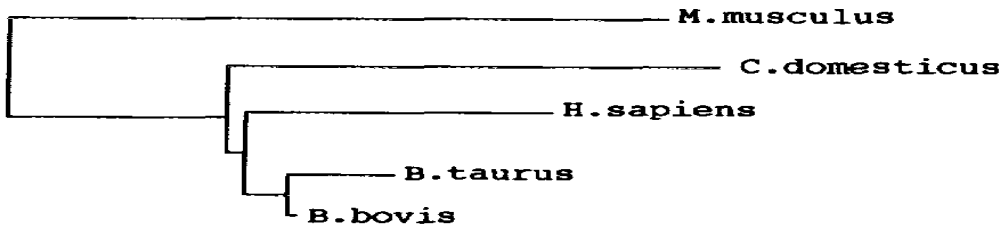


Figure 7. Phylogenetic tree of mammalian β_2GPI proteins
From Sellar et al. 1994 [102]. Human (H. sapiens); bovine (B. taurus and B. bovis); canine (C. domesticus) and mouse (M. musculus). The branch lengths indicate the evolutionary distances between the different.

1.2.3 *APOH* Polymorphisms

Complete DNA sequence variation in *APOH* has been reported recently in 46 Caucasian Americans and 48 African Americans chromosomes and a larger Pittsburgh cohort of Caucasian Americans (n = 452) and African blacks (n = 81) [106]. A total of 150 SNPs, including 146 from the SeattleSNPs Program for Genome Application (University of Washington-Fred Hutchinson Cancer Research Center Variation Discovery Resource website - http://pga.gs.washington.edu/data/apoh/) and one tri-allelic polymorphism were identified. Eight of them were present in the coding region (Exon 3 → Ser88Asn; Exon 5 → Ile122Thr, Arg135His, Ser140Ser, Ala141Asp; Exon 7→ Val247Leu, Cys306Gly; Exon 8 → Trp316Ser), 14 in the 5′-region and two in the 3′-region (17273T>C and 17314T>C). Of these, 16 were unique to Caucasian Americans, 62 to African Americans, and 68 were present in both groups. The observed number of SNPs (130 *vs.* 84), SNP density (6.5 SNPs per kb *vs.* 4.2 SNPs per kb)

and inferred haplotypes (34 *vs.* 16), were higher among African Americans than Caucasian Americans. Race-specific linkage disequilibrium (LD) pattern of SNPs identified 17 informative tagSNPs among Caucasian Americans and 35 in African Americans. The present study is focused on the 14 SNPs present in the 5′- region, five of which are found only in African American or black population (Table 2). The -1284 C>G polymorphism is present only in the Caucasian Americans or white population.

Table 2. Positions and minor allele frequencies of *APOH* Promoter SNPs

SNP ID	Ethnicity (W = Whites B = Blacks)	SNP	African Americans (N = 24)	African Blacks (N = 81)	Caucasian Americans (N = 23)	American Whites (N = 425)
-1284 (rs8178818)	W	C>G	0	0	0.02	0.001
-1219 (rs8178819)	W/B	G>A	0.02	0.07	0.04	0.1
-1190 (rs3760290)	W/B	G>C	0.43	0.34	0.28	0.38
-1076 (rs8178896)	B	G>A	0.02	NA	0	NA
-1055 (rs8178897)	B	T>G	0.02	NA	0	NA
-759 (rs8178820)	W/B	A>G	0.12	0.09	0.2	0.24
-742 (rs8178821)	W/B	delT	0.11	NA	0.2	NA
-700 (rs3760291)	W/B	C>A	0.11	0.08	0.2	0.24
-643 (rs3760292)	W/B	T>C	0.17	0.1	0.17	0.13
-627 (rs8178898)	B	A>C	0.07	NA	0	NA
-581 (rs8178899)	B	A>C	0.02	NA	0	NA
-363 (rs8178900)	B	C>T	0.12	NA	0	NA
-38	W/B	G>A	0	NA	0	NA
-32 (rs8178822)	W/B	C>A	0.05	NA	0.07	NA

From Chen & Kamboh, 2006 [106]. The A of the ATG of the initiator methionine codon is denoted nucleotide +1, the nucleotide 5′ to +1 is numbered −1.NA = not applicable

1.2.4 *APOH* Protein Polymorphisms

1.2.4.1 Quantitative Polymorphisms of β_2-glycoprotein I

The normal concentration of β_2GPI in serum is ~20mg/100ml. Early studies by Cleve et al. (1968) [107] proposed that the variation in the β_2GPI concentration values as observed in family and population data was controlled by two autosomal codominantly expressed alleles, Bg^N (normal) and Bg^D (deficiency). Bg^N Bg^N homozygous individuals had a β_2GPI concentration between 16-30mg/100ml; Bg^N Bg^D heterozygous individuals were intermediate between 6-14 mg/100ml; and Bg^D Bg^D homozygous individuals had the lowest at <5 mg/100ml. Furthermore, the frequency of the Bg^D deficiency allele was found to be much higher in mongoloid and black populations (>20%) as compared with whites (~6%) [108].

1.2.4.2 Qualitative Polymorphisms of β_2-glycoprotein I

Genetically determined structural polymorphism of β_2GPI as detected by isoelectric focusing (IEF) and immunoblotting provided evidence for two kinds of β_2GPI variation [108]. The first is the variation in sialic acid moieties which serves as a major determinant of intraindividual glycoprotein microheterogeneity. The second kind of variation has been observed among individuals plasma samples and reveals structurally different isoforms with different isoelectric points, presumably representing an altered polypeptide chain owing to amino acid substitution resulting in a given variant isoform. The four primary isoforms, therefore, can be accounted for by the presence of three altered polypeptide chains resulting from the substitutions of three aa residues on different positions on the normal chain. As originally revealed by IEF *APOH*

structural locus has three common alleles namely, *APOH* 1*, *APOH* 2* (most common or wild-type) and *APOH* 3* and a fourth rare allele, *APOH* 4,* that was hypothesized to explain the qualitative β_2GPI variation (Figure 8). The distribution pattern of *APOH* allele frequencies revealed (1) identical frequencies of the *APOH* 1* and *APOH* 3* alleles in U.S. Whites, (2) a lower frequency of the *APOH* 1* allele in U.S. Blacks as a consequence of admixture, and (3) the polymorphic occurrence of the *APOH* 4* as a unique marker present only in the black population.

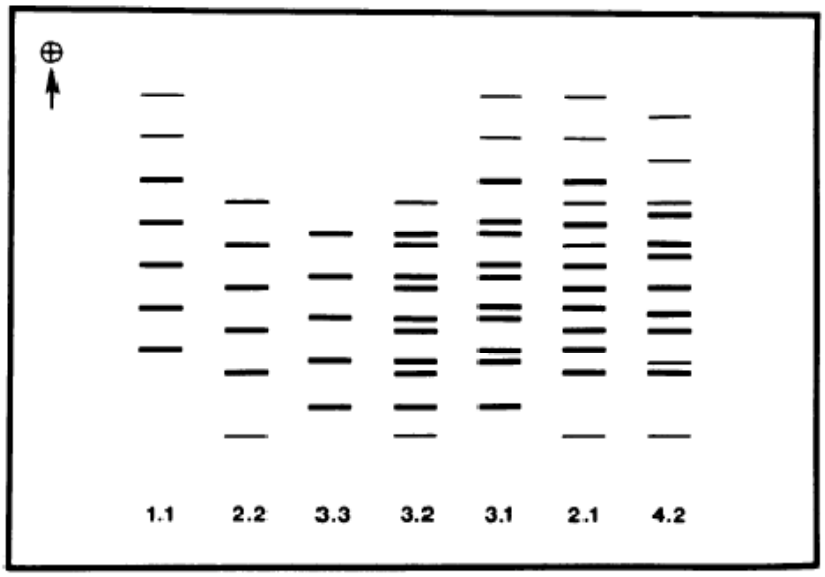


Figure 8. Schematic representation of β_2GPI phenotypes detected by IEF
From Kamboh et al. 1988 [108]

Three IgG monoclonal antibodies (mAb) to human β_2GPI, designated 3G9, 1B4, and 3D11, have been produced. In contrast to mAbs 3G9 and 1B4 which recognize both free and phospholipid-bound β_2GPI and react with all β_2GPI allelic isoforms, the 3D11 was specific only for one form of the *APOH*3* allele, called *APOH*3*^{W}*,* that does not bind to anionic phospholipids [109]. IEF and immunoblotting results provided evidence for the equal reactivity

with all four *APOH* allelic products for the 3G9 and 1B4 mAbs, whereas the 3D11 recognized only the *APOH*3* allele product [110]. The *APOH* 3* allele was also found to be heterogeneous and could be distinguished into *APOH* 3^W* (reactive) and *APOH* 3^B* (non-reactive) based on its differential reactivity with monoclonal 3D11 antibody [110]. The *APOH* 3^W* allele product that reacted with 3D11 did not bind to anionic phospholipid precluding the production of APA. This suggests that the 3D11 epitope lies within the lipid binding site and is unavailable when β_2GPI binds to phospholipids. Since the 3D11 reacted only with *APOH*3* allele present mostly in Whites and Chinese, but not in Blacks, this variant plays a significant role in the ethnic-specific variation in the cofactor activity of β_2GPI. Molecular basis of β_2GPI protein polymorphisms, *APOH* 1* and *APOH* 3^W* alleles has been reported .due to two missense mutations, G341A (Ser88Asn) in exon 3 and Trp316Ser in exon 8, respectively [111]. The IEF pattern of the most common phenotype in chimpanzees was identical to the human *APOH*3^B* polymorphism [105]. Another phenotype which was distinct from any reported human isoforms was designated as *APOH*4* allele, which corresponded to a missense mutation at codon Lys210Glu (A>G) in exon 6 that did not affect the binding of β_2GPI to cardiolipin. The correlation between SNPs in the coding region of *APOH* and protein polymorphisms have been reported by comparing human and chimpanzee β_2GPI amino acid sequences [112]. The human and chimpanzee protein sequence difference lie in the coding regions, involving three samesense mutations at codons 57, 75, and 130, and two missense mutations at codons 122 and 135. The wild-type human β_2GPI protein consists of isoleucine and arginine at codons 122 and 135, respectively, but the chimpanzee β_2GPI has threonine and histidine at the two respective sites. An evolutionary pathway of observed *APOH* haplotypes was suggested by screening a large African population (n = 755), wherein the *APOH* 3* allele comprising the five-site haplotype (122Thr-135His-

141Ala-247Val-316Trp) was the ancestral haplotype from which all subsequent allelic isoforms (*APOH*1*: **88Asn**; *APOH*2*: **122Ile-135Arg**-141Ala-**247Leu**-316Trp; *APOH*3*^{W}: 122Thr-135His-141Ala-247Val-**316Ser**; *APOH*3*^{B}: 122Thr-135His-**141Asp**-247Val-316Trp) were derived (Figure 9). A revised nomenclature was proposed based on the evolutionary pathway wherein the previously identified *APOH* 4* allele was designated as *APOH* 3*^{B} (122Thr-135His-**141Asp**-247Val-316Trp) seen in Blacks only, and the *APOH* 3*^{W} (122Thr-135His-141Ala-247Val-**316Ser**) seen mainly in Whites, remained the same. Association of *APOH* protein polymorphisms with prevalence of anti-β_2GPI antibodies revealed a gender-specific genotypic effects of *APOH* 3* and *APOH* 3*^{B} polymorphisms associated with higher anti-β_2GPI levels only in males. Evidence for the third domain of β_2GPI to be involved in binding with cardiolipin, as revealed by the binding abilities of the naturally occurring haplotypes (carrying mutations at codons 122 and 135) of *APOH* 3* and *APOH* 3*^{B} has been established.

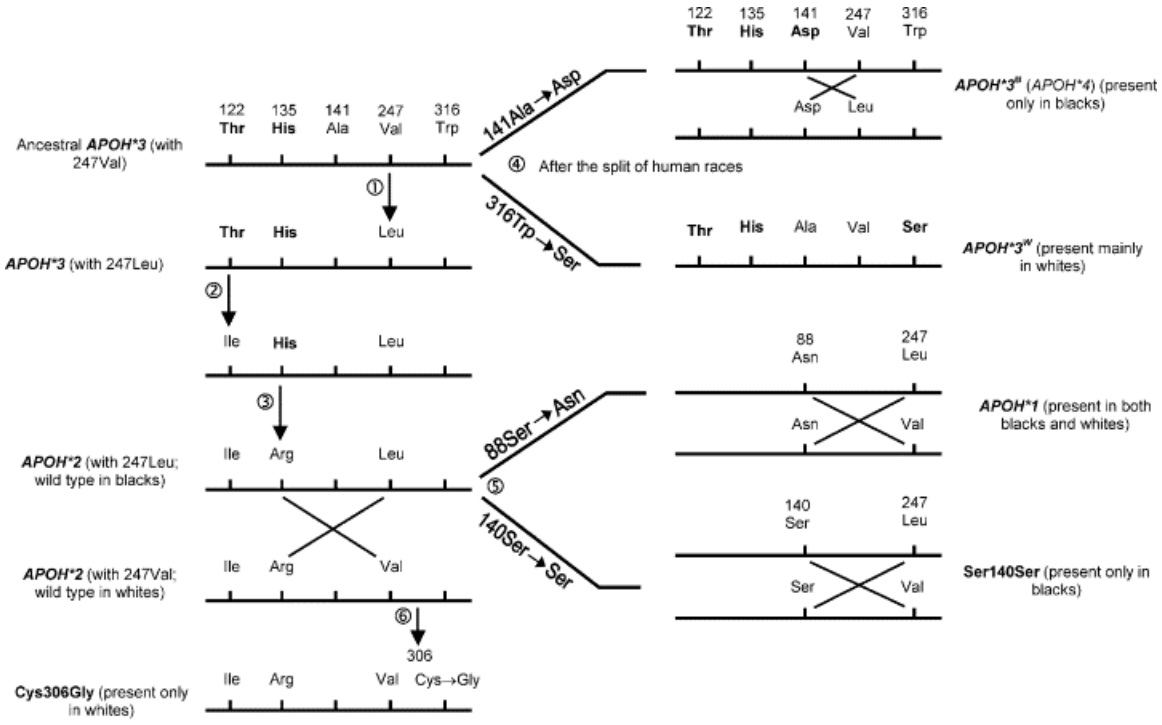


Figure 9. Evolutionary pathways of *APOH* haplotypes
*From Kamboh et al. 2004 [112]. The evolutionary steps that may have led to the formation of present day haplotypes are numbered in sequential order from 1 to 6. Crosses between two haplotypes indicate possible recombinant events. Bold letter amino acids (Thr, His, Asp, Ser) indicate the characteristic amino acids of the APOH*3, APOH*3^{B} and APOH*3^{W} haplotypes.*

1.2.5 Effect of *APOH* Polymorphisms on Plasma β_2-glycoprotein I levels

There is a wide range of interindividual variation in β_2GPI plasma levels, ranging from immunologically undetectable to as high as 35 mg/dL with a mean value of 20 mg/dL in Caucasians and 15 mg/dL in AA, which may have clinical relevance in β_2GPI-related pathways [113]. Family and heritability data have provided strong support that β_2GPI plasma variation is under genetic control but the exact molecular basis of this variation remains largely unknown. A

low level of plasma β_2GPI is thought to be a risk factor for thrombosis [114] and it may also affect the production of APA, therefore it is important to determine the molecular basis of β_2GPI plasma variation. Population-based genetic association studies in non-Hispanic Whites from Colorado indicated a significant association of *APOH* protein polymorphisms with plasma β_2GPI levels in both men and women ($P < 0.0001$) that accounted for 11.4% and 13.6% of the variation seen in men and women, respectively. Compared with the *APOH*1* and *APOH*2* alleles, the *APOH*3* allele was associated with significantly lower plasma β_2GPI levels. More specifically, the $APOH*3^W$ form which is defined by the codon Cys316Gly polymorphism explained about 10% of the variation in plasma β_2GPI levels, indicating that this polymorphism is a major determinant of plasma β_2GPI levels [115]. Previous studies in 222 white SLE women have also confirmed that the codon 306 and codon 316 polymorphisms show significant ($P < 0.0001$) gene-dosage effects on plasma β_2GPI levels accounting for about 30% and 13% of the variation, respectively [113]. The variant alleles of Gly306 and Ser 316 were associated with lower levels of β_2GPI than their corresponding wild-type alleles. Apart from common genetic variation in *APOH* coding sequences, a functionally important point mutation (-32C>A) was identified in the 5′-flanking region at the transcription initiation site of *APOH* that was associated with significantly lower β_2GPI plasma levels ($P < 0.0001$) in a sample population consisting of 232 lupus patients [116]. Functional relevance was also reported by *in vitro* studies that showed two-fold lower luciferase reporter gene expression (a 696-bp long fragment of 5′ region of *APOH* from nucleotide -622 to +74 was tagged to pGL3 - Basic luciferase constructs) in COS-1 cells associated with the -32A minor allele and northern blot results that revealed this SNP affects *APOH* transcription. Consistent with the luciferase results, the two-fold difference observed between the -32C and -32A alleles was similar also between the AA (9.4 mg/dL) and CC

(18.5mg/dL) genotypes for plasma β_2GPI levels. Further evidence for its effect on β_2GPI transcription was demonstrated by EMSA results indicating an allele specific (-32A having lower affinity than -32C) binding to transcription initiation factor (TFIID) and also to transcription factors in mouse liver nuclear extracts. The plasma β_2GPI concentrations were found to be significantly higher in patients positive for APA than in patients negative for APA (18.5±4.0 mg/dl *vs* 17.1+3.8 mg/dl; $P = 0.02$) [113]. The distribution of the Trp3l6Ser polymorphism was significantly different between the APA-positive and APA-negative groups. The frequency of the mutant allele (Ser316) was significantly lower in the APA-positive group than the APA-negative group (3.1% *vs.* 12.1% $P < 0.04$), indicating that the Ser316 mutation is protective against the production of phospholipid- β_2GPI dependent APA. Due to the almost complete LD between SNPs -32C>A and Trp316Ser, the -32C>A SNP also showed a similar significant protective effect against the occurrence of APA. The carrier frequency of the -32A allele was almost four-fold lower in the APA-positive group than the APA-negative group (4.8% vs. 16.6%; $P = 0.019$). In univariate analysis, both the -32C>A and Trp316Ser SNPs showed significant association with β_2GPI plasma levels. However, in multivariate analysis, the effect of the Trp316Ser was lost, indicating that the -32C>A is the functional variant. The Cys306Gly SNP, however, exhibited an independent significant effect on β_2GPI plasma levels in both univariate and multivariate analyses.

1.2.6 Functions of β_2GPI

1.2.6.1 β_2GPI and Lipid Metabolism

Although a role for β_2GPI has been implicated in a variety of physiological pathways, no precise metabolic function has yet been assigned to it. Approximately 35% by weight plasma β_2GPI is associated with various lipoprotein classes (very low-density lipoproteins (VLDL - 16%), high-density lipoproteins (HDL - 17%), low-density lipoprotein (LDL - 2%) and chylomicrons), and the remaining 65% is present in the lipid-free 1.21 density fraction [78, 117]. Being a constituent of these lipoprotein particles, its function in lipid metabolism has been investigated in detail [118]. β_2GPI was first thought to be involved mainly in triglyceride metabolism since much of the circulating protein is bound to lipoproteins, and it was postulated that when stimulated by apoC-II, β_2GPI activates lipoprotein lipase (LPL) and increases its enzymatic activity about 45% in triglyceride metabolism [119]. The association of *APOH* polymorphisms in relation to serum lipoprotein concentrations exhibited a sex-specific effect with females having higher triglyceride levels [120]. A recent study comprehensively investigated the association of variation in *APOH* with lipid traits in hepatic cholesterol transport (HCT), dietary cholesterol transport (DCT), and reverse cholesterol transport (RCT) [121]. The study population consisted of families from the Genetic Epidemiology Network of Arteriopathy (GENOA) multicenter study that includes African Americans, Mexican Americans, and European Americans. The strongest associations were found for DCT traits (triglyceride and apoE levels) in Mexican Americans with a nonsynonymous variant (SNP 14917, Cys306Gly) that may alter β_2GPI protein folding in a region involved in phospholipid binding.

1.2.6.2 β_2GPI and Atherosclerosis

Oxidative stress and low-density lipoprotein (LDL) modification (oxidized low-density lipoprotein; oxLDL) are early pro-atherogenic events [122-124]. The oxidation products of LDL are cytotoxic, chemotactic, and proinflammatory, and promote the formation of macrophage-derived foam cells. Increased cardiovascular morbidity and mortality due to the premature or accelerated development of atherosclerosis has been reported in patients with systemic autoimmune diseases such as SLE and APS [125]. A growing body of data has implicated β_2GPI-mediated immune response in the pathogenesis of atherosclerosis. β_2GPI binds significantly to oxLDL than to native LDL [126] to form oxLDL/β_2GPI complexes (Figure 10). The nature of the binding has been further characterized and the oxLDL-derived ligands specific for β_2GPI have been identified and synthesized.

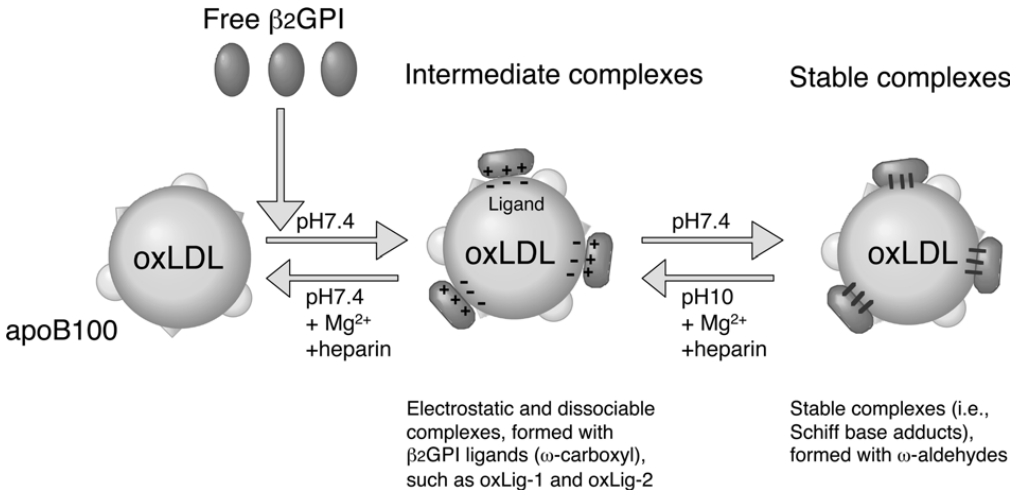


Figure 10. OxLDL/β_2GPI complex formation
From Matsuura et al. 2006 [127]. β_2GPI interacts with oxLDL in a two-step reaction that is calcium independent. Initially, β_2GPI binds to oxLDL via the specific, high affinity ligands oxLig-1 and -2. Stable complexes between the protein and oxidized lipid form at neutral pH. The latter complexes are non-dissociable at the neutral pH, but can be dissociated with Mg2+ or heparin at pH 10.

Circulating oxLDL/β_2GPI complexes are significantly elevated in sera of patients with systemic autoimmune and/or atherosclerotic disease such as APS, SLE, and type 2 diabetes mellitus as compared to healthy controls [128]. However, these complexes were not associated with either SLE disease activity or any major clinical manifestation of APS. Circulating oxLDL/β_2GPI complexes have been implicated as atherogenic autoantigens, and their presence might represent a risk factor or an indirect but significant contributor to thrombosis (chronic inflammation of the vasculature) and atherosclerosis (oxidative stress) that occurs in autoimmune patients. SLE and APS patients produce autoantibodies (anti-β_2GPI) to this complex and the resulting circulating immune complexes are etiologically important in the development of atherosclerosis in these patients [129]. Studies have reported IgG anti-oxLDL/β_2GPI antibodies

to be significantly higher in SLE and systemic sclerosis (SSc) patients when compared to the control population [130]. A positive predictive value (PPV) of IgG anti-oxLDL/ β_2GPI antibodies for total thrombosis (arterial and venous) in patients with secondary APS was 92% and for arterial thrombosis 89%. These observations provided concrete explanation for the accelerated (premature) development of atherosclerosis in autoimmune patients. IgG anti-oxLDL/β_2GPI antibodies seem to represent a distinct subset of APA (i.e., anti-β_2GPI) that appear to be useful serologic markers for atherothrombotic risk in autoimmune patients with high specificity for APS [127]. The physiologic relevance of these findings was demonstrated *in vitro* by the enhanced macrophage uptake of IgG immune complexes of oxLDL/β_2GPI (Figure 11). The participation of macrophage FcγRI receptors in the uptake of oxLDL-containing complexes seems to be particularly important to explain the development of foam cells and autoimmune-mediated atherosclerotic plaques. Circulating IgG anti- β_2GPI antibodies facilitate the antigen presentation by macrophages to specific T cells. Interestingly, *in vitro* stimulation of macrophages by oxLDL/β_2GPI complexes together with anti-β_2GPI antibodies led to the expression of membrane CD36 as well as FcγRI [131]. These observations suggest that IgG immune complexes containing oxLDL/β_2GPI provide not only FcγRI- but also scavenger receptor-mediated uptake of oxLDL/β_2GPI complexes by macrophages. Thus, oxLDL/β_2GPI complexes represent a major atherogenic autoantigen, and IgG anti-β_2GPI autoantibodies may facilitate antigen presentation and foam cell formation in APS.

Interestingly, β_2GPI could play a dual role in the regulation of uptake of oxLDL by macrophages [126]. β_2GPI is primarily anti-atherogenic but the immune response against β_2GPI converts it to proatherogenic. In the absence of APA, β_2GPI inhibits the uptake of oxLDL and cholesterol accumulation in macrophages resulting in anti-atherogenic effect [132]. On the other

hand, in the presence of APA, β_2GPI increased oxLDL uptake, which indicates a pro-atherogenic effect [126, 127, 133].

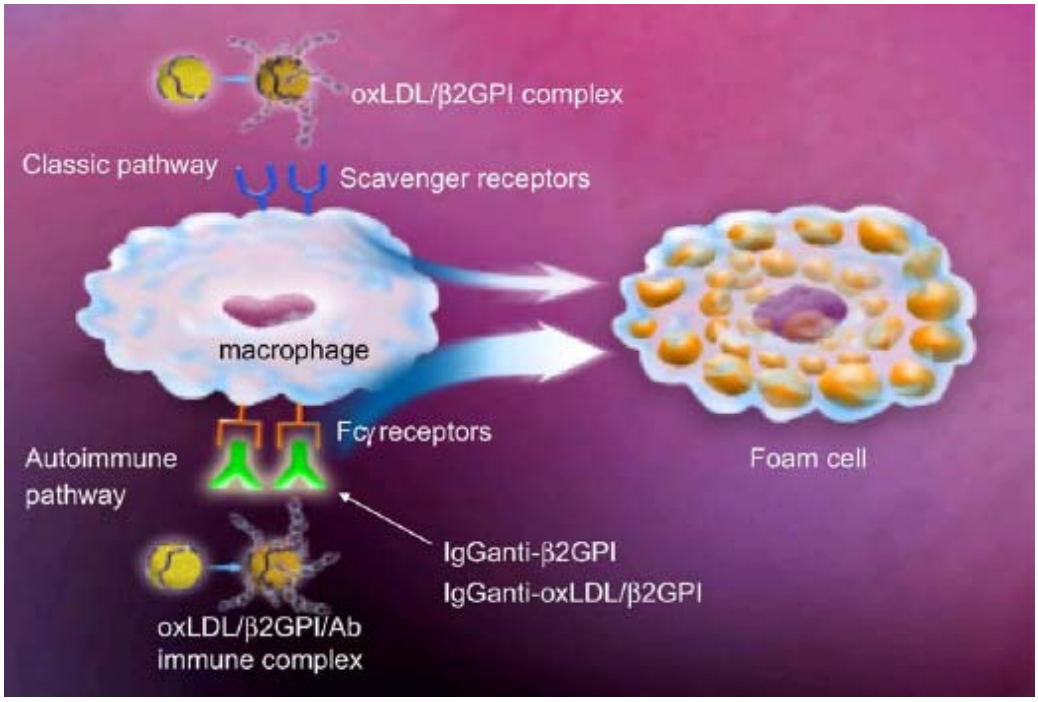


Figure 11. Proposed mechanisms of foam cell formation in immune-mediated atherosclerosis
From Matsuura and Lopez, 2008 [134]

1.2.6.3 β_2GPI and Blood Coagulation

The role of β_2GPI in the coagulation system both in healthy people and patients with APS is being increasingly elucidated. The involvement of β_2GPI in the coagulation cascade seems multifaceted because the protein can exert both procoagulant and anticoagulant activities (Figure 12). β_2GPI exerts anticoagulant activity by inhibiting the contact activation of the intrinsic coagulation pathway, both tenase and prothrombinase activity on platelets or phospholipids vesicles, it inhibits activation of factor X and XII [135] and modulates ADP-induced platelet

aggregation [136]. On the other hand, β_2GPI exhibits procoagulant activities by reduction of activated protein C [137, 138]. Several *in vivo* experimental models have demonstrated a role of β_2GPI in thrombotic complications. Mice lacking β_2GPI have been produced by gene targeting; plasma from these knockout mice exhibited impaired thrombin generation *in vitro* [139]. These experiments provide direct evidence of the role of β_2GPI in the direct coagulation cascade, at a stage upstream of thrombin generation. Despite the regulatory functions of β_2GPI in the coagulation cascade, genetic deficiency of β_2GPI does not represent a major risk factor of either thrombosis or bleeding in humans [140]. β_2GPI appears to not only interact with factors of the coagulation cascade, but also participate in the regulation of intrinsic fibrinolysis [141, 142]. Fibrinolytic reactions involve the formation of plasmin generated from plasminogen and the hydrolytic cleavage of fibrin to fibrin degradation products by plasmin (Figure 13). Plasmin conversion from plasminogen by tissue plasminogen activator (tPA) occurs upon activation of extrinsic fibrinolysis for the thrombolysis against intravascular blood clots. Impaired fibrinolysis is a contributing factor for the development of thrombosis. β_2GPI is proteolytically cleaved between Lys317 and Thr318 (nicked- β_2GPI) by activated factor X and plasmin in domain V being unable to bind phospholipids. This cleavage may occur *in vivo* on the surface of activated endothelial cells or platelets. Once the nicked form of β_2GPI is generated, it loses its antigenicity for pathogenic APA. Recently, increasing attention is focused on the role of nicked- β_2GPI as a regulator of extrinsic fibrinolysis pathway. Nicked- β_2GPI has been found in large quantities in plasma of patients with disseminated intravascular coagulation (DIC), a pathological state characterized by massive thrombin generation and fibrinolytic turnover [143]. The detection of nicked- β_2GPI in plasma represents a sensitive marker of cerebral ischemic events and might be a useful screening tool in the assessment of patients at risk of ischemic stroke [142]. Nicked β_2GPI

has been shown to be a physiologic inhibitor of fibrinolysis and that plasmin cleavage of β_2GPI is part of the negative feedback pathway of extrinsic fibrinolysis [144].

Anticoagulant
1. Inhibition of prothrombinase activity (thrombin generation)
2. Inhibition of Factor X activation
3. Inhibition of Factor XII activation
4. Inhibition of platelets aggregation
Procoagulant
1. Inhibition of protein C activation
2. Inhibition of inactivation of activated Factor V by activated protein C
3. Inhibition of protein Z anticoagulant pathway

Figure 12. β_2GPI and blood coagulation
From Atsumi et al. 2005 [144]

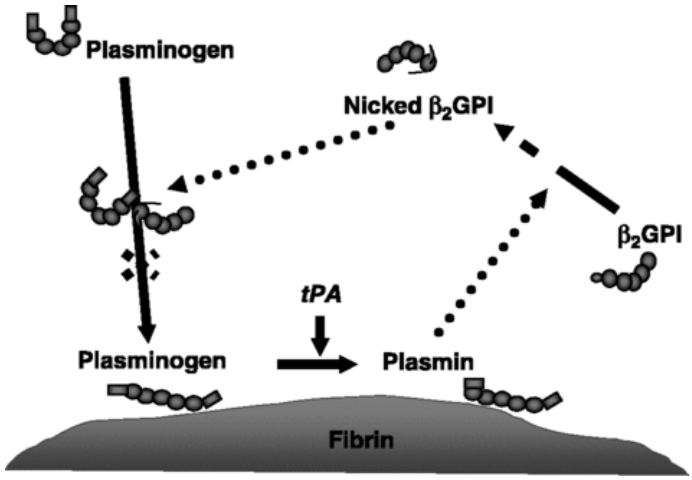


Figure 13. Role of β_2GPI in the negative feedback pathway of extrinsic fibrinolysis
From Atsumi et al. 2005 [144]

1.2.6.4 β_2GPI and Antiphospholipid Syndrome

The antiphospholipid syndrome (APS, Hughes' syndrome) is a thrombophilic disorder that manifests clinically with arterial and/or venous thrombosis and/or pregnancy morbidity, associated with the presence of APA. APS could occur independently in most cases as a primary entity or occur as a secondary condition in association with other autoimmune diseases, particularly SLE [141]. Among these autoantibodies, LAC and aCL are included in the list of biological criteria defining the APS [145]. Accumulating evidence from human and animal studies (NZW×BXSB F1 mice, an APS mouse model) [146] indicates that β_2GPI represents the major target antigen for APA and it plays a critical role in the pathogenesis of APS [147]. In classical aCL immune assays, anti- β_2GPI antibodies are involved in the aCL reactivity, which is directed mainly against the β_2GPI–cardiolipin complex. Despite a high level of correlation between aCL and anti- β_2GPI assays, the presence of anti- β_2GPI antibodies alone has been

described in some SLE patients with clinical manifestations of APS. Controversial results have been reported regarding the APA-specific epitopes of β_2GPI that are crucial in the development of APS. The positively charged (multiple lysine) region between cys281–cys288 is highly conserved in domain V and a critical phospholipid binding site that is essential for β_2GPI - dependent autoantibody binding [148]. Although short peptides containing sequences from domain V of β_2GPI inhibited binding of human monoclonal antibodies to β_2GPI, polyclonal antibodies from human APS patients did not bind to these peptides [149]. In a recent study using anti- β_2GPI antibodies from APS patients with deleted mutants [150] and single-point mutated domain I of β_2GPI, it was shown that domain I of β_2GPI contains significant epitopes for the anti- β_2GPI antibodies found in the APS [151]. The charged surface patch defined by residues 40–43 on domain I of β_2GPI contributes to an immunodominant epitope for anti- β_2GPI antibodies [152]. Anti- β_2GPI autoantibodies from most patients are intrinsically of low affinity and bind to β_2GPI only in the presence of negatively charged phospholipids or on pre-irradiated plates The binding of low affinity anti- β_2GPI was dependent on antigen density and dimerisation of β_2GPI induces a significant increase in anti- β_2GPI binding affinity enabling binding of anti- β_2GPI autoantibodies to β_2GPI coated on a normal plate. Once cell activation exposes anionic PL, the dimerisation of β_2GPI then facilitates binding to β_2GPI cell surface receptor (ApoER2 for platelet cells) and cell signaling (Figure 14) [141].

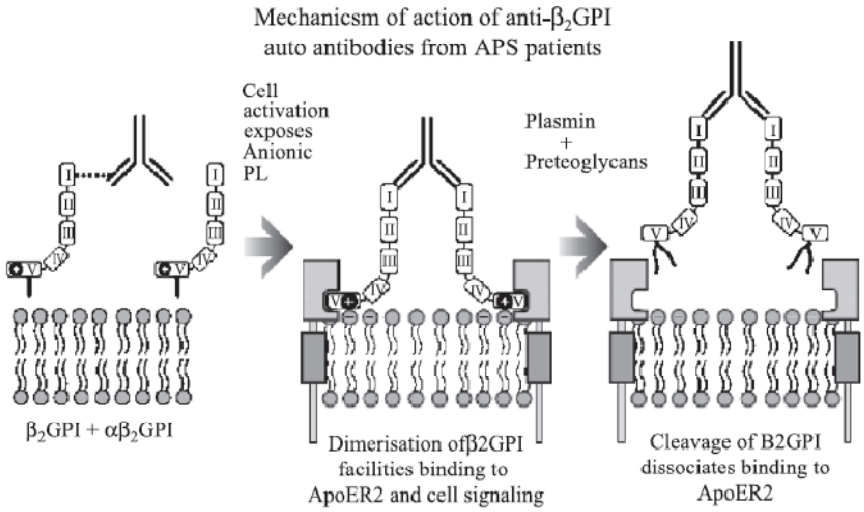


Figure 14. Role of β_2GPI in APS
From Miyakis et al. 2004 [141]

β_2GPI constitutes the principal antigenic target in APS-related fetal loss. APA reacting with β_2GPI can inhibit the proliferation and secretory activity of human placental trophoblast cells, confirming the involvement of β_2GPI in pregnancy loss associated with APS [141]. The role of β_2GPI in pregnancy failure has been further elucidated following experiments on genetically modified mice lacking β_2GPI [139]. β_2GPI knockout mice are able to carry pregnancies to term, with reproductive outcomes indistinguish able from that of the control mice. Direct interaction between β_2GPI, FXI and plasmin in the coagulation and fibrinolysis cascades, have given impetus to the ongoing research directed towards the role of β_2GPI in the thrombotic events of APS [141].

1.2.6.5 β_2GPI and Systemic Lupus Erythematosus

Some β_2GPI -dependent APA exert lupus anticoagulant (LA) activity, which seems to depend on APA epitope specificity. The term LA has been defined as APA that prolong the clotting time of phospholipid-dependent coagulation tests. A number of studies have provided evidence that the persistent finding of β_2GPI -dependent APA, which express high LA activity, is a real risk factor for thrombosis [153]. The most common and best characterized APA that occur in a variety of autoimmune diseases, particularly SLE are anti-aCL/β_2GPI antibodies, anti-phosphatidylserine /prothrombin antibodies (anti-PS/PT) and LA activity that are detected by conventional enzyme-linked immunosorbent assay (ELISA) and *in vitro* phospholipid-dependent coagulation reactions, respectively. Several clinical studies have established that the presence of aCL/ β_2GPI, anti-PS/PT and/or LA activity is associated with clinical events such as arterial and/or venous thromboembolic and obstetric complications in SLE and APS patients. Elevated blood levels of APA in autoimmune diseases such as in SLE, in which levels of these autoantibodies are raised in 20–60% of patients, have been associated with recurrent venous and arterial thromboses, fetal loss, thrombocytopenia and neurological disorders. Serum anti- β_2GPI antibodies are strongly associated with thrombosis in SLE patients [154]. Measurement of anti- β_2GPI IgG is clinically useful in identifying patients with SLE at higher risk for venous and arterial thrombosis [155]. Persistent positivity increased the association of IgG anti- β_2GPI with venous thrombosis and anti- β_2GPI IgM with arterial thrombosis. IgA anti- β_2GPI was not significantly associated with APS manifestations. Atherosclerosis obliterans (ASO), ischaemic heart disease (IHD) and CVD are major causes of mortality in patients with SLE. Recently, the presence of aCL/ β_2GPI contributing to the risk of development of ASO, which may represent an important mechanism

for the pathogenesis of IHD in patients with SLE [156]. No association between anti-β_2GPI antibodies and lupus nephritis either in patients with SLE or in patients with primary APS with nephritis was reported [157]. Patients with SLE develop subclinical atherosclerosis significantly more frequent than the general population. Coexistence of APS and presence of APA are risk factors for subclinical atherosclerosis development in patients with SLE. Thickened intima-media in patients with SLE is significantly associated with an increased risk of cardiovascular manifestations. Reports showing association between IgG anti- oxLDL/ β_2GPI and intima media thickness (IMT) of carotid arteries in primary APS (PAPS) and hence contribute to the development of autoimmune-mediated atherosclerosis [129]. oxLDL binds to endogenous β_2GPI and that these complexes (oxLDL/ β_2GPI) can be found circulating in the blood stream of patients with various chronic diseases, such as SLE, APS, chronic renal disease, diabetes mellitus. IgG autoantibodies against oxLDL/ β_2GPI were significantly elevated in about 40% of patients with APS as compared to SLE patients without APS. Further, these antibodies showed a stronger correlation with arterial thrombosis than venous thrombosis and pregnancy morbidity. The coexistence of oxLig-1/ β_2GPI autoantibodies with oxLDL/ β_2GPI complexes, suggest that these two elements interact perhaps forming circulating immune complexes (oxLDL/β_2GPI /antibody) (Figure 15). Such immune complexes in serum samples of SLE and/or APS has been detected [133]. This observation along with the increased macrophage uptake of oxLDL/ β_2GPI complexes in the presence of anti- oxLDL / β_2GPI antibodies [126, 133], provide a possible explanation for the accelerated development of atherosclerosis in autoimmune patients.

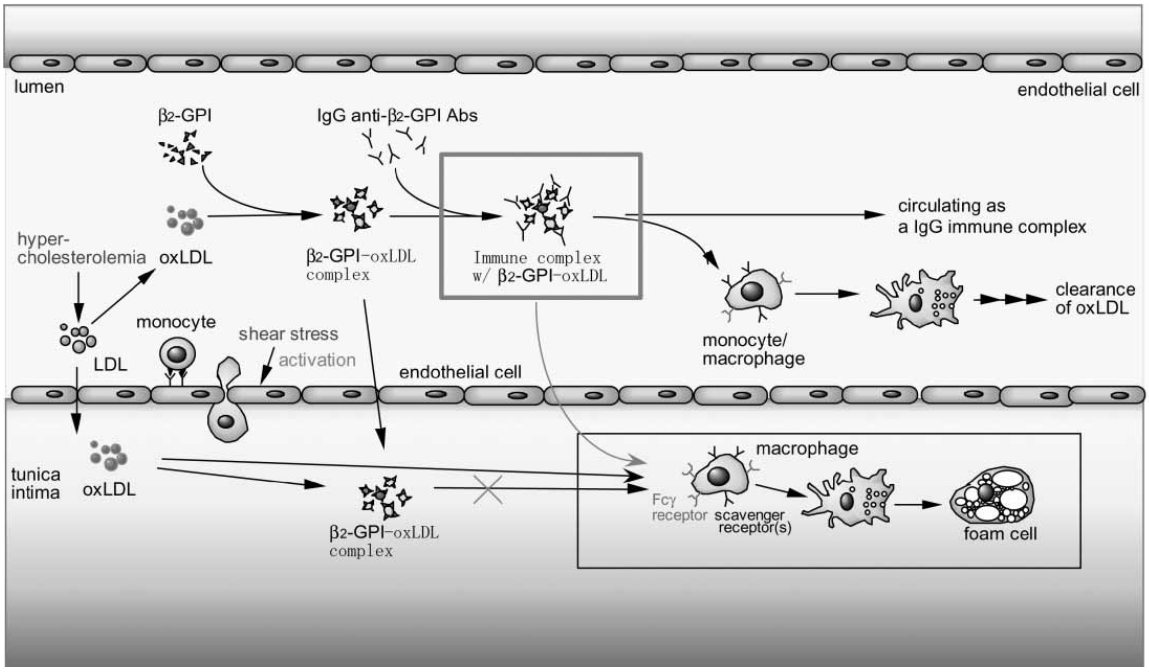


Figure 15. Development of thrombosis in SLE and/or APS patients by circulating IgG oxLDL/β_2GPI immune complexes
From Matsuura and Lopez, 2004 [158]

2.0 RATIONALE AND SIGNIFICANCE

Genome-wide comparisons indicate that studying the coding regions alone will not be sufficient for explaining the biological complexity of complex diseases. Genetic variants and the epigenetic differences of *cis*-regulatory elements are also crucial to elucidate many complicated biological phenomena [159]. Regulatory polymorphisms are DNA elements that modify the expression level of a transcript or its isoforms. Recently, there has been a considerable upsurge of interest in the influence of *cis*-acting polymorphisms and *trans*-acting elements on gene transcription in human diseases (Figure 16).

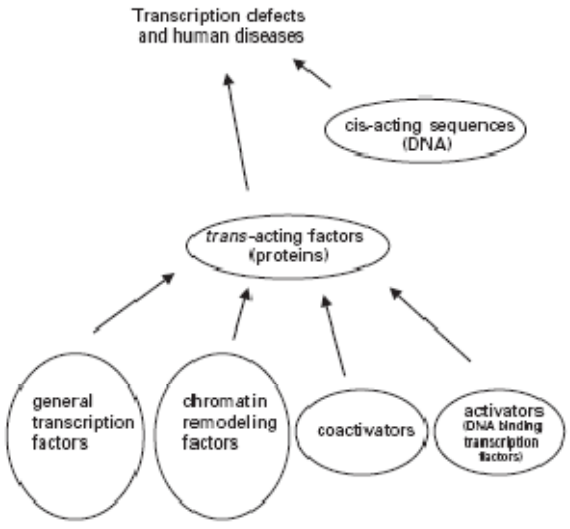
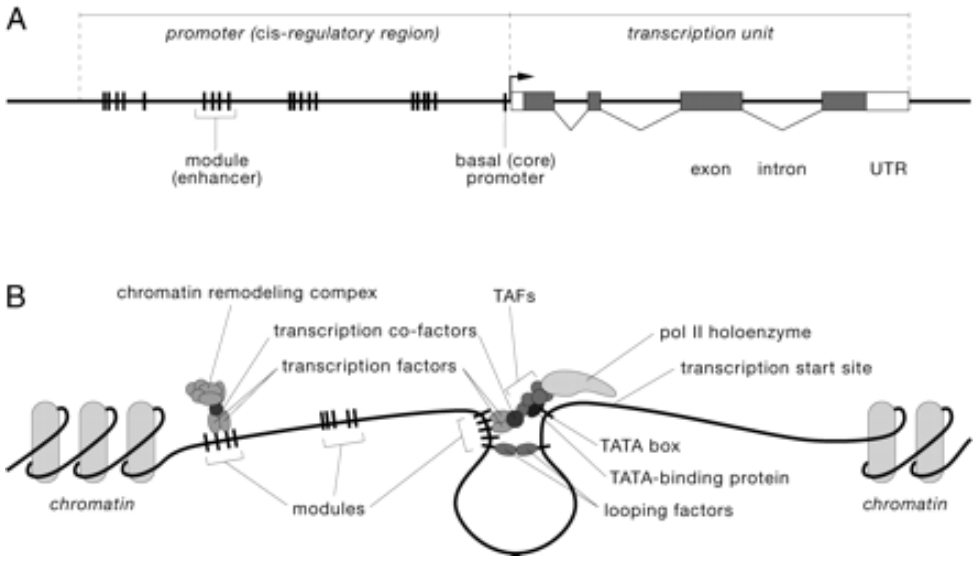


Figure 16. *Cis*- and *Trans*- Transcriptional Regulation and Human Diseases
From Villard, 2004 [160]

C*is*-acting regulatory factors (such as DNA polymorphisms and methylation) in the flanking DNA sequence of the gene may explain up to 25 to 35% of interindividual differences in gene expression. In contrast to coding sequence polymorphisms, where the consequences of non-synonymous variation may be resolved at the level of the protein phenotype, defining specific functional regulatory polymorphisms has proved problematic. Current techniques can detect expression differences as low as 1.2-fold between samples or alleles; the phenotypic consequences (if any) of such small differences are likely to depend on the function of the gene in question. Most known regulatory polymorphisms are located in gene promoter regions and function by altering gene transcription. Promoters are the best characterized transcriptional regulatory sequences in complex genomes [161]. Identification and characterization of these regulatory elements are vital in understanding the complex network of human gene regulation. It has long been known that mutations in non-coding regions which affect gene expression can cause human genetic disease [162]. Promoter sequences are potential sources of polymorphisms affecting gene expression, phenotypic variation and possibly susceptibility to common diseases [163]. Variations in the promoter DNA sequence may potentially alter the affinities of existing protein-DNA interactions or recruit new proteins to bind to the DNA, altering the specificity and kinetics of the transcriptional process (Figure 17).



A) Promoter structure and function B) Promoter operation in transcription

Figure 17. Transcriptional regulation of eukaryotic promoter
From Wray et al. 2003 [164]

Evidence for the medical importance of *cis*-acting polymorphisms has been provided by recent positional cloning of susceptibility genes that are not associated with protein coding or splice-site polymorphisms for common diseases such as stroke [165] and type 2 diabetes [166]. Classic examples include variants located within the highly conserved proximal promoter element (the "TATA" box) associated with thalassemias [167] and a SNP in the proximal Sp1 site of the human LDL receptor promoter as a cause of familial hypercholesterolemia [168]. A review of 107 genes with functional polymorphisms in the 5`-flanking region shows that 63% are associated with two-fold or greater allelic differences in their rates of transcription and 10% had 10-fold or more differences, indicating that functional *cis*-regulatory variation is widespread in the human genome [169]. A large number of putative functional SNPs present in promoter regions of candidate genes have been analyzed using traditional experimental techniques that

assay protein-DNA interactions, such as gel-shift assays, and those measuring gene expression using reporter gene assays. The most widely used experimental tool to interrogate the significance of specific putative regulatory polymorphisms are reporter gene assays in which cells are transiently transfected with allele- or SNP-specific promoter constructs. Using both luciferase- gene reporter and electrophoretic mobility shift assays Belanger et al. (2005) [170] reported four promoter SNPs associated with the cell-cycle check point genes, *E2F1*, *HDAC1*, *RB1* and *CCND1* to influence transcriptional activity in an allele specific manner. Systematic surveys of promoter haplotypes by reporter gene analysis on chromosomes 21 [162] and 22q11 [171] show functional variation among 18% and 20% of polymorphisms tested, respectively; a proportion similar to that reported on analysis of allele-specific expression for other genomic regions. Some examples in which promoter variation has been linked with disease and altered expression of gene include *APOE* and Alzheimer's disease [172], functional polymorphisms in the promoter region of the *β*-fibrinogen gene influencing plasma fibrinogen levels, which is a major risk factor for CHD, stroke, and peripheral arterial disease [173]; VLDL response element in the promoter region of the human plasminogen activator inhibitor-1 gene was implicated in impaired fibrinolysis of hypertriglyceridemia which increases risk of CHD and other atherothrombotic disorders [174]. Several studies have suggested associations of interleukin-10 (*IL-10*) and *IL* -18 promoter SNPs [175] and *TNF-α* in SLE disease susceptibility [176]. Two functional polymorphisms in the CRP gene (-409G>A and -390C>T>A) were identified by *in vitro* assays, wherein the *CRP*-promoter haplotype, -409G/-390T showed highest promoter activity in luciferase reporter assays [177]. The important functional role of *CRP* promoter polymorphisms having a significant influence on CRP levels in SLE patients has been recently reported [178]. Recent studies proposed a role to the *MBL* in the SLE physiopathogenesis with

distinct *MBL-2* polymorphisms, both at the coding and the promoter regions, associated with low serum MBL levels and with SLE susceptibility, as well as with clinical and laboratory typical features of the disease, cardiovascular events, and infections [179]. Genome-wide association studies identified a new genetic locus (*BLK–C8orf13* on chromosome 8) for SLE susceptibility which involved a promoter variant associated with reduced expression of *BLK* and increased expression of *C8orf13* [51]. Although novel high-throughput stratagies and *in silico* analysis make it relatively easier to identify different classes of *cis*-DNAs, they are limited with regard to the underlying mechanism of how these variations affect gene expression and contribute to phenotypic variances. Promoters are involved in initiating transcription and are therefore among the many important *cis*-acting elements that regulate gene expression that might harbor functionally relevant polymorphisms. The proposed study lends credibility to the hypothesis that polymorphism within promoters may be a common source of phenotypic variation and possibly susceptibility to common disease, particularly SLE.

3.0 OBJECTIVES

Since variation in promoter region could affect gene expression and disease risk, the objective of this study, in part, was to characterize the putative *APOH* promoter. For this purpose, we have subcloned a ~1.4 kb genomic fragment of the human putative *APOH* promoter region and characterized the proximal promoter region using traditional techniques such as *in vitro* promoter – reporter gene assay, electrophoretic mobility shift assay and 5′ deletion analysis. The association of genetic variation in the coding region of *APOH* with SLE risk has been examined extensively; however, a comprehensive analysis of *APOH* promoter SNPs has not been reported yet. Therefore, this study also aims to test the association of *APOH* promoter SNPs with SLE risk and/or SLE-related clinical phenotypes in a cohort of SLE cases and controls. The *APOH* promoter polymorphisms were also investigated for association with difference in plasma β_2GPI levels among individuals. All eight *APOH* promoter SNPs previously reported to be present in Caucasians (-1284C>G, -1219G>A, -1190G>C, -759 A>G, - 700C>A, -643T>C, -38G>A, and -32C>A) and five present in African Americans (-1076G>A, -1055T>G, -627A>C, -581A>C and -363C>T) were examined [106]. The insertion/deletion polymorphism (-742delT) could not be characterized due to repetitive sequences in the surrounding region.

4.0 SPECIFIC AIMS

Following are the specific aims of the project:

Specific Aim 1: To characterize the putative *APOH* functional promoter and its *cis*-acting regulatory elements by 5′-deletion analysis.

Specific Aim 2: To functionally characterize the *APOH* promoter polymorphisms by dual-luciferase reporter gene assay and electrophoretic mobility shift assay.

Specific Aim 3: To perform genetic association of *APOH* promoter SNPs with SLE risk and/or SLE-related clinical phenotypes (autoimmune-mediated cardiovascular, lupus nephritis, occurrence of APA) in SLE cases and controls.

Specific Aim 4: To determine the genetic association of *APOH* promoter SNPs with plasma β_2GPI levels in a SLE cohort.

5.0 PATIENTS AND METHODS

5.1 SUBJECTS

Peripheral blood samples were obtained from 393 (345 white and 48 black) women with SLE and 496 (454 white and 42 black) healthy control women. SLE subjects were derived from the Pittsburgh Lupus Registry and were 18 years of age or older (mean age ± standard deviation (SD) = 43.45 ± 11.40 for whites and 41.69 ± 12.11 for blacks), and met the 1982 and the 1997 revised American College of ACR classification criteria for definite or probable SLE [180, 181]. The spectrum of ACR criteria for our Caucasian patient population is shown in Table 3. Blood samples from Caucasian control subjects (mean age ± SD = 45.28 ± 13.14 for whites and 49.39 ± 16.36 for blacks) with no history of SLE were obtained from Central Blood Bank of Pittsburgh. This study was approved by the University of Pittsburgh Institutional Review Board and all participants provided written informed consent. A second cohort of Caucasian female SLE patients (n = 109) and controls (n = 81) from the Chicago SOLVABLE (Study of Lupus Vascular and Bone Longterm Endpoints) study was used to replicate the most significant SLE disease association and the most significant carotid plaque association (cases only) detected in the Pittsburgh cohort. Detailed description of the Chicago study population is reported elsewhere [182].

Table 3. Clinical characteristics of Caucasian SLE patients

Clinical features	Occurrence
skin (malar rash and discoid rash)	55%
photosensitivity	59%
oral ulcers	54 %
arthritis	91%
serositis	45%
renal involvement	30%
neurologic involvement	9%
hematologic involvement	52%
immunologic involvement	72%
antinuclear antibody	98%

Diagnosis criteria for lupus nephritis among SLE patients was based on the presence of either 1) renal biopsy showing lupus nephritis, 2) at least 2 readings of proteinuria > 0.5 gm/24 hours or 3 + protein by dipstick, or 3) red blood cell casts. Two main clinical parameters that were used to define subclinical CVD in a subgroup of SLE patients (n = 245) were the measurements of carotid IMT and carotid plaque index. B-mode carotid ultrasound was performed using a Toshiba SSA-270 A scanner (Tustin, CA) equipped with a 5-mHz linear array imaging probe as described previously [23]. Sonographers scanned the right and left common carotid artery, carotid bulb, and the first 1.5 cm of the internal and external carotid arteries. Plaque was defined as a distinct area protruding into the vessel lumen that was at least 50% thicker than the surrounding areas. The average carotid IMT was measured across 1-cm segments of both the right and left sides of the near and far walls of the distal common carotid artery and the far wall of the carotid bulb and internal carotid artery. Values from each location were then averaged to produce an overall measure of carotid IMT.

5.2 MEASUREMENT OF ANTIPHOSPHOLIPID ANTIBODIES

Serum samples were screened in duplicate for the presence of aCL (IgG aCL positivity defined as >15 IgG phospholipid units, IgM aCL positivity defined as >10 IgM phospholipid units; kits obtained from Incstar, Stillwater, MN), LAC (by partial thromboplastin time or Russell's viper venom time with mix), and anti-β_2GPI (Quantalite β_2GPI screen, INOVA Diagnostics, San Diego, CA). Additional information regarding the methods that were used to measure these three APA is provided elsewhere [105, 113].

5.3 GENOTYPING

QIAamp kit (Qiagen, Chatsworth, CA) was used to isolate genomic DNA from buffy coat. The *APOH* promoter was sub-divided into smaller PCR fragments containing specific SNPs as listed in Table 4. For two of the amplicons (Promoter 1 and Promoter 2), the PCR involved three primers: two amplification primers (forward and reverse) that are specific to the sequence of interest, one of which is tagged to the universal biotinylated primer. For the rest, PCR was performed with either the forward or the reverse primer biotinylated (Table 4). For each fragment, 1 nanogram of genomic DNA was amplified in a 50 µL reaction using the cycling conditions as shown in Table 5. Genotyping was performed using standard PCR procedures. A 5 µL aliquot of the amplified PCR product was verified by gel electrophoresis (2% gel stained with ethidium bromide). The robustness of the genotyping results was tested by randomly selecting about 10% of the samples for repeated assays and check for concordance.

Table 4. Primer pairs used for PCR amplification of genomic *APOH*

APOH Promoter PCR Fragments	SNPs	PCR Primers
Promoter 1	-1284C>G -1219G>A -1190G>C	Universal Biotinylated Tag 5′ GCTGCTCCGGTTCATAGATT Forward + Tag - 5′ AGCGCTGCTCCGGTTCATAGATTTCTGCCTGACAGATGGA GATT Reverse - 5′ CACACCTGAAGCCTTTCC
Promoter 2	-759A>G -700C>A -643T>C	Universal Biotinylated Tag 5′ GCTGCTCCGGTTCATAGATT Forward - 5′ CGAACCCTCTCAAGCAACA Reverse + Tag - 5′ AGCGCTGCTCCGGTTCATAGATTTTGCAAGCTCCTATAGCT CCA
Promoter 3	-627A>C -581A>C	Forward - 5′ GGCCCATTATCTTATCCTACTCAA Reverse - 5′ Biotin - TGCAAGCTCCTATAGCTCCAGTTA
Promoter 4	-1076G>A -1055T>G	Forward - 5′ AAAGGAAAGGCTTCAGGTGTG Reverse - 5′ Biotin - CCCAACTTAAGGCCAACCA
Promoter 5	-38G>A -32C>A	Forward - 5′ Biotin - GTGGGTCTCAGAGTTCCATTCAA Reverse - 5′ CCTGACATATACGAAGGGGTTGGA

APOH genomic promoter reference sequence is shown in Figure 21

Table 5. PCR conditions

PCR reaction	Promoter 1	Promoter 2	Promoter 3	Promoter 4 & 5
10X Buffer MgCl2 dNTPs Forward Primer Reverse Primer Universal Tag Primer *Taq* Polymerase	6.0 µL 4.8 µL 3.0 µL (Tag) 0.06 µL 0.6 µL 0.6 µL 0.3 µL	6.0 µL 4.8 µL 3.0 µL 0.6 µL (Tag) 0.06 µL 0.6 µL 0.3 µL	5.0 µL 2.0 µL 2.5 µL 0.3 µL 0.3 µL --- 0.25 µL	5.0 µL 2.0 µL 2.5 µL 0.5 µL 0.5 µL --- 0.25 µL
Cycling conditions	95°C for 5 min. – 1 cycle 95°C for 30 sec | 60°C for 30 sec | x 45 cycles 72°C for 30 sec | 72°C for 5 min. – 1 cycle		95°C for 5 min. – 1 cycle 95°C for 30 sec | 60°C for 30 sec | x 35 cycles 72°C for 30 sec | 72°C for 5 min. – 1 cycle	

10X PCR buffer (100mM Tris-HCl, pH=8.3, 500mM KCl), 50 mM $MgCl_2$, 1.25mM of each dNTP , 20 µmol/L PCR primers, 20µmol/L biotinylated universal tag sequence, and 1.25 units of Taq DNA polymerase (Invitrogen), sec = second, min = minute

5.3.1 Pyrosequencing

Pyrosequencing, a non-electrophoretic DNA sequencing platform for the analysis of SNPs, was used to genotype each genomic DNA sample for all *APOH* promoter SNPs. Table 6 shows the sequence of the primers for each pyrosequencing reaction. Primers were designed using Pyrosequencing Assay Design Software version 1.0.6 (Biotage, Charlottesville, VA). The principle of pyrosequencing analysis using a universal biotinylated primer for mutation detection and SNP genotyping has been described elsewhere [183]. For each pyrosequencing reaction, 10-20 μL of each biotinylated PCR product (depending on product yield) was aliquoted into the wells of a 96-well plate. To bind the products to sepharose beads, 80 μL of binding reaction mix was added to each well. The binding reaction mix consists of 40 μL Binding Buffer (Biotage, Charlottesville, VA), 18 μL high purity water, and 2 μL Streptavidin-Sepharose beads (Amersham Biosciences, Piscataway, NJ). The binding reaction mix and PCR products were mixed at 1400 rpm at room temperature for at least ten minutes. Each pyrosequencing primer (Table 6) was diluted to 1:10 in annealing buffer, and 10 μL of the annealing mix (including pyrosequencing primer) was added to each well of a PSQ™ HS 96-well plate, resulting in 5 pmol of pyrosequencing primer per well. Negative controls (i.e. pyrosequencing primer only and biotinylated primer and Pyrosequencing primer without template) were included on each plate to confirm that background signal was negligible. Sepharose-bound PCR products were captured on the probes of the Pyrosequencing Vacuum Prep Tool (Biotage, Charlottesville, VA). The beads were washed in 70% ethanol (5 secs), followed by denaturation solution (0.2 M NaOH) (5 secs), and then washing buffer (10 secs). The vacuum was released, and the probes were immersed in the PSQ HS 96-well plate containing the annealing solution, and the beads were released by gentle shaking. The plate was then incubated on a heat block at 90°C for 2 minutes and allowed

to cool to room temperature prior to reading. Plates were read on a PSQ HS Pyrosequencer using PyroGold reagents and analysis software version 1.2 in allele quantification (AQ) mode.

Table 6. Pyrosequencing primers

APOH Promoter PCR Fragments	SNPs	Pyrosequencing Primers
Promoter 2	-1284C>G -1219G>A -1190G>C	5′ CTAAAATTCCCTGAGACAC 5′ AGCTCTGAATAAATAACCTC 5′ TTAATGGCTGCCTCC
Promoter 3	-759A>G -700C>A -643T>C	5′ TCAGCACTGGCCC 5′ CCCAAGTTGTTAATTTCA 5′ CTAGACAGATCCAAGACATA
Promoter 4	-627A>C -581A>C	5′ ACATATTAAGAATGGATGAG 5′ AGAAGATAGAGAATTCAAGG
Promoter 5	-1076G>A -1055T>G	5′ TCAGGTGTGCTCCAA 5′ TTGTTGGTATGGGGA
Promoter 6	-38G>A -32C>A	5′ TCACACTGGCACTACC

5.3.1.1 Principle of Pyrosequencing

In the initial step, biotin-labeled PCR amplicons are immobilized by binding to streptavidin-coated Sepharose beads, and DNA is denatured to produce single-stranded DNA template for the Pyrosequencing assay. Next, the reaction is incubated with four catalytic enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase, and the substrates, adenosine 5′ phosphosulfate (APS) and luciferin. In a cascade of enzymatic reactions (Figure 18), visible light is generated that is proportional to the number of incorporated nucleotides. The cascade begins with a nucleic acid polymerization reaction in which pyrophsophate (PPi) is released as a result of nucleotide incorporation by polymerase, in a quantity equimolar to the amount of nucleotide incorporated. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5'

phosphosulfate. ATP then drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. In the next step, unincorporated dNTPs and excess ATP are continuously degraded by, the nucleotide degrading enzyme, apyrase, leading to loss of light emission. After the degradation is completed, the next dNTP is added and a new Pyrosequencing cycle is started. Finally, the sequential incorporation of every nucleotide is converted to light, which is detected by the PSQ 96 instrument and displayed as a peak in pyrosequencing readouts (pyrogram), enabling the sequence of the template strand to be determined and SNPs to be genotyped. Each peak height is proportional to the number of nucleotides incorporated. Genotypes are assigned by both system-generated and manual calls using the pyrosequencing software (PSQ-96MA). An example of the theoretical histograms along with the actual pyrograms of the three possible genotypes for the complementary (c) strands of *APOH* promoter -38G>A and -32C>A SNPs are shown in Figure 19.

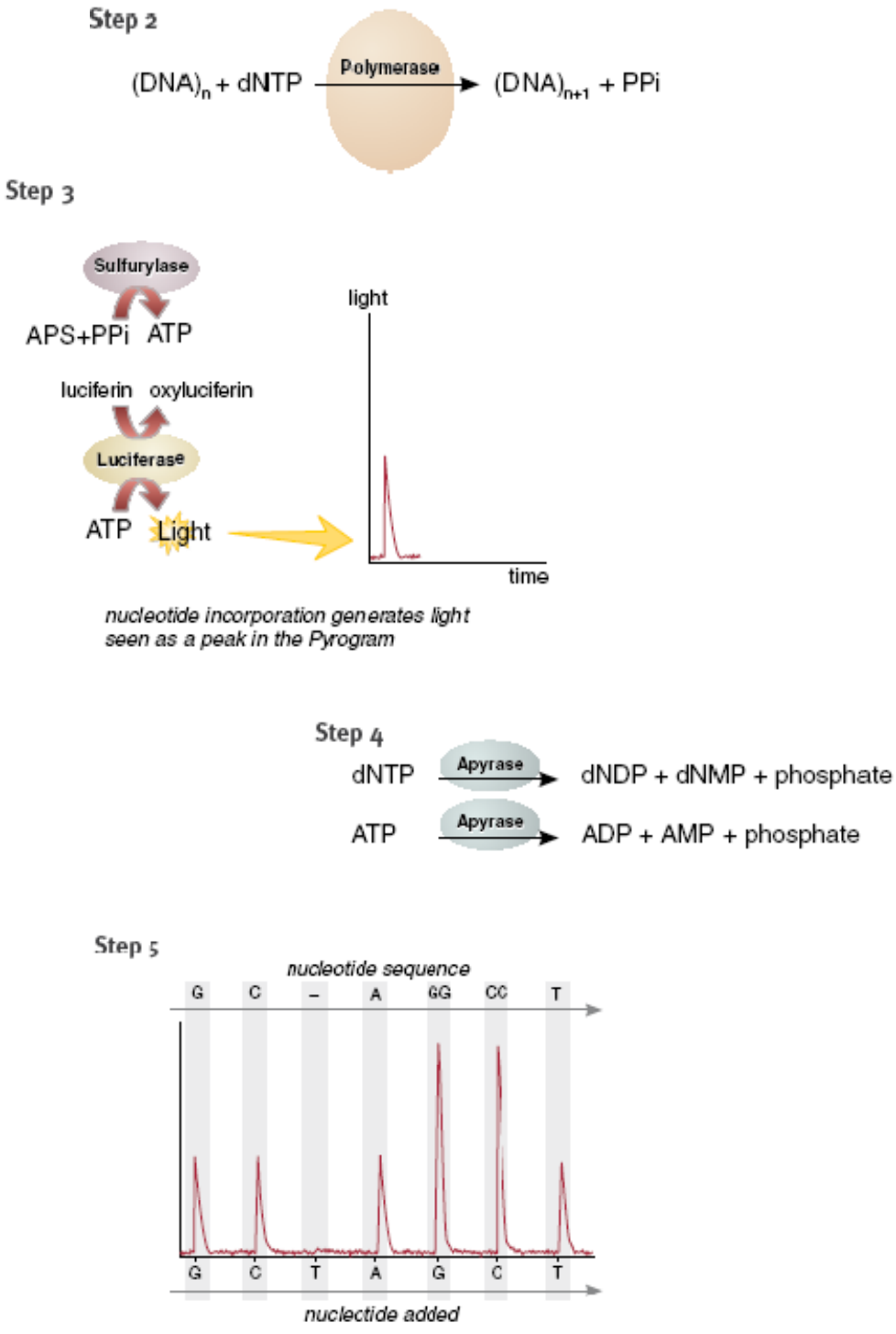
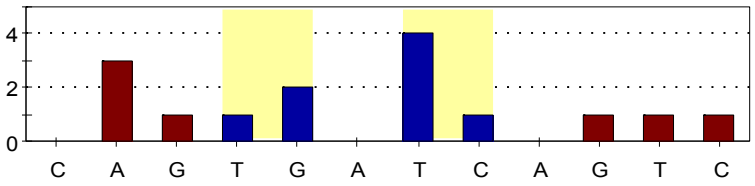
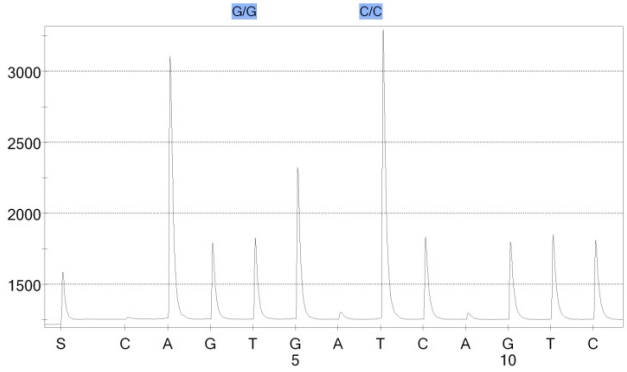


Figure 18. Principle of pyrosequencing
From Biotage, 2006



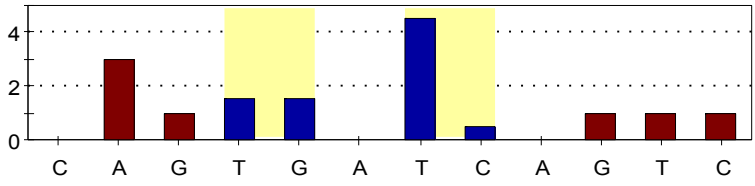
Theoretical Histogram: c(-32C>A): GG

c(-38G>A): CC



Actual Pyrogram: c(-32C>A): GG

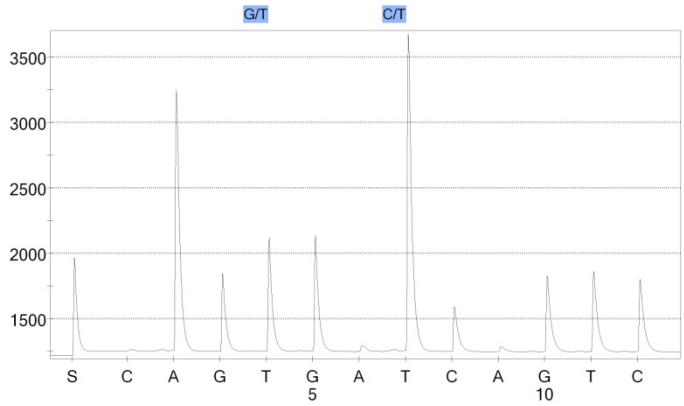
c(-38G>A): CC



Theoretical Histogram: c(-32C>A): GT

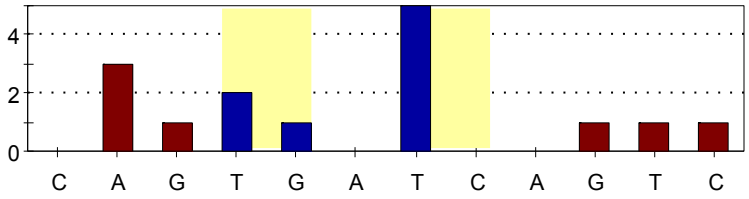
c(-38G>A): CT

Figure 19. (Continued Below)

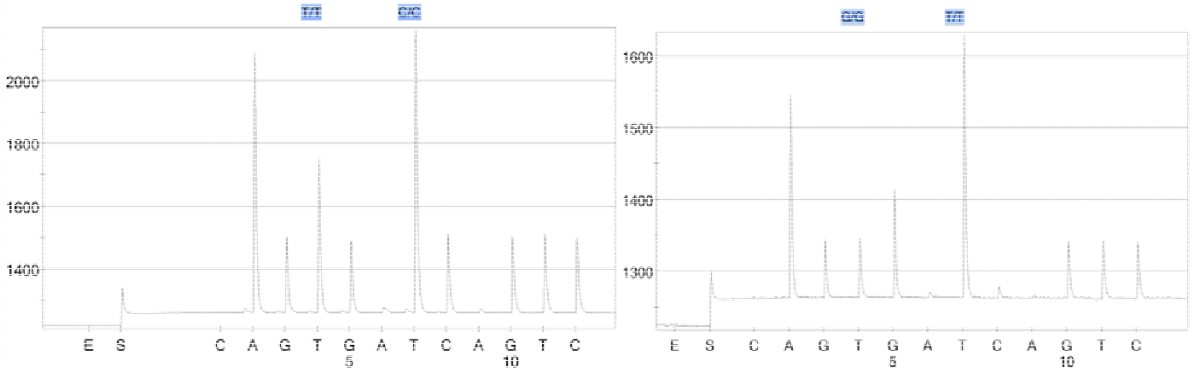


Actual Pyrogram: c(-32C>A): GT

c(-38G>A): CT



Actual Pyrogram: c(-32C>A): TT c(-38G>A): TT



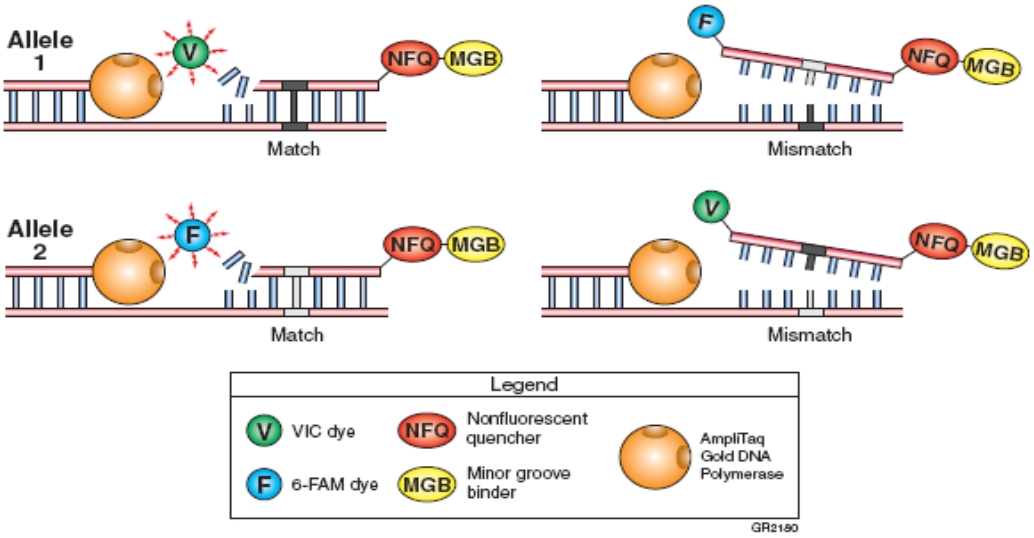
Theoretical Histogram: c(-32C>A): TT

c(-38G>A): TT

Figure 19. *APOH* Promoter 6 -32C>A (antisense: G>T) & -38 G>A (antisense: C>T)

5.3.2 TaqMan

The *APOH* promoter SNP -643T>C (rs3760292) was genotyped in the Chicago SLE sample using a custom TaqMan allelic discrimination genotyping assay (Assay ID: C___2658405_10) (Applied Biosystems, Foster City, CA). Standard ABI protocol for TaqMan SNP Genotyping Assay was followed that utilizes 3′ minor groove binder (MGB) probe-based technology consisting of two unlabeled PCR primers, one VIC dye-labeled probe, and one FAM dye-labeled probe for allelic discrimination (Figure 20). The MGB molecule binds to the minor groove of the DNA helix, improving hybridization-based assays by stabilizing the MGB-probe/template complex. Allele detection is achieved with exonuclease cleavage of a 5′ allele-specific dye label, which generates the permanent assay signal A non-fluorescent quencher (NFQ) is also implemented to eliminates background fluorescence and improve signal strength, and therefore call accuracy. PCR was performed on a using a PTC-200 Peltier Thermal Cycler (MJ Research) and an ABI PRISM® 7900HT Sequence Detection System was used to run the samples and analyze the results. The cycling conditions involve an initial hold for 10 min at 95 °C, followed by 40 amplification cycles (15 secs at 95 °C, 1 min at 60 °C).



A substantial increase in...	Indicates...
VIC dye fluorescence only	homozygosity for Allele 1.
FAM dye fluorescence only	homozygosity for Allele 2.
both fluorescent signals	Allele 1-Allele 2 heterozygosity.

Figure 20. Schematic representation of TaqMan probe-target matches and mismatches
From Applied Biosystems, 2004

5.4 *IN VITRO* ANALYSIS

5.4.1 Construction of *APOH* Promoter Luciferase Reporter Gene Vector (Wild-type)

A 1,418 bp fragment of the human *APOH* 5´-flanking region (-1375/+43 nucleotides from the translation initiation codon ATG) containing the promoter and the first untranslated exon (Figure 21, *http://pga.gs.washington.edu/data/apoh/apoh.ColorFasta.htm [106]*) was PCR amplified using forward (5'-TGGCAGCACACTCTTCTTAT-3') and reverse (5´-

GTTCTCGAGTTTTCTCTGCC-3′) primers. This *APOH* promoter fragment was amplified from an individual who had wild-type alleles for all 14 promoter SNPs included in this study. The PCR conditions were denaturing at 95°C for two minutes, followed by 35 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for one minute, before a final extension at 72°C for 10 minutes. The PCR-generated fragment was cloned into the pCR-2.1-TOPO vector (Invitrogen Corporation, Carlsbad, CA) using the supplier's standard protocol. The size and orientation of the DNA insert was confirmed by restriction analysis (*Hin*dIII and *Sac*I). The promoter fragment was then excised out of the TOPO vector using enzymes *Kpn*I and *Eco*RV and ligated into the *Kpn*I-*Sma*I restricted pGL3-Basic firefly luciferase (*Luc*) reporter plasmid (Figure 22) and transformed into top 10 chemically competent cells (Invitrogen Corporation, Carlsbad, CA), with positive clones then selected. Following transformation, the reporter construct pGL3-*APOH* promoter was confirmed by sequencing. This constituted the original wild-type *APOH* promoter construct which contained the major alleles for all 14 *APOH* promoter SNPs.

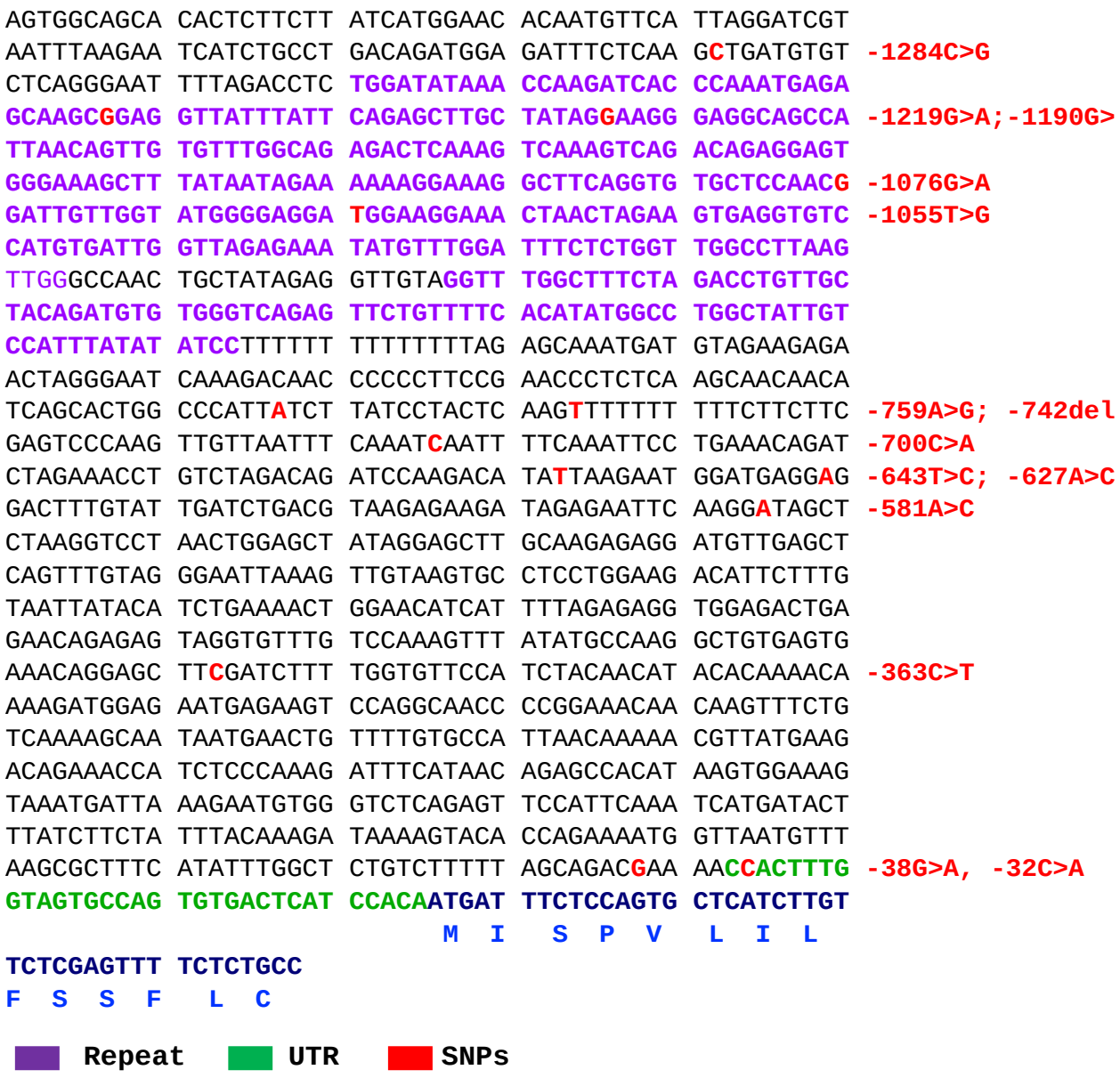


Figure 21. *APOH* promoter fragment (1,418 bp) containing the 14 SNPs

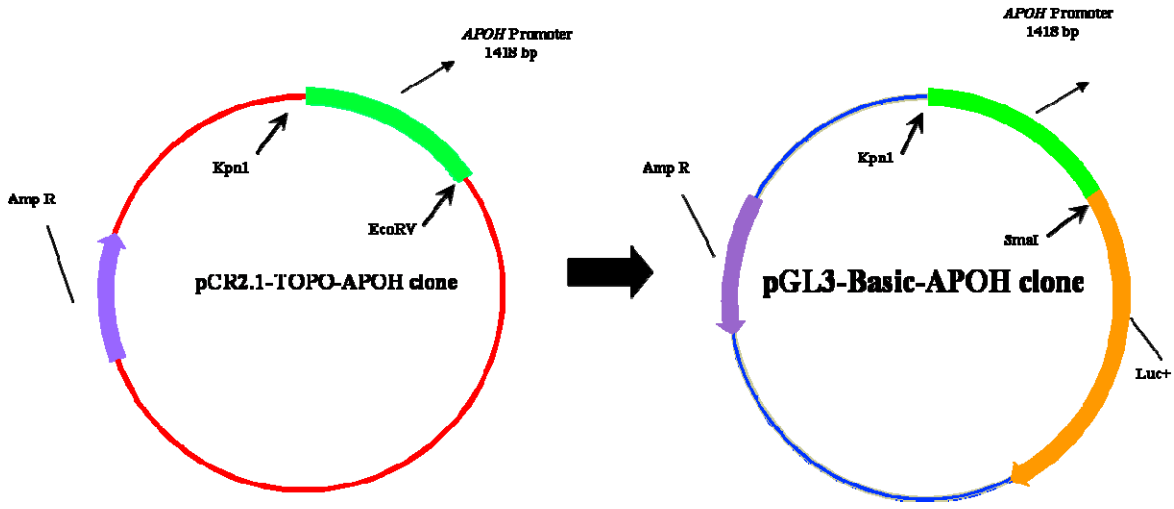


Figure 22. Construction of *APOH* promoter luciferase reporter gene vector (wild-type)

5.4.2 Construction of *APOH* Promoter Deletion Mutants

Experimental Design: Examination of the 5′-flanking region helps localize previously unrecognized and functionally important positive/negative regulatory sequences. Deletion analysis with promoter-reporter fusion constructs is based on the assumption that elimination of important *cis*-elements will result in reduced or abolished reporter gene expression. For this purpose, we subcloned series of 5′- deletion mutants of the ~ 1.4 bp *APOH* promoter fragment into a new *Luc* reporter vector pGL3-Basic. The construction process involved excising the desired length deletion fragments from the initial wild-type construct and inserting them into an empty pGL3-Basic vector with no insert. For this purpose, the original wild-type construct carrying the 1,418 bp *APOH* promoter fragment served as a parental template for designing PCR primers to amplify several truncated *APOH* promoter fragments. Due to the presence of a large amount of repeat sequence within the ~1.4 kb fragment we designed five *APOH* deletion mutant constructs differing in approximately 200 bp between each fragment as shown in Figure 23.

APOH Deletion fragment 1 (*APOH* del FR #1): It is the largest of all 5 fragments (858 bp). The position of this region with respect to the translational start site is +43 to -815.

APOH Deletion fragment 2 (*APOH* del FR #2): This fragment contains 618 bp. The location of this deletion mutant from the translational start site is +43 to -575.

APOH Deletion fragment 3 (*APOH* del FR #3): The third fragment (368 bp) position with respect to the translational start site is +43 to -325.

APOH Deletion fragment 4 (*APOH* del FR #4): The fourth fragment is further truncated to position -166 and is sized 209 bp.

APOH Deletion fragment 5 (*APOH* del FR #5): It is the smallest of all 5 fragments (109 bp). The position of this region with respect to the translational start site is +43 to -65.

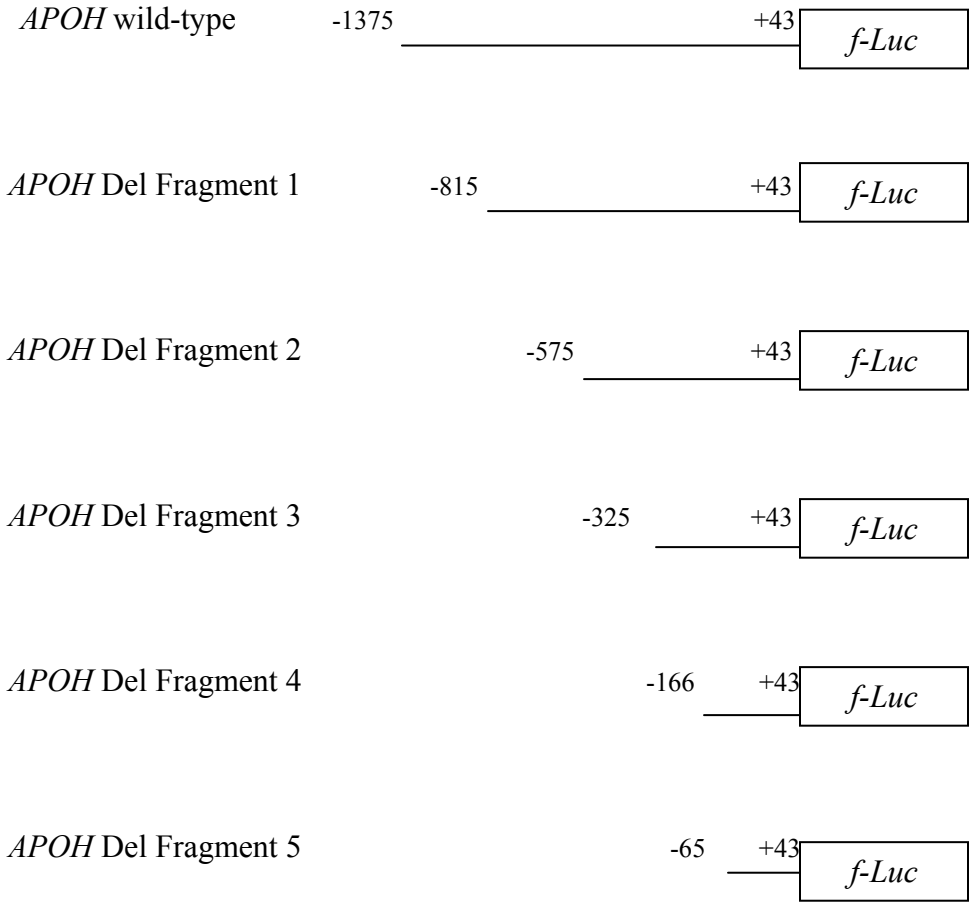


Figure 23. Schematic diagram to illustrate the *APOH* deletion mutant constructs
f-Luc = Firefly luciferase reporter gene of pGL3-Basic vector

Construction of Deletion Mutants: The initial construct comprised of the plasmid pGL3-Basic containing the 1418 bp fragment of the 5′-flanking region of *APOH* served as a template for PCR in order to amplify the five truncated fragments. Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) was used to design PCR primers containing linker sites for the restriction enzymes - *Kpn*I and *Bam*HI at the 5′ and 3′ ends of each deleted fragment respectively. Table 7 gives the list of PCR primers containing the *Kpn*I and *Bam*HI

sites. The PCR conditions used were, denaturing at 95°C for 5 minutes, followed by 35 cycles of denaturing at 95°C for 45 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, before a final extension at 72°C for 10 minutes.

Table 7. PCR primers containing the *Kpn*I and *BamH*I sites for *APOH* promoter deletion analysis

Deletion Mutants	Forward Primer (5´ to 3´)	Reverse Primer (5´ to 3´)
APOH del FR# 1	GCTCGGTACCCAAAGACAAC	CATGGATCCGGCAGAGAAA
APOH del FR# 2	CTCGGTACCCTAAGGTCCTAACT	TCATGGATCCGGCAGAGAA
APOH del FR# 3	GCTCGGTACCAAAGATGGAG	CATGGATCCGGCAGAGAA
APOH del FR# 4	GCTCGGTACCAAAGAATGTG	CATGGATCCGGCAGAGAA
APOH del FR# 5	CTCGGTACCCATATTTGGCTCT	TCATGGATCCGGCAGAGA

Bases highlighted in red indicate: Kpn1 site and blue: BamH1 site

The PCR products were gel purified (Qiagen, Valencia, CA) and then excised from the original pGL3-Basic vector by double digestion with *Kpn*I and *Bam*HI restriction enzymes. The digested fragments were again gel purified. The promoter-less pGl3-Basic vector (Promega Corporation, Madison, WI) was digested with *Kpn*I and *Bgl*II, gel purified and Calf Intestinal Alkaline Phosphatase (CIP) treated in order to prevent self ligation of the empty vector. The *APOH*-PCR DNA was then ligated to the gel purified and CIP-treated pGL3-Basic vector by T4 DNA ligase to generate the fusion vector construct carrying *APOH*-upstream truncated sequence fused to the in-frame luciferase reporter gene (Figure 24). The ligated product was then transformed into competent *E. coli* followed by screening of recombinant plasmids by colony PCR technique. The positive clones carrying the PCR fragment inserted into the pGL3-Basic vector were further confirmed by DNA sequencing and restriction digestion.

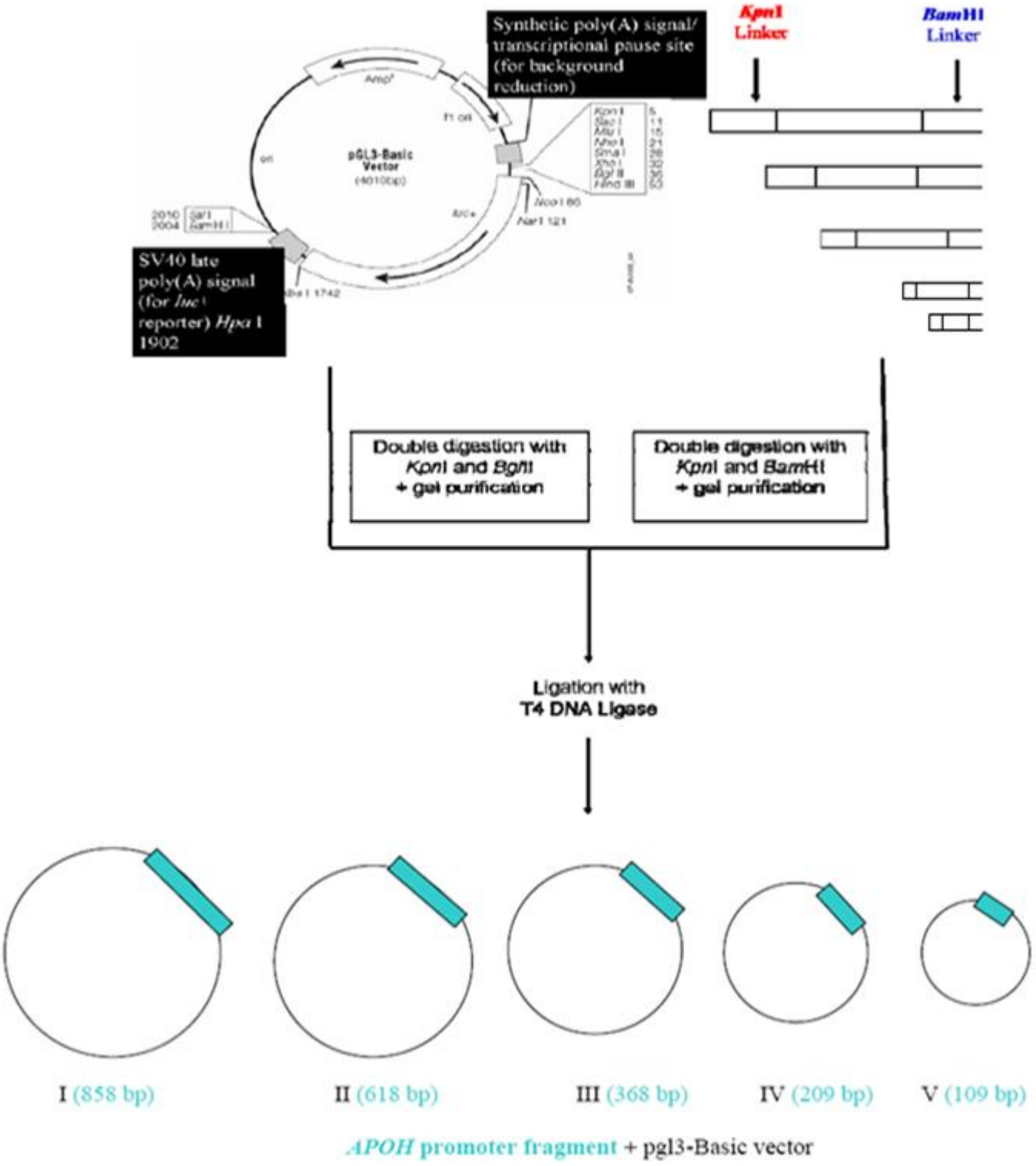


Figure 24. Construction of *APOH* promoter deletion mutants

5.4.3 Site-Directed Mutagenesis

Constructs bearing mutant/minor alleles for each *APOH* promoter variant were generated by PCR using the wild-type *APOH* promoter/luciferase report construct (~1.4 kb 5' region of *APOH* promoter inserted into the pGL3-Basic luciferase reporter vector) as template according to the manufacturer's protocol in the QuickChange II Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). Primers were designed according to manufacturer's instruction using the PrimerX software (http://www.bioinformatics.org/primerx/index.htm) as listed in Table 8. The basic procedure (Figure 25) utilizes a supercoiled double-stranded plasmid DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. *PfuTurbo* DNA polymerase extends the oligonucleotide primers, each complementary to opposite strands of the vector, during PCR. The PCR conditions used were denaturing at 95°C for 30 seconds, followed by 16 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for one minute, and extension at 68°C for six minutes, before a final extension at 68°C for 10 minutes. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following the thermal cycling, the PCR product was incubated with 1 μL *Dpn* I enzyme for an hour at 37°C. *Dpn* I, a restriction endonuclease specific for methylated and hemimethylated DNA, is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. Then 4 μL of the digestion was transformed into XL1-Blue supercompetent cells and positive clones were cultured to prepare minipreps of DNA using Qiaprep Spin Miniprep Kit. The success/presence of mutant clones and the fidelity of the *Pfu* DNA polymerase were verified by sequencing the plasmid DNA. A pWhitescript 4.5-kb control

plasmid was used to test the efficiency of mutant plasmid generation using this kit. Following transformation, positive control colonies were screened for the β-galactosidase (β-gal^{+}) phenotype of blue color on media containing IPTG (Isopropyl-Beta-d-Thiogalactopyranoside) and X-gal.

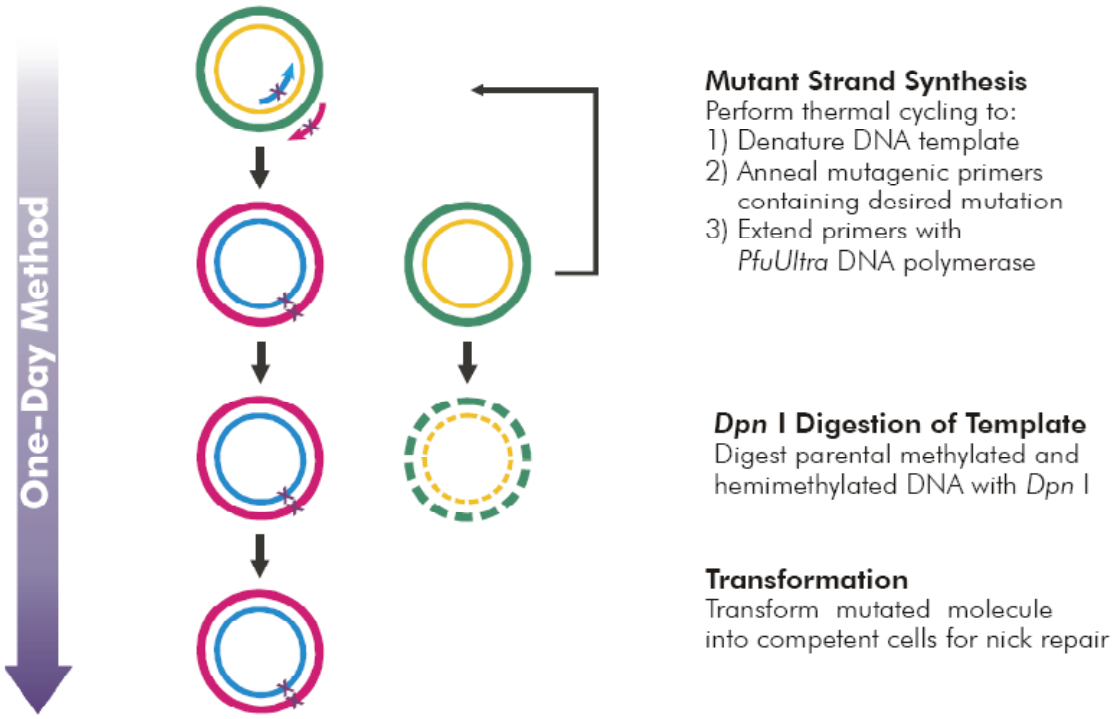


Figure 25. Overview of Quikchange II site-directed mutagenesis method
From Stratagene, 2007

Table 8. PCR primers for site-directed mutagenesis of *APOH* promoter SNPs

APOH Promoter SNPs	PCR Primers for Mutagenesis (sense primer sequence (5'-3') / antisense primer sequence (5´-3´))
-1284C>G	GGAGATTTCTCAAG**G**TGATGTGTCTCAGGG / CCCTGAGACACATCA**C**CTTGAGAAATCTCC
-1219G>A	CCCAAATGAGAGCAAGC**A**GAGGTTATTTATTCAG / CTGAATAAATAACCTC**T**GCTTGCTCTCATTTGGG
-1190G>C	GAGCTTGCTATAG**C**AAGGGAGGCAGC / CTGCCTCCCTT**G**CTATAGCAAGCTC
-1076G>A	CAGGTGTGCTCCAAC**A**GATTGTTGGTATGG / TACCAACAATC**T**GTTGGAGCACACCTG
-1055T>G	GGTATGGGGAGGA**G**GGAAGGAAACTAAC / GTTAGTTTCCTTCC**C**TCCTCCCCATACC
-759A>G	CAGCACTGGCCCATT**G**TCTTATCCTACTCAAG / CTTGAGTAGGATAAGA**C**AATGGGCCAGTGCTG
-700C>A	CCCAAGTTGTTAATTTCAAAT**A**AATTTTCAAATTCCTGAAAC / GTTTCAGGAATTTGAAAATT**T**ATTTGAAATTAACAACTTGGG
-643T>C	GACAGATCCAAGACATA**C**TAAGAATGGATGAGGAG / CTCCTCATCCATTCTTA**G**TATGTCTTGGATCTGTC
-627A>C	CATATTAAGAATGGATGAGG**C**GGACTTTGTATTGATCTGACG / CGTCAGATCAATACAAAGTCC**G**CCTCATCCATTCTTAATATG
-581A>C	GAAGATAGAGAATTCAAGG**C**TAGCTCTAAGGTCCTAAC / GTTAGGACCTTAGAGCTA**G**CCTTGAATTCTCTATCTTC
-363C>T	GTGAAACAGGAGCTT**T**GATCTTTTGGTGTTCC / GGAACACCAAAAGATC**A**AAGCTCCTGTTTCAC
-38G>A	CTCTGTCTTTTTAGCAGAC**A**AAAACCACTTTGGTAGTGC / GCACTACCAAAGTGGTTTTT**T**GTCTGCTAAAAAGACAGAG
-32C>A	CTTTTTAGCAGACGAAAAC**A**ACTTTGGTAGTGCCAGTG / CACTGGCACTACCAAAGT**T**GTTTTCGTCTGCTAAAAAG

Bases highlighted in red indicate introduced point mutations

5.4.4 Cell Culture, Transient Transfection and Dual-Luciferase Reporter Gene Assay

Dual-Luciferase reporter gene assays were performed between wild-type and mutant constructs carrying minor allele at individual SNP sites for each of the *APOH* promoter polymorphisms. Reporter assays were also performed between the wild-type and mutant H3 (minor allele only at SNP -643T>C) and H7 (minor alleles at SNPs -643T>C, -1190G>C and -759A>G) haplotype pGL3-basic firefly luciferase reporter vectors. The wild-type and mutant constructs were used to transiently co-transfect COS-1 cells along with the *Renilla* luciferase control vector (pRL-TK) (Promega). COS-1 (African green monkey kidney) cells were obtained from the American Type

Culture Collection (ATCC CRL-1650, Rockville, MD) and cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen) supplemented with 10% fetal bovine serum, 2mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin and were harvested at 50 to approximately 70% confluence. HepG2 cells (Human hepatocellular liver carcinoma; ATCC HB-8065) were grown in Eagle's minimal essential medium (EMEM, ATCC) supplemented with 10% fetal bovine serum, and penicillin/streptomycin. A day prior to transfection, 1.6 x 10^5 cells were seeded in each well of a 12-well plate with 1mL of antibiotic-free DMEM/EMEM media. Transfection of cells was performed utilizing Lipofectamine 2000 reagent as per manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA). Briefly, Opti-MEM I reduced serum media (Invitrogen Corporation, Carlsbad, CA) was used to plate 60-80% confluent cells in 12-well culture plates and each well was then transiently co-transfected with 1.5 µg of the pGL3 vector (with /without *APOH* promoter) and 50 ng of the pRL-TK vector (Promega Corporation, Madison, WI) using the cationic lipid-based transfection lipofectamine 2000 (4 µl/ml) reagent (Invitrogen Corporation, Carlsbad, CA, USA). The pRL-TK vector encoded for the *Renilla* luciferase transcribed by a HSV-TK promoter which was used as internal control to normalize firefly luciferase expression. After 5–7 hours (hrs) of exposure to the transfection medium, cells were incubated with serum-containing DMEM/ EMEM media and incubated for 48 hrs before dual-luciferase reporter assays (Promega Corporation, Madison, WI) were performed. Upon completion of 48 hrs, the cells were lysed in 100 µL passive lysis buffer (Promega Corporation, Madison, WI). Cell lysate was added to the luciferase substrate (dual luciferase reporter system, Promega) and firefly and *Renilla* luciferase light outputs were measured with either TD-20/20 Luminometer (Turner Design, Sunnyvale, CA) or the Tecan Infinite 200 plate reader (Tecan Trading, Switzerland) according to the manufacturer's

instructions. The luciferase data (firefly/*renilla*) was used in the statistical analysis and normalized to the average activity of the promoter-less empty vector to yield data reflecting fold-activity increase over baseline levels for each *APOH* promoter construct. Triplicate wells for each transfection condition were assayed (intra-experiment variation), and three independent transfections were carried out (inter-experiment variation).

5.4.4.1 Principle of Dual-Luciferase Reporter Assay

In the Dual-Luciferase reporter assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is simultaneously initiated by adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent also produces a stabilized signal from the *Renilla* luciferase, which decays slowly over the course of the measurement. In the DLR. Assay System, both reporters yield linear assays with no endogenous activity of either reporter in the experimental host cells. Both firefly and *Renilla* luciferases have dissimilar enzyme structures and substrate requirements. Firefly luciferase is a 61kDa monomeric protein that functions as a genetic reporter immediately upon translation. Photon emission is achieved through oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+} and O_2 (Figure 26).The luminescent reaction catalyzed by *Renilla* luciferase (36kDa monomeric protein) utilizes O2 and coelenterate-luciferin (coelenterazine; Figure 9).

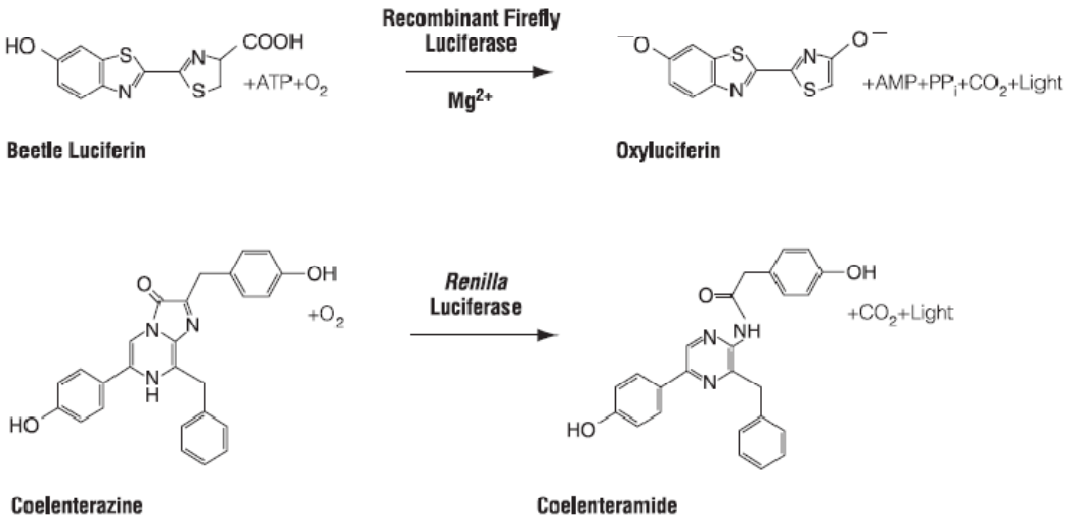


Figure 26. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferases

5.4.5 Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSA) were performed to analyze the binding of nuclear proteins from HepG2 cell nuclear extracts to oligonucleotides comprising of *APOH* promoter SNPs. Four single-stranded ~30-mer oligonucleotides corresponding to the wild-type or mutant allele were custom ordered (Sigma Genosys, TX and Operon Biotechnologies, AL) for each *APOH* SNP (Table 9). To make double-stranded probes and competitors, complementary oligos of equal amount were heated at 95°C for 5 min and then annealed for an hour at room temperature. The wild-type oligonucleotide was 5'-end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (New England Biolabs, MA) and purified by the QIAquick Purification kit (Qiagen, Valencia, CA). To allow DNA-protein binding, the mixture of unlabeled and labeled

oligos were incubated with 1 μL (5.68μg) of human HepG2 cell nuclear extracts for 20 minutes at room temperature in gel shift binding buffer (1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM TRIS-HCl pH 7.5, 20% glycerol). For the competition experiments, unlabeled competitor DNA was added in 1x, 5x, 20x, 50x, and 100x excess volumes of the labeled probe and was incubated with the HepG2 nuclear extract (Active Motif, CA) for 10 min before the addition of the labeled probe The DNA-protein complexes were then separated on 5% polyacrylamide gel at 120 volts for two hours, the gels were dried and exposed overnight for autoradiography on X-ray films (Kodak). For setting up of EMSA experimental procedures, an earlier published positive shift assay for the *APOH* promoter SNP -32C>A [116] was reproduced and used as a positive control.

Table 9. EMSA oligonucleotide sequences of *APOH* promoter SNPs

APOH Promoter SNPs	EMSA Oligonucleotides (sense primer sequence (5'-3') / antisense primer sequence (5´-3´))
-1284C	GATTTCTCAAG**C**TGATGTGTCTCAGGGA / TCCCTGAGACACATCA**G**CTTGAGAAATC
-1284G	GATTTCTCAAG**G**TGATGTGTCTCAGGGA / TCCCTGAGACACATCA**C**CTTGAGAAATC
-1219G	AAATGAGAGCAAGC**G**GAGGTTATTTATT / AATAAATAACCTC**C**GCTTGCTCTCATTT
-1219A	AAATGAGAGCAAGC**A**GAGGTTATTTATT / AATAAATAACCTC**T**GCTTGCTCTCATTT
-1190G	AGCTTGCTATAG**G**AAGGGAGGCAGC / GCTGCCTCCCTT**C**CTATAGCAAGCT
-1190C	AGCTTGCTATAG**C**AAGGGAGGCAGC / GCTGCCTCCCTT**G**CTATAGCAAGCT
-1076G	GGTGTGCTCCAAC**G**GATTGTTGGTATGGGG / CCCCATACCAACAATC**C**GTTGGAGCACACC
-1076A	GGTGTGCTCCAAC**A**GATTGTTGGTATGGGG / CCCCATACCAACAATC**T**GTTGGAGCACACC
-643T	GACAGATCCAAGACATA**T**TAAGAATGG / CCATTCTTA**A**TATGTCTTGGATCTGTC
-643C	GACAGATCCAAGACATA**C**TAAGAATGG / CCATTCTTA**G**TATGTCTTGGATCTGTC
-32C	CAGACGAAAAC**C**ACTTTGGTAGT / ACTACCAAAGT**G**GTTTTCGTCTG

Bases highlighted in red indicate SNP location

5.5 STATISTICAL ANALYSIS

Allele frequencies were determined by direct allele counting. Concordance of the genotype distribution to Hardy-Weinberg equilibrium was tested using χ^2 goodness-of-fit test for each polymorphism. LD pattern was determined using the Haploview version 3.32 (http://www.broad.mit.edu/mpg/haploview/). To control for multiple testing concerns, we performed two types of "gate-keeper" analyses to determine whether there was evidence that any of these SNPs influenced risk of SLE: (1) multiple regression incorporating all SNPs and (2) haplotype analyses. The multiple regression analysis was performed under additive model for genotypic effects, that is, major allele homozygote = 0, heterozygote = 1, minor allele homozygote = 2. Subsequent to observing associations with SLE risk, we performed additional

analyses to determine if any of the promoter SNPs also influenced specific clinical manifestations of SLE to gain insights into possible mechanisms of action. Again, to control for multiple testing concerns, we initially assessed possible associations of all SNPs with clinical variables assessed in patients only (the cases who were negative for these variables were treated as controls) using a multiple regression analysis. In these analyses, the genotypic effects were modeled as dominant (i.e., major homozygote *vs.* heterozygote + minor homozygote) because of the small sample size. Only for those SNPs that showed significant association in multiple regression analysis, we performed follow-up single-site analysis of the genotype (using Fisher's exact test or analysis of deviance when adjusting for covariates) and allele frequencies (standard *Z*-test of 2 binomial proportions). Carotid plaque was categorized as plaque positive (degree of plaque equal to or greater than 1) and plaque negative (degree of plaque equal to zero). The carotid IMT data was transformed to reduce the effects of non-normality. The covariates considered for analysis of variance (ANOVA), multiple regression analysis, and odds ratio (OR) calculation were age, body mass index (BMI), ever smoking, lipid profile (high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol and triglycerides) and C-reactive protein (CRP) levels whenever they were significant for a given phenotype. All computations were performed using the R statistical software package (version 2.3.1, http://www.r-project.org). The haplotype analysis was performed using both EH (Estimate Haplotype-frequencies) (version 1.2, http://linkage.rockefeller.edu/ott/eh.htm) and Haploview programs to check for overall haplotype distribution differences and individual haplotype associations, respectively. A *P* value of less than 0.05 was considered as suggestive evidence of association. Student's *t*-test was used to determine statistical significance of the expression

difference between the wild-type and mutant constructs. Power analysis was performed using the QUANTO software (http://hydra.usc.edu/GxE).

6.0 RESULTS

6.1 5´ SERIAL DELETION ANALYSIS OF *APOH* PROMOTER

In order to localize the active promoter regions and to identify regions that are important for regulation of the human *APOH* activity, the wild-type 1,418 bp 5'–flanking region of *APOH* was amplified from genomic DNA and used as template to create a series of five different deletion constructs containing 5' truncated fragments of *APOH* promoter fused upstream to a promoterless firefly luciferase (*Luc*) gene of the pGL3-Basic reporter vector (Figure 23). The sequence of each construct was verified by sequencing (data not shown) and confirmed by alignment with the human genomic DNA database. For COS-1 cells, as shown in Figures 27-29, the wild-type region extending from −1375 to +43 had promoter activity since luciferase activity of the reporter gene was ~4.5 fold that of the empty vector. A 5′ deletion of the promoter sequence to −815 (mutant 1, -815/+43), and further deletion to -575 (mutant 2, -575/+43) increased promoter activity; however there was no significant difference in luciferase activity between the wild-type, mutant 1, and mutant 2 constructs (Experiment III: wild-type *vs*. mutant 1; $P = 0.260$, wild-type *vs.* mutant 2; $P = 0.135$). Successive removal of nucleotides from −575 (mutant 2, -575/+43), to −325 (mutant 3, -325/+43), enhanced promoter activity appreciably (Experiment III: wild-type *vs.* mutant 3; $P = 0.019$), suggesting the possibility of negative regulatory elements within the −575/−325 regions. The mutant 3 construct (-325/+43) conferred

maximum luciferase activity in COS-1 cells. Decrease in promoter activity was observed for further deletion of sequence from -325 to -166 (mutant 4, -166/+43), however not to a significant extent (Experiment III: $P = 0.04$). But when the sequence from −166 to −65 was removed (mutant 5, -65/+43), promoter activity dropped significantly ($P < 0.001$) compared to the wild-type. This suggests the presence of a critical element in the region extending from −166 to −65.

Although COS-1 transiently transfected cells have been widely used in studying *APOH* expression, we replicated the deletion analysis using human hepatoma cell line (HepG2) since the liver is a major site of synthesis of β_2GPI. The magnitude of expression efficiency was much higher for HepG2 cells as compared to COS-1 cells because hepatocytes are the predominant cell type in the liver (Figures 30-31). Transfection of HepG2 cells revealed an overall similar trend as seen in COS-1 cells with mutant 3 (-325/+43) showing the highest promoter activity. A slight difference in trend was observed for the wild-type, mutant 1 (-815/+43), and mutant 2 (-575/+43) constructs, wherein mutant 1 was lower than the wild-type for HepG2 but not in COS-1 cells. Although, there was no significant difference between the three fragments (wild-type, mutant 1 and mutant 2) for both cell lines, the inconsistency in trend can be attributed due to the cell-type related factors influencing the basal and inducible *APOH* expression. Consistent with COS-1 cells, deletion from -166 (mutant 4, -166/+43) to -65 (mutant 5, -65/+43) almost abolished promoter activity by ~ 98% decrease in the promoter-luciferase activity when compared to the wild-type ($P < 0.001$, Table 11), indicating that the region between – 166 and − 65 contains *cis*-elements that are critical for transcription of *APOH.*

The average and standard deviation (SD) values along with the % increase/decrease with wild-type and significance of difference for each experiment is listed in Table 10 and Table 11 for COS-1 cells and HepG2 cells, respectively. Thus, using both COS-1 and HepG2 cell lines,

we have identified the region ~166 bp upstream of the translation start site as the basal promoter of the human *APOH* and also the presence of key *cis*-acting elements located in the regions between -166 and -65 from ATG codon to regulate *APOH* expression.

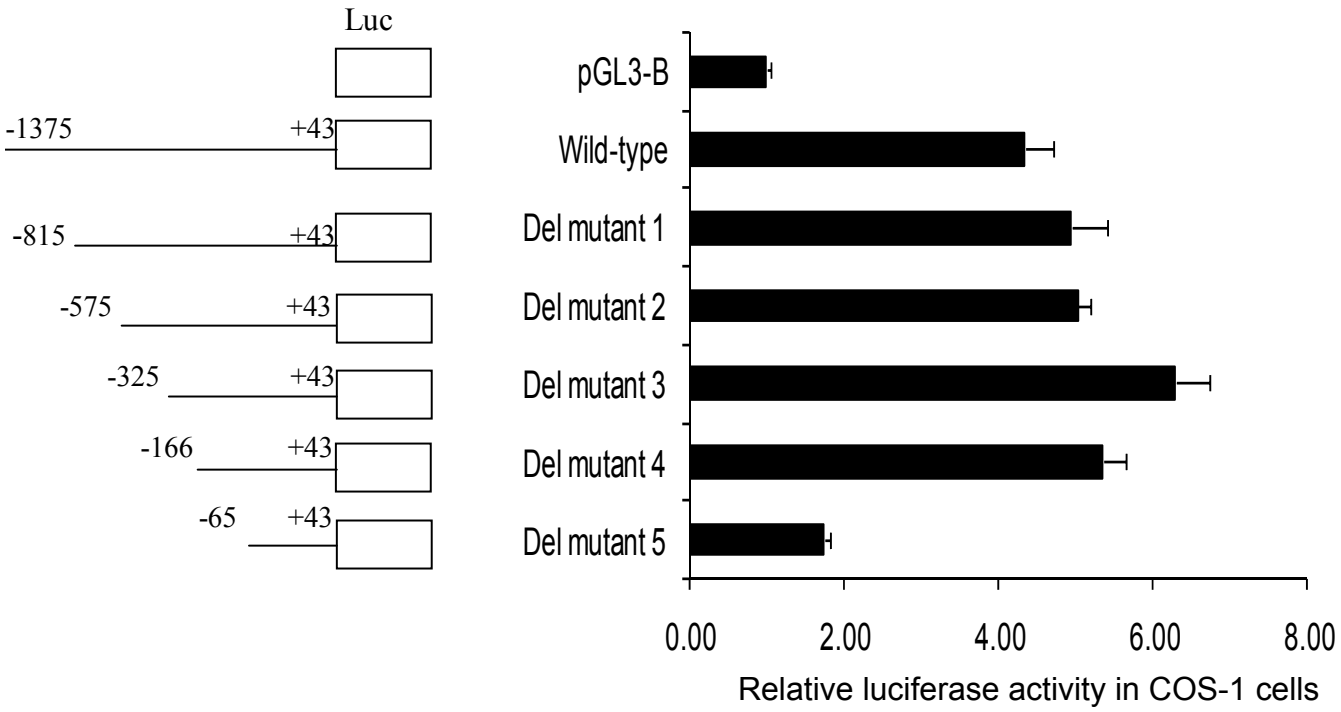


Figure 27. Dual-luciferase of *APOH* promoter deletion experiment I In Cos-1 cells

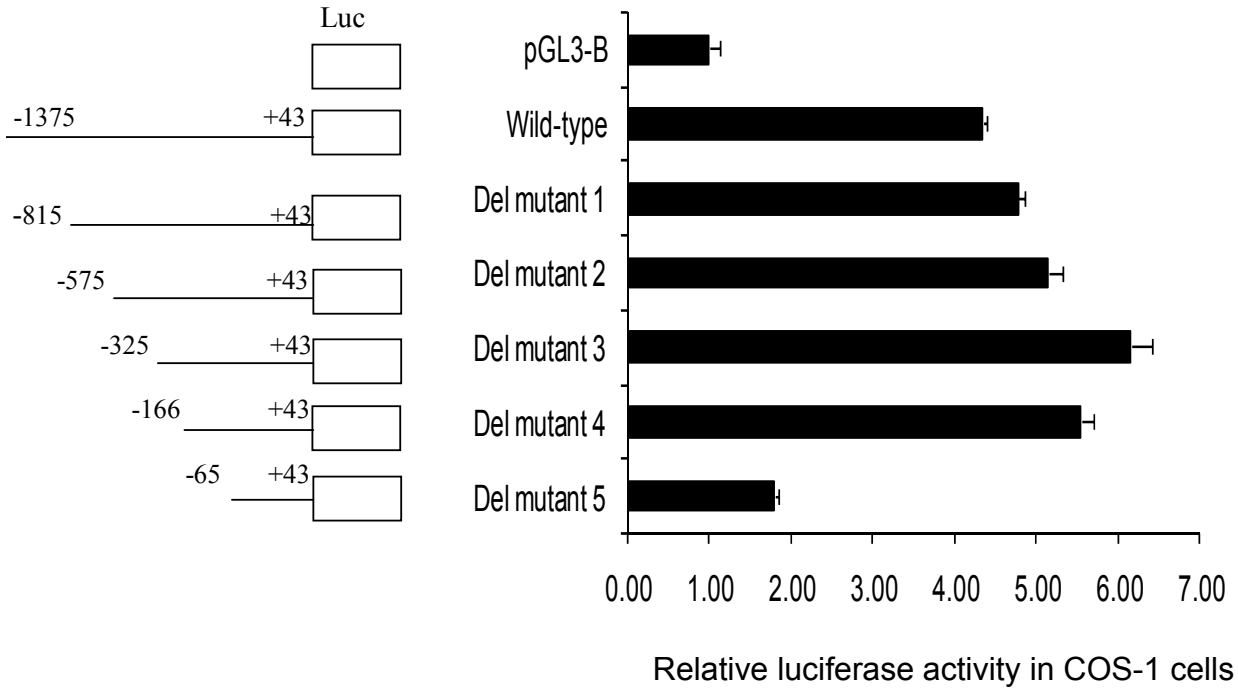


Figure 28. Dual-luciferase of *APOH* promoter deletion experiment II in COS-1 cells

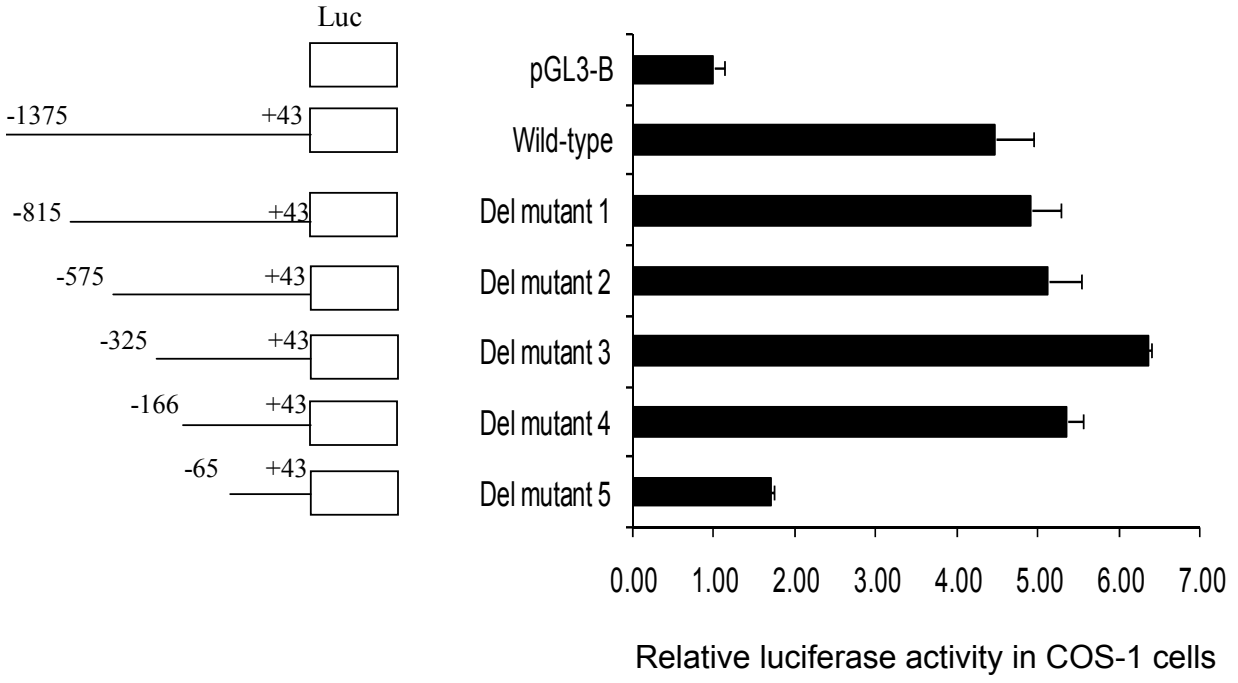


Figure 29. Dual-luciferase of *APOH* promoter deletion experiment III in COS-1 cells

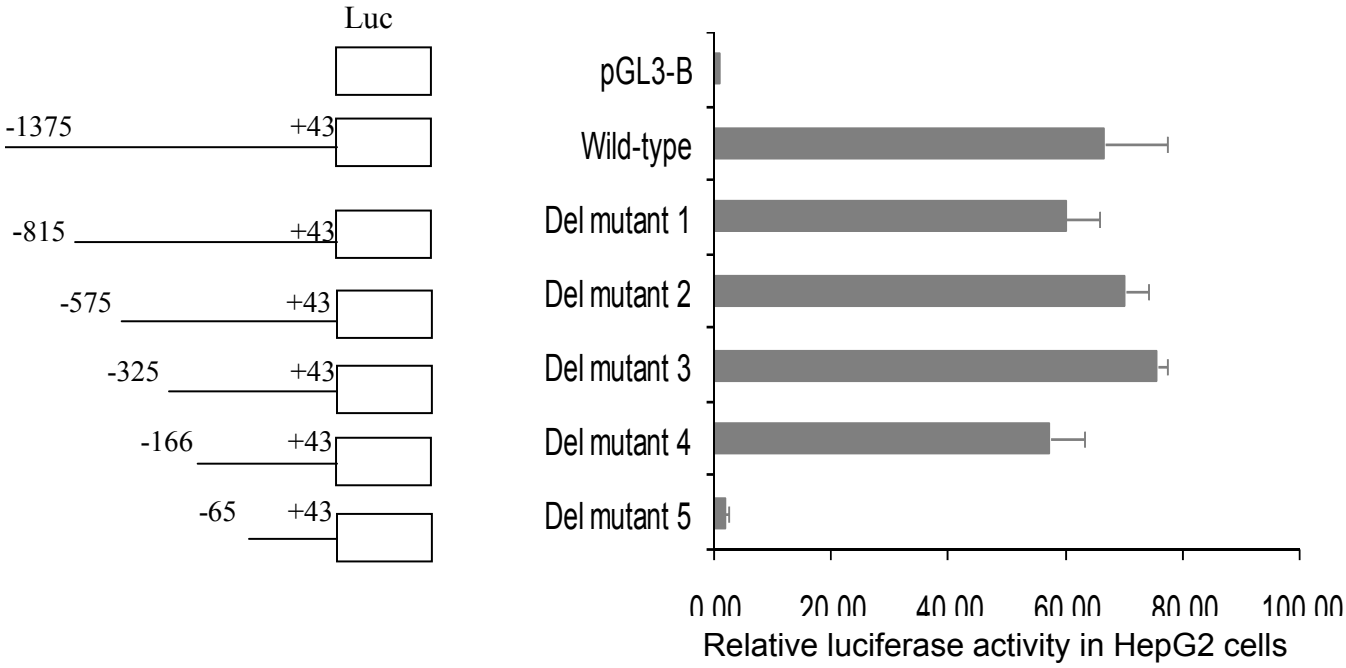


Figure 30. Dual-luciferase of *APOH* promoter deletion experiment I in HepG2 cells

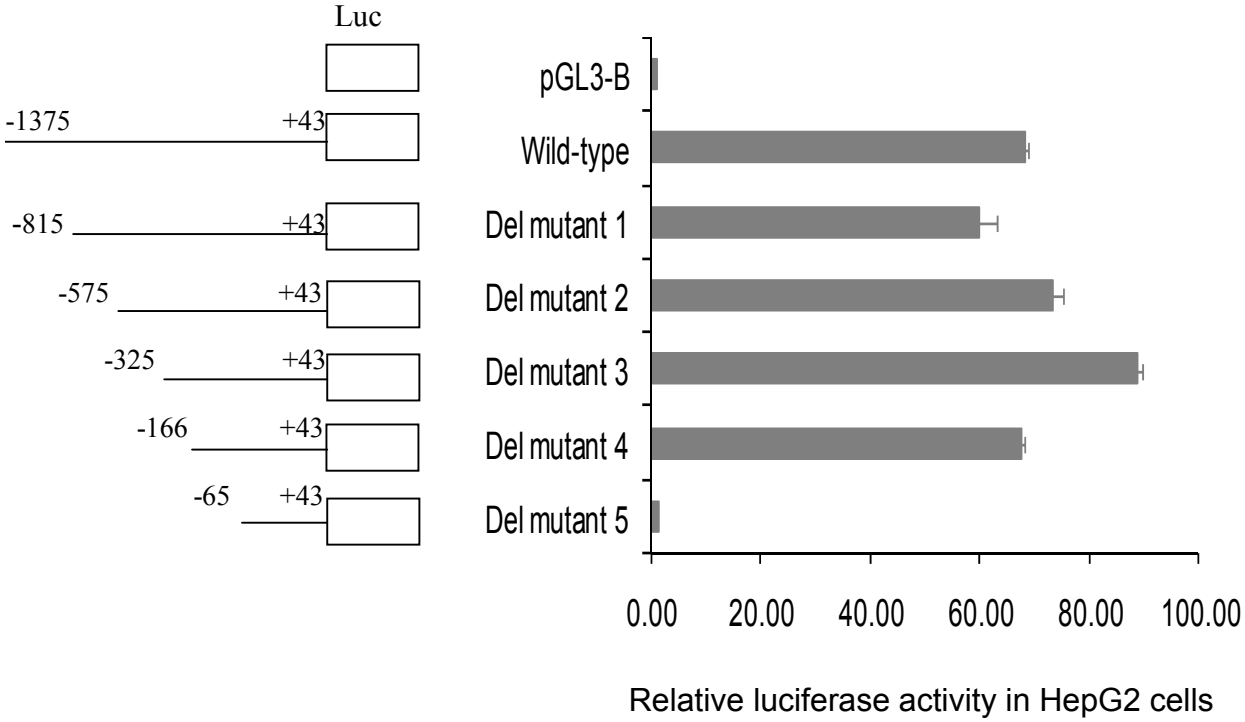


Figure 31. Dual-luciferase of *APOH* promoter deletion experiment II in HepG2 cells

Table 10. Data values (average and SD) of *APOH* promoter deletion in COS-1 cells

Experiment I			
Constructs	**Average**	**SD**	**% Increase (+) / Decrease (-)**
pGL3-Basic	1.00	0.04	--
Wild-type	4.35	0.35	--
Mutant 1	4.95	0.46	+13.84
Mutant 2	5.03	0.15	+15.67
Mutant 3	6.29	0.43	+44.73
Mutant 4	5.35	0.29	+23.01
Mutant 5	1.74	0.07	-60.00
Experiment II			
Constructs	**Average**	**SD**	**% Increase (+) / Decrease (-)**
pGL3-Basic	1.00	0.13	--
Wild-type	4.34	0.05	--
Mutant 1	4.78	0.07	+10.17
Mutant 2	5.15	0.17	+18.77
Mutant 3	6.16	0.25	+42.03
Mutant 4	5.54	0.16	+27.62
Mutant 5	1.79	0.05	-58.63
Experiment III			
Constructs	**Average**	**SD**	**% Increase (+) / Decrease (-)**
pGL3-Basic	1.00	0.12	--
Wild-type	4.47	0.47	--
Mutant 1	4.92	0.36	+10.05
Mutant 2	5.13	0.39	+14.73
Mutant 3	6.36	0.03	+42.33
Mutant 4	5.36	0.20	+19.92
Mutant 5	1.70	0.04	-61.90

% Increase/Decrease of each mutant is as compared to the wild-type

Table 11. Data values (average and SD) of *APOH* promoter deletion in HepG2 cells

Experiment I				
Constructs	**Average**	**SD**	**% Increase (+) / Decrease (-)**	***P* - values**
pGL3-Basic	1.00	0.19	--	--
Wild-type	66.70	10.64	--	--
Mutant 1	60.00	5.75	-10.04	0.392
Mutant 2	73.51	9.62	+10.20	0.457
Mutant 3	75.57	1.68	+13.30	0.284
Mutant 4	57.26	5.87	-14.15	0.250
Mutant 5	1.84	0.35	-97.24	0.009
Experiment II				
Constructs	**Average**	**SD**	**% Increase (+) / Decrease (-)**	***P* - values**
pGL3-Basic	1.00	0.09	--	--
Wild-type	68.23	0.59	--	--
Mutant 1	59.84	3.32	-12.30	0.013
Mutant 2	73.31	2.08	+7.45	0.015
Mutant 3	88.74	0.99	+30.06	0.000
Mutant 4	67.64	0.43	-0.86	0.233
Mutant 5	1.31	0.07	-98.08	0.000

% Increase/Decrease of each mutant is as compared to the wild-type

6.2 FUNCTIONAL CHARACTERIZATION OF *APOH* PROMOTER SNPS

6.2.1 Dual-luciferase activity of individual *APOH* promoter SNPs in COS-1 cells

In order to investigate the differential allele-specific effect on promoter activity, pGl3-Basic – *APOH* promoter constructs harboring individual point mutations for each of the 12 *APOH* promoter SNPs (-1284C>G, -1219G>A, -1190G>C, -1076G>A, -1055T>G, -759 A>G, -700C>A, -627A>C, -643T>C, -363C>T -38G>A, and -32C>A) were generated. The summary of the results for each luciferase activity along with the average ± SD, % decrease as compared with

the wild-type construct and significance of difference between the wild-type and mutant *APOH* promoter constructs is listed in Table 12. The relative luciferase activity assessed in three independent experiments performed in triplicate for all of the *APOH* promoter SNP constructs is illustrated in Figures 32-43.

Our results indicate that three *APOH* promoter SNPs (-1219G>A, -643T>C, and -32C>A) possess a functional activity, because they significantly affected the production of the luciferase gene ($P < 0.0001$). All the three mutant alleles, -1219A, -643C, and -32A showed ~ 51%, ~40% and ~ 36% mean reduction of gene expression respectively when compared with the wild-type alleles (Table 12). None of the SNPs present in African Americans (-1076G>A, -1055T>G, -627A>C, -581A>C and -363C>T) showed statistically significant reduction in gene expression (Table 12). The -32C>A SNP showed similar significant reduction effect as reported earlier by [116]; however the extent of reduction was slightly less in this study (~36% *vs.* ~50%) which can be attributed due to difference in constructs and experimental setup.

Table 12. Dual-luciferase of individual *APOH* promoter SNPs in COS-1 cells

SNPs	Wild-type Allele (Mean ± SD)	Mutant Allele (Mean ± SD)	% Decrease	*P*-value
-1284C>G	C	G		
	5.06 ± 0.10	4.16 ± 0.36	17.79	0.014
	5.27 ± 0.06	4.56 ± 0.34	13.47	0.023
	5.55 ± 0.46	4.64 ± 0.46	16.40	0.075
-1219G>A	G	A		
	2.86 ± 0.05	1.40 ± 0.01	51.05	< 0.001
	4.10 ± 0.21	2.06 ± 0.16	49.76	< 0.001
	3.70 ± 0.12	1.81 ± 0.08	51.08	< 0.001
-1190G>C	G	C		
	3.01 ± 0.19	2.16 ± 0.03	28.24	< 0.01
	2.79 ± 0.19	1.98 ± 0.23	29.03	< 0.01
	3.93 ± 0.50	2.84 ± 0.08	27.74	< 0.01
-1076G>A	G	A		
	10.01 ± 0.38	9.13 ± 0.86	8.79	0.178
	10.86 ± 0.53	9.98 ± 0.60	8.10	0.129
	8.40 ± 0.47	7.74 ± 0.07	7.86	0.075
-1055T>G	T	G		
	4.66 ± 0.18	3.44 ± 0.17	26.18	< 0.01
	7.66 ± 0.53	6.13 ± 0.04	19.97	< 0.01
	3.49 ± 0.09	2.53 ± 0.14	27.51	< 0.01
-759A>G	A	G		
	5.28 ± 0.29	4.57 ± 0.11	13.45	0.017
	4.82 ± 0.27	4.27± 0.18	11.41	0.042
	4.90 ± 0.12	4.38 ± 0.50	10.61	0.155
-700C>A	C	A		
	4.65 ± 0.05	4.31 ± 0.10	7.31	< 0.01
	4.90 ± 0.17	4.58 ± 0.33	6.53	0.214
	4.27 ± 1.32	3.99 ± 0.51	6.56	0.745
-643T>C	T	C		
	19.91 ± 1.68	11.94 ± 0.15	40.03	0.001
	5.73 ± 0.07	3.20 ± 0.24	44.15	< 0.001
	10.79 ± 0.88	6.26 ± 0.39	41.98	0.002
-627A>C	A	C		
	3.09 ± 0.15	2.85 ± 0.11	7.77	0.086
	6.72 ± 0.31	6.18 ± 0.12	8.04	0.049
	5.75 ± 0.23	5.12 ± 0.01	10.96	0.009
-363 C>T	C	T		
	3.82 ± 0.34	3.34 ± 0.25	12.57	0.117
	2.96 ± 0.49	2.42 ± 0.40	18.24	0.212
	2.88 ± 0.16	2.44 ± 0.26	15.28	0.065
-38G>A	G	A		
	4.56 ± 0.15	3.62 ± 0.15	20.61	0.002
	3.95 ± 0.20	3.21 ± 0.17	18.73	0.009
	3.81 ± 0.09	3.16 ± 0.03	17.06	< 0.001
-32C>A	C	A		
	18.91 ± 0.38	11.92 ± 0.39	36.96	< 0.001
	15.79 ± 1.03	10.32 ± 0.17	34.64	< 0.001
	16.71 ± 0.92	10.56 ± 0.06	36.80	< 0.001

% Decrease and significance (P - values) of each mutant is as compared with wild-type

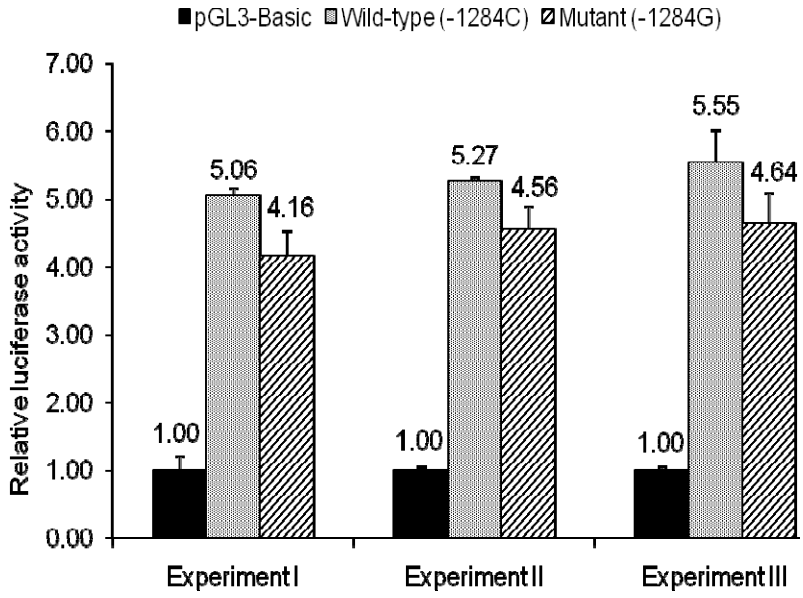


Figure 32. Dual-luciferase of *APOH* promoter -1284C>G SNP

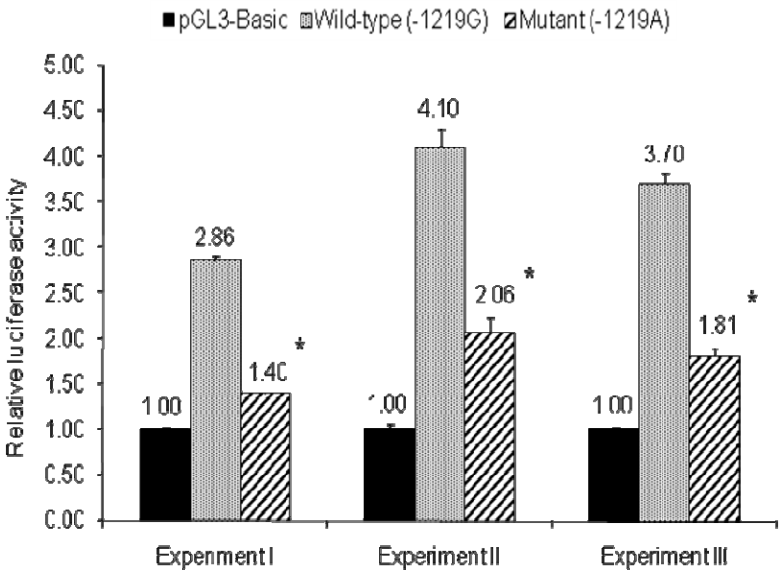


Figure 33. Dual-luciferase of *APOH* promoter -1219G>A SNP
(* *P* < 0.0001)

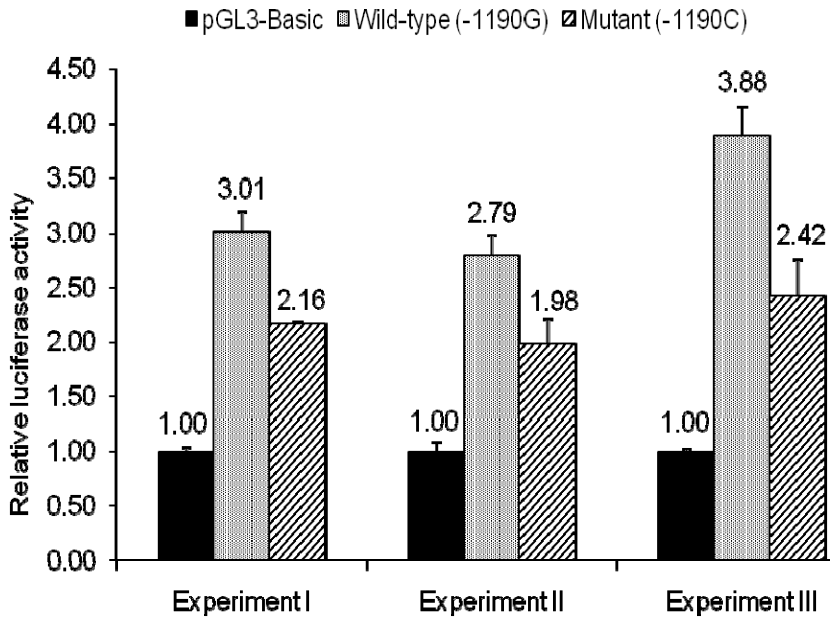


Figure 34. Dual-luciferase of *APOH* promoter -1190G>C SNP

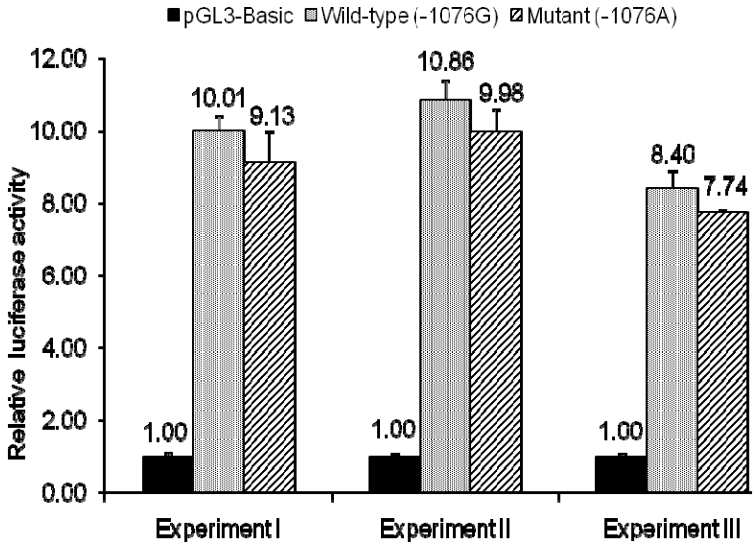


Figure 35. Dual-luciferase of *APOH* promoter -1076G>A SNP

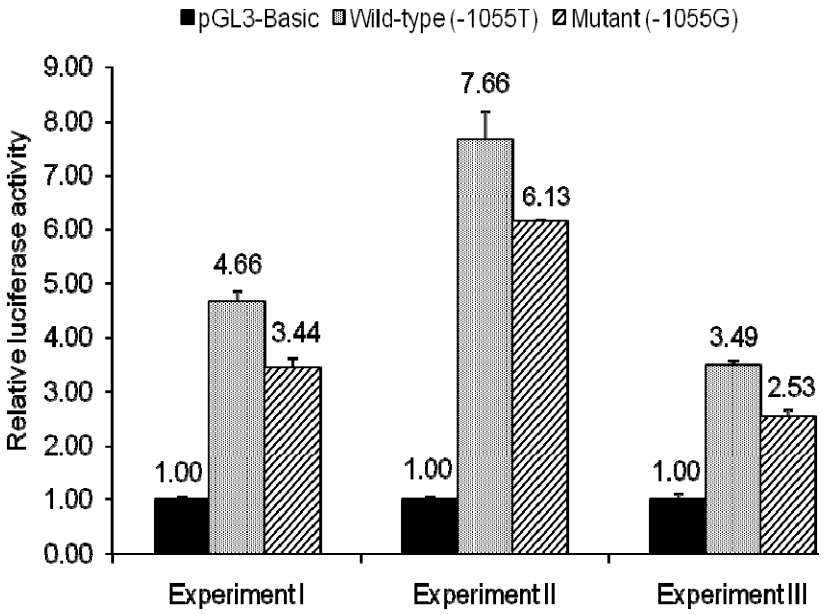


Figure 36. Dual-luciferase of *APOH* promoter -1055T>G SNP

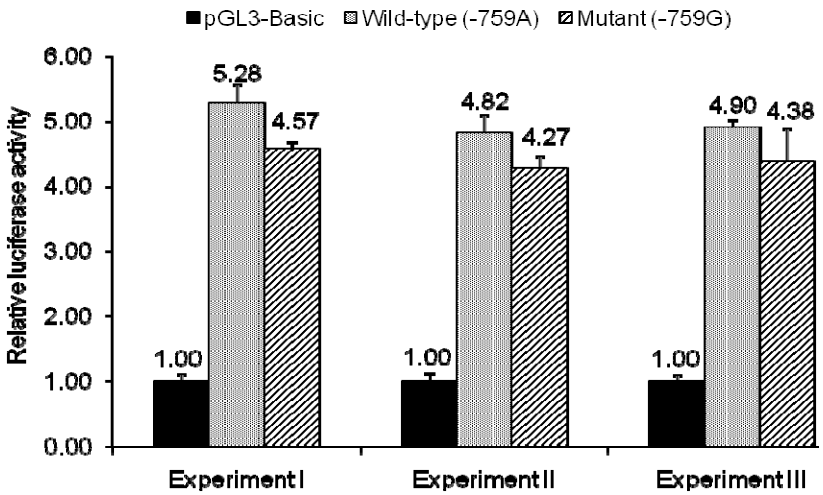


Figure 37. Dual-luciferase of *APOH* promoter -759A>G SNP

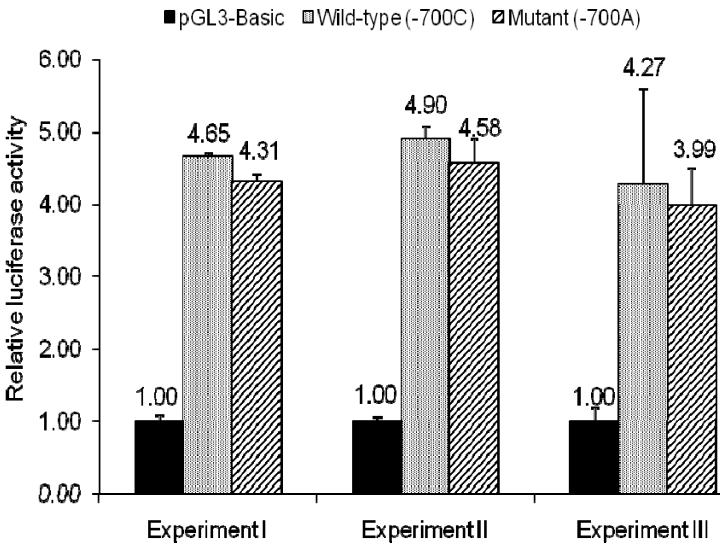


Figure 38. Dual-luciferase of *APOH* promoter -700C>A SNP

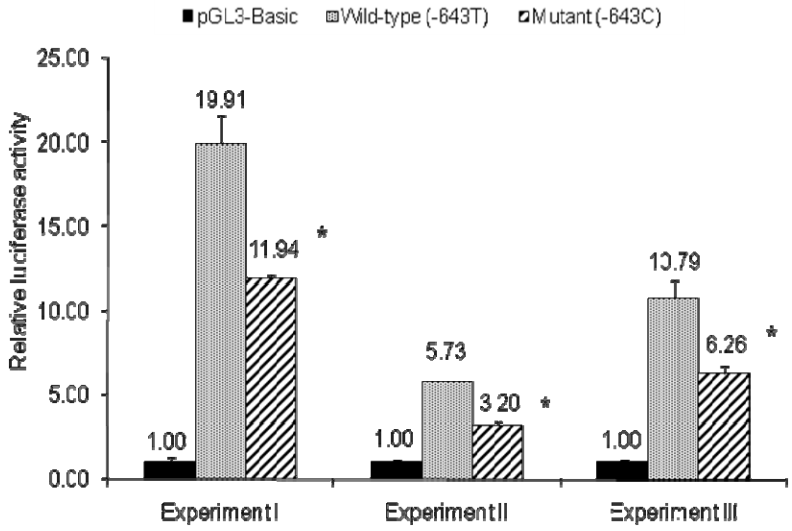


Figure 39. Dual-luciferase of *APOH* promoter -643T>C SNP
(* *P* < 0.0001)

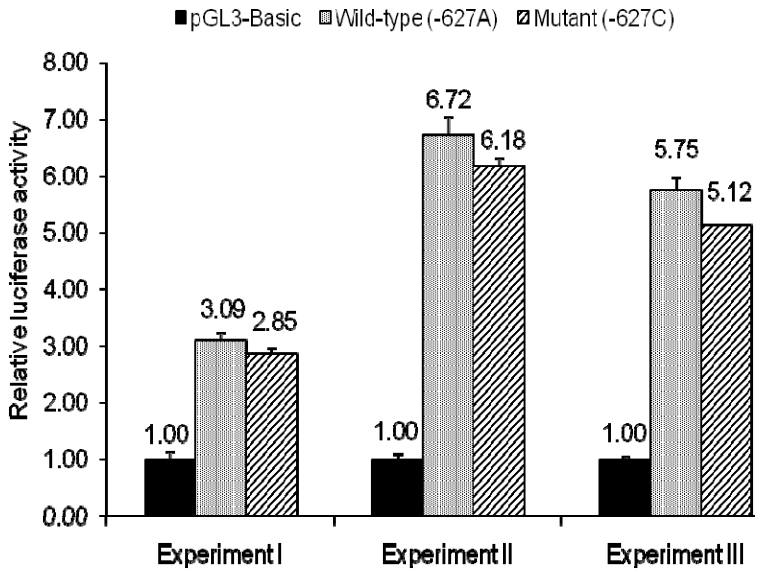


Figure 40. Dual-luciferase of *APOH* promoter -627A>C SNP

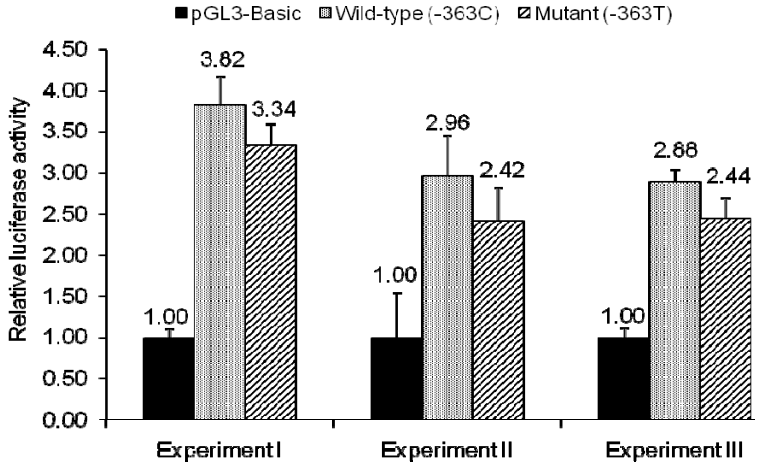


Figure 41. Dual-luciferase of *APOH* promoter -363C>T SNP

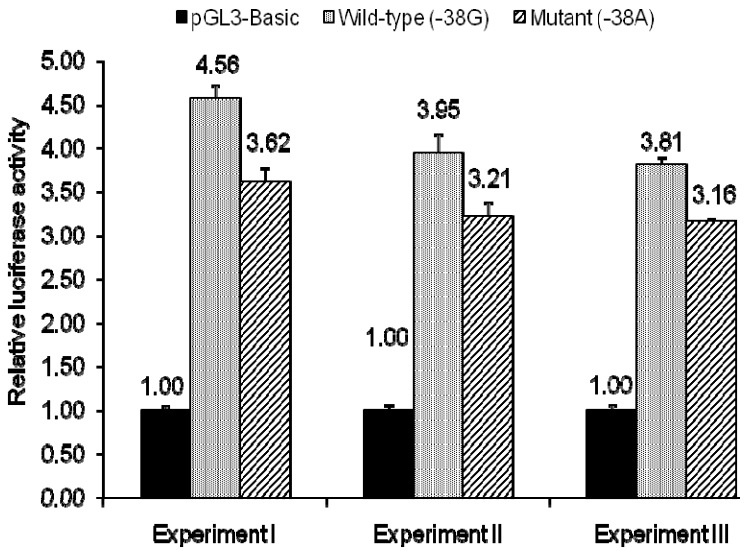


Figure 42. Dual-luciferase of *APOH* promoter -38G>A SNP

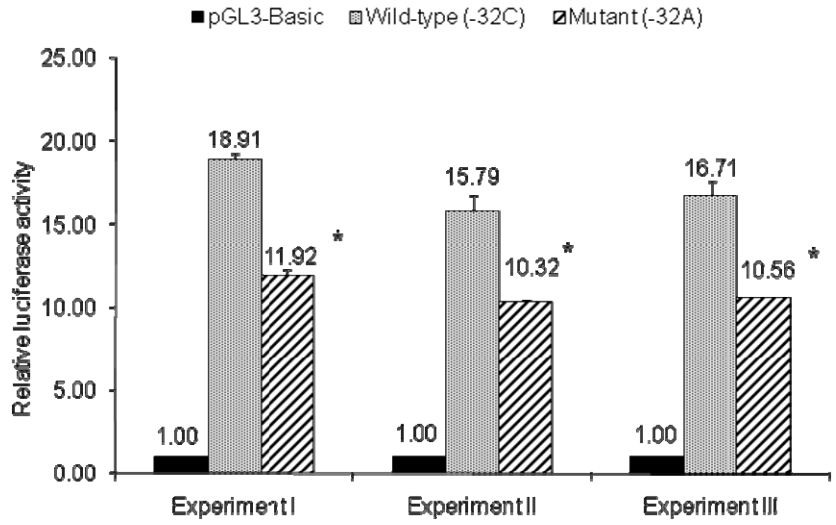


Figure 43. Dual-luciferase assay of *APOH* promoter -32C>A SNP
(* *P* < 0.0001)

6.2.2 Electrophoretic mobility shift assays of *APOH* promoter SNPs

To determine whether the *APOH* promoter polymorphisms affect the binding activity of nuclear factors, synthetic wild-type and mutant oligonucleotides were incubated with HepG2 cell nuclear extracts and subjected to EMSA. We assessed by EMSA whether a promoter SNP in a predicted transcription factor binding site (TFBS) would modify the binding of protein to the polymorphic DNA sequences. Upon incubation of radiolabeled oligonucleotides specific for wild-type and mutant alleles with HepG2 nuclear extracts, for each of the five promoter SNPs tested (-1284C>G, -1219G>A, -1190G>C, -643T>C and -1076G>A), DNA-protein complexes were observed (Figures 44-48), indicating that a nuclear factor(s) binds these sequences. Competition assays using increasing amounts of unlabeled wild-type oligonucleotides lead to decrease in level of the labeled product as a consequence of increasing non-labeled oligomer concentration. This is an indication of the efficiency of displacement, thereby reflecting the relative stability and specificity of the formed DNA-protein complex. For all of the five of the *APOH* promoter SNPs processed similarly, none of them showed differential allelic shifts in HepG2 cell lines.

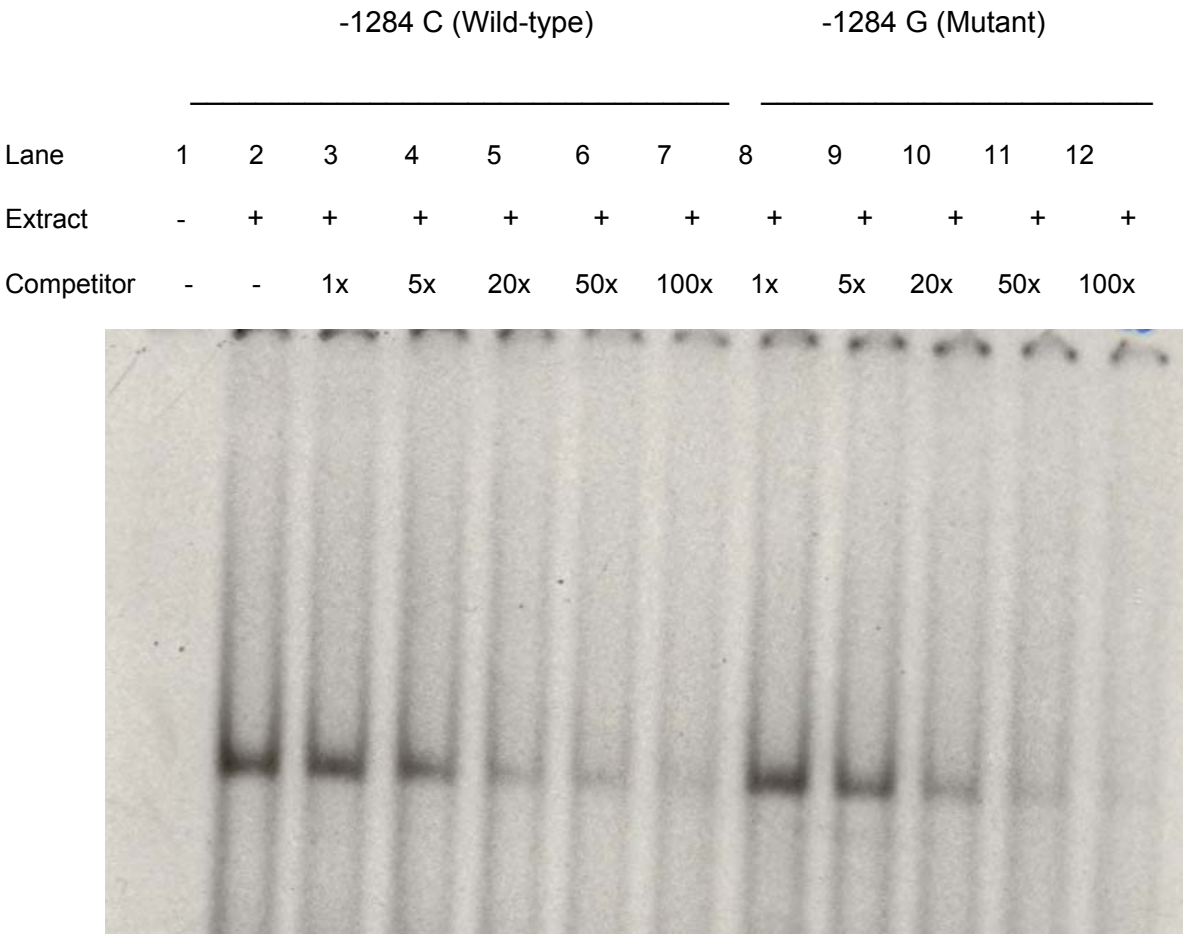


Figure 44. EMSA of -1284C>G SNP

Each sample contains a mixture of 5 µg of nuclear extract derived from human HepG2 cell nuclear extract and 30xmer ^{32}P-labeled wild-type oligonucleotide containing C allele. Arrowhead indicates specific DNA-protein complex associated with the -1284C>G polymorphic site. Lane 1, labeled oligonucleotide without nuclear extract from HepG2 cells; 2, labeled oligonucleotide with nuclear extracts. Lanes 3 to 7 have increasing amounts of C oligo competitor (1x, 5x, 20x, 50x, 100x, respectively); lanes 8 to 12 have increasing amounts of G oligo competitor (1x, 5x, 20x, 50x, 100x, respectively).

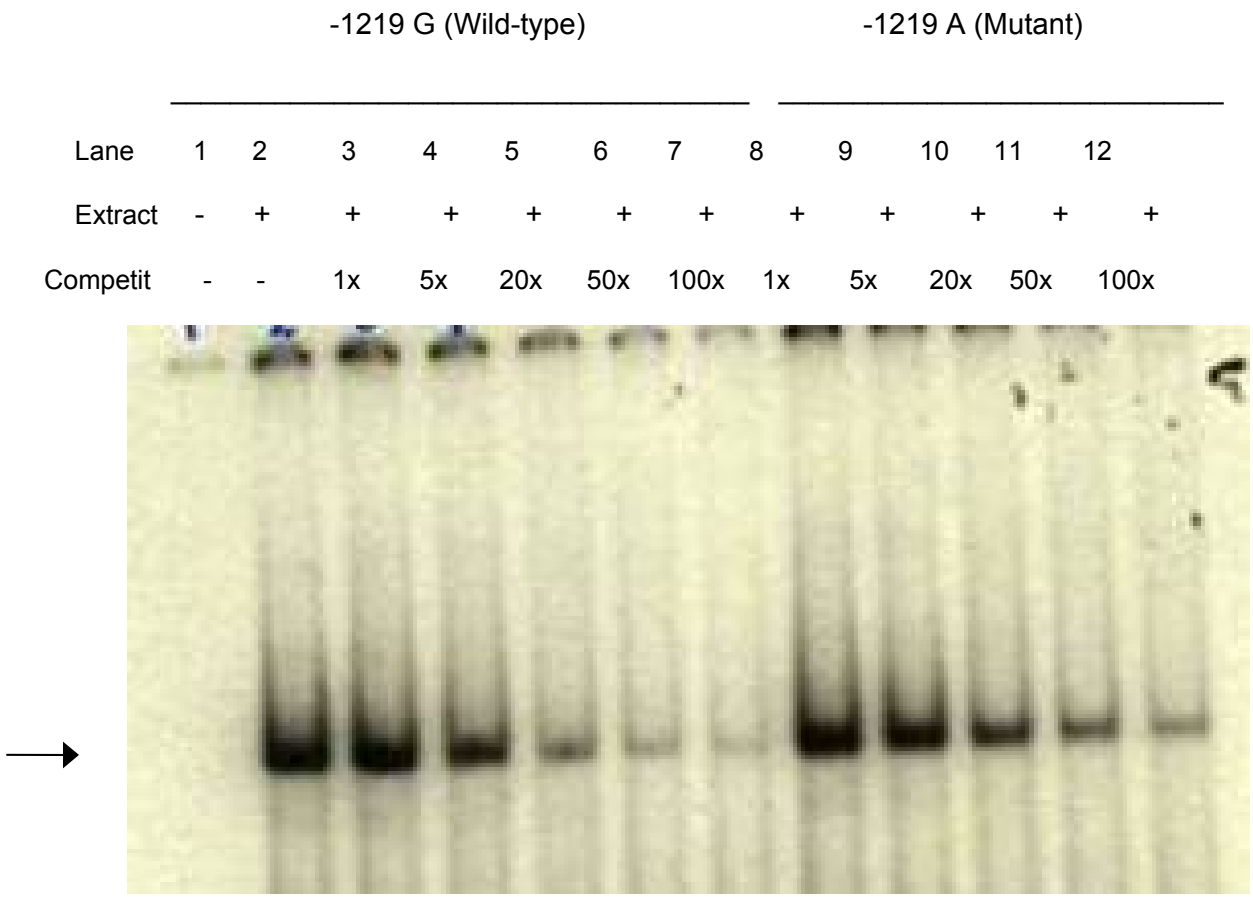


Figure 45. EMSA of -1219G>A SNP

Each sample contains a mixture of 5 µg of nuclear extract derived from human HepG2 cell nuclear extract and 30xmer ^{32}P-labeled wild-type oligonucleotide containing G allele. Arrowhead indicates specific DNA-protein complex associated with the -1219G>A polymorphic site. Lane 1, labeled oligonucleotide without nuclear extract from HepG2 cells; 2, labeled oligonucleotide with nuclear extracts. Lanes 3 to 7 have increasing amounts of G oligo competitor (1x, 5x, 20x, 50x, 100x, respectively); lanes 8 to 12 have increasing amounts of A oligo competitor (1x, 5x, 20x, 50x, 100x, respectively).

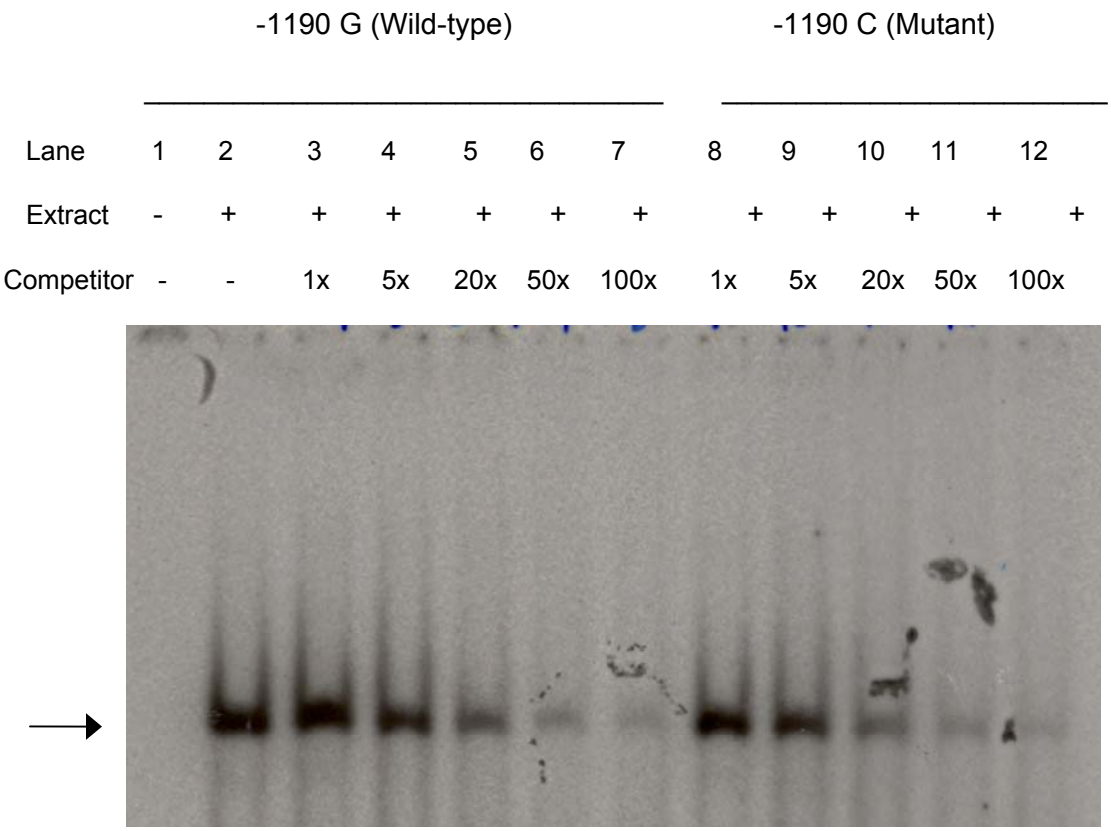


Figure 46. EMSA of -1190G>C SNP

Each sample contains a mixture of 5 µg of nuclear extract derived from human HepG2 cell nuclear extract and 30xmer ^{32}P-labeled wild-type oligonucleotide containing G allele. Arrowhead indicates specific DNA-protein complex associated with the -1190G>C polymorphic site. Lane 1, labeled oligonucleotide without nuclear extract from HepG2 cells; 2, labeled oligonucleotide with nuclear extracts. Lanes 3 to 7 have increasing amounts of G oligo competitor (1x, 5x, 20x, 50x, 100x, respectively); lanes 8 to 12 have increasing amounts of C oligo competitor (1x, 5x, 20x, 50x, 100x, respectively).

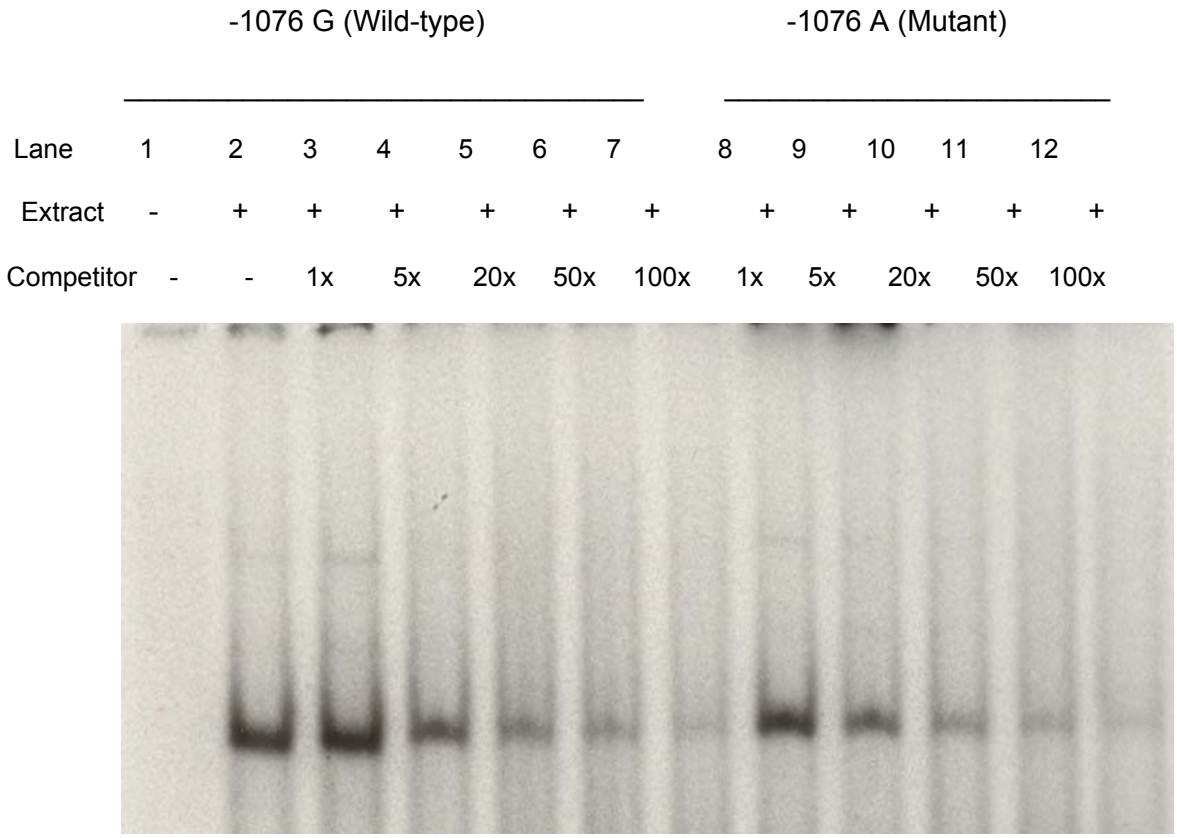


Figure 47. EMSA of -1076G>A SNP

Each sample contains a mixture of 5 µg of nuclear extract derived from human HepG2 cell nuclear extract and 30xmer ^{32}P-labeled wild-type oligonucleotide containing G allele. Arrowhead indicates specific DNA-protein complex associated with the -1076 G>A polymorphic site. Lane 1, labeled oligonucleotide without nuclear extract from HepG2 cells; 2, labeled oligonucleotide with nuclear extracts. Lanes 3 to 7 have increasing amounts of G oligo competitor (1x, 5x, 20x, 50x, 100x, respectively); lanes 8 to 12 have increasing amounts of A oligo competitor (1x, 5x, 20x, 50x, 100x, respectively).

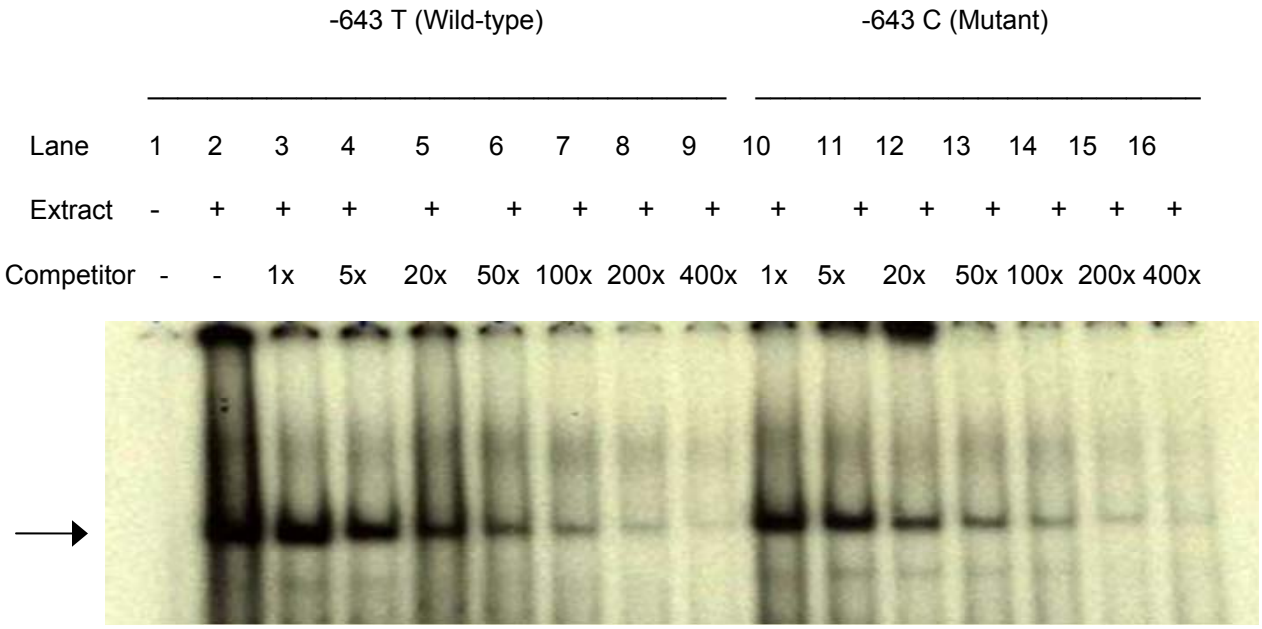


Figure 48. EMSA of -643T>C SNP

Each sample contains a mixture of 5 µg of nuclear extract derived from human HepG2 cell nuclear extract and 30xmer ^{32}P-labeled wild-type oligonucleotide containing T allele. Arrowhead indicates specific DNA-protein complex associated with the -643T>C polymorphic site. Lane 1, labeled oligonucleotide without nuclear extract from HepG2 cells; 2, labeled oligonucleotide with nuclear extracts. Competition assay was performed by adding excess cold oligonucleotides containing either the -643 T allele (lanes 3 to 9) or the C allele (lanes 10 to 16). Lanes 3 to 9 have increasing amounts of T oligo competitor (1x, 5x, 20x, 50x, 100x, 200x, and 400x, respectively); lanes 10 to 16 have increasing amounts of C oligo competitor (1x, 5x, 20x, 50x, 100x, 200x, and 400x, respectively).

6.3 GENETIC ASSOCIATION OF *APOH* PROMOTER SNPS WITH SLE RISK AND/OR SLE-RELATED CLINICAL PHENOTYPES

6.3.1 Linkage disequilibrium of *APOH* promoter SNPs in whites

Pairwise LD analysis (quantified by D' and r^2) using seven SNPs showed similar patterns in cases and controls (data not shown), therefore only the results from combined case + control cohort are presented in Figures 49-50. There was a strong LD with high correlation ($D' = 0.92$, $r^2 = 0.79$) between -759A>G and -700C>A SNPs, hence one of these SNPs (-700C>A) was excluded from subsequent multiple regression and haplotype analyses.

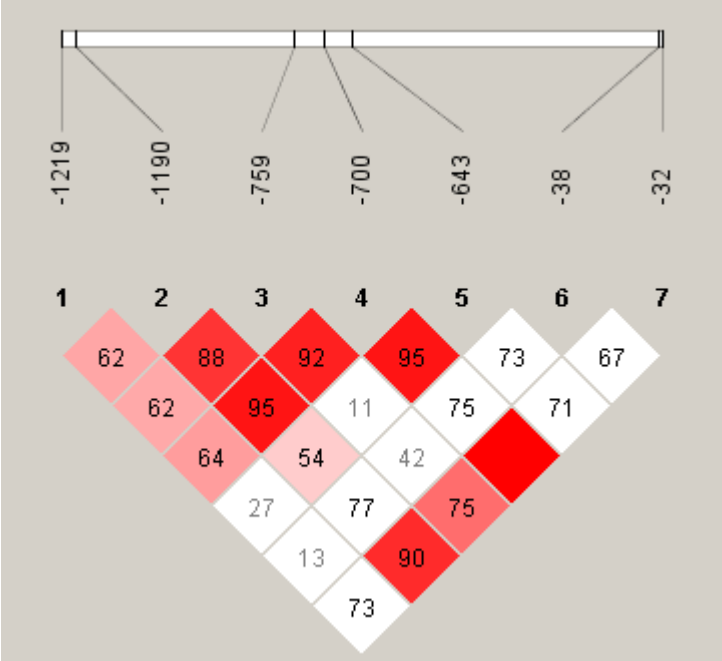


Figure 49. Pairwise LD analysis (D') of white cases + controls of *APOH* promoter SNPs

Each square indicates the level of LD between two variants. The colors are defined as: red (high LD), LOD ≥ 2 and D' = 1; blue, LOD < 2 and D' = 1; white, LOD < 2 and D'<1. The top of the figure shows the genomic position of the SNPs.

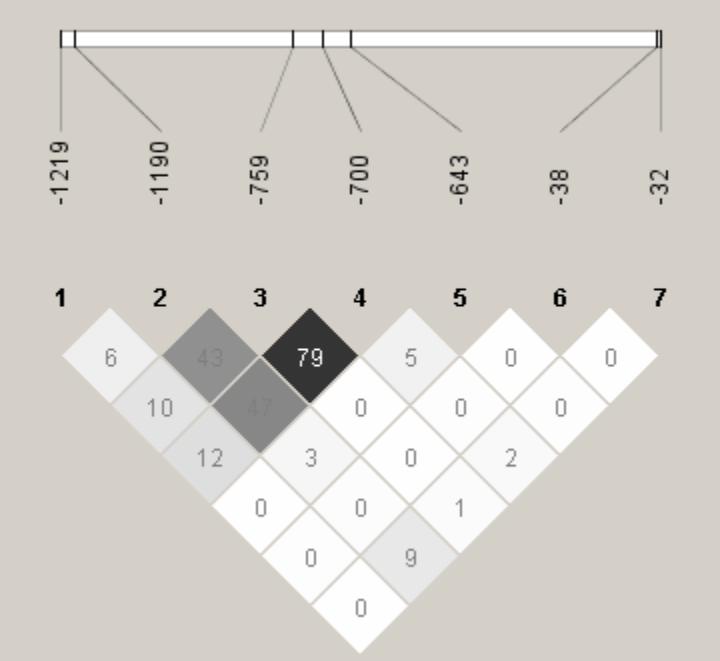


Figure 50. Pairwise LD analysis (r^2 x 100) of white cases + controls of *APOH* promoter SNPs

The intensity of gray is proportional to r^2, with the darkest gray being the highest r^2 value.

6.3.2 *APOH* promoter polymorphisms and race

The genotype and allele frequencies of the *APOH* promoter polymorphisms between the white cases + controls (n =799) and black cases + controls (n = 90) are given in Table 13. The genotype distributions of all SNPs were in accordance with Hardy-Weinberg equilibrium expectations in both races. Both genotype distribution and allele frequency were significantly different between races ($P < 0.001$), for the -759A>G and -700C>A SNPs, that are in strong LD, with whites showing a higher frequency of the variant -759G (25.5% *vs.* 10.8%) and -700A

(24.1% *vs.* 10.3%) alleles than blacks, respectively. Significant difference in allele frequencies were also seen for the -1219G>A ($P = 0.012$) and -643T>C ($P = 0.005$) SNPs.

Table 13. Distribution of *APOH* promoter SNPs between whites and blacks

SNP*	Genotypes/ Alleles	Whites n (%)	Blacks n (%)	SNP*	Genotypes/ Alleles	Whites n (%)	Blacks n (%)
-1284C>G	Genotype			-700C>A	Genotype		
(rs8178818)	CC	795 (99.8)	88 (100.0)	(rs3760291)	CC	458 (57.8)	70 (80.5)
	CG	2 (0.25)	0 (0.0)		CA	288 (36.3)	16 (18.4)
	GG	0 (0.0)	0 (0.0)		AA	47 (5.9)	1 (1.2)
	total	797	88		total	793	87
		**P* = 1.000				***P* < 0.001**	
	Allele				Allele		
	C/G	0.999/0.001	1.000/0.000		C/A	0.759/0.241	0.897/0.103
		***P* = 0.157				****P* < 0.001**	
-1219G>A	Genotype			-643T>C	Genotype		
(rs8178819)	GG	662 (83.6)	80 (90.9)	(rs3760292)	TT	573 (72.1)	72 (81.8)
	GA	119 (15.0)	8 (9.1)		TC	195 (24.5)	16 (18.2)
	AA	11 (1.4)	0 (0.0)		CC	27 (3.4)	0 (0.0)
	total	792	88		total	795	88
		**P* = 0.219				**P* = 0.065	
	Allele				Allele		
	G/A	0.911/0.089	0.955/0.045		T/C	0.843/0.157	0.909/0.091
		****P* = 0.012**				****P* = 0.005**	
-1190G>C	Genotype			-38G>A	Genotype		
(rs3760290)	GG	294 (37.0)	40 (45.5)		GG	763 (96.0)	86 (97.7)
	GC	393 (49.5)	37 (42.1)		GA	30 (3.8)	2 (2.3)
	CC	107 (13.5)	11 (12.5)		AA	2 (0.3)	0 (0.0)
	total	794	88		total	795	88
		**P* = 0.302				**P* = 0.807	
	Allele				Allele		
	G/C	0.618/0.382	0.665/0.335		G/A	0.979/0.021	0.989/0.011
		***P* = 0.211				***P* = 0.254	
-759A>G	Genotype			-32C>A	Genotype		
(rs8178820)	AA	444 (55.9)	70 (79.6)	(rs8178822)	CC	697 (87.7)	76 (86.4)
	AG	295 (37.2)	17 (19.3)		CA	95 (12.0	11 (12.5)
	GG	55 (6.9)	1 (1.1)		AA	3 (0.4)	1 (1.1)
	total	794	88		total	795	88
		***P* < 0.001**				**P* = 0.412	
	Allele				Allele		
	A/G	0.745/0.255	0.892/0.108		C/A	0.936/0.064	0.926/0.074
		****P* < 0.001**				***P* = 0.616	

SNPs are numbered according to their positions relative to the first base of the start codon (ATG)
***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

6.3.3 *APOH* promoter polymorphisms and SLE

The genotype distributions in SLE cases and controls did not deviate significantly from Hardy–Weinberg equilibrium for all the polymorphisms examined (data not shown). Minor allele frequencies **(**MAFs) of the *APOH* promoter SNPs that we observed in our control population (-1284G: 0.1%, -1219A: 9.9%, -1190C: 37.6%, -759G: 24.1%, -700A: 24.4%, -643C: 13.8%, -38A: 2.5%, -32A:6.2%) were similar to those previously reported in the literature (Chen and Kamboh, 2006) and/or in public databases. Due to its rare presence (MAF < 0.01), -1284C>G SNP was excluded from multiple regression and haplotype analyses.

Multiple regression analysis (Table 14) of the remaining 6 SNPs showed significant association of the -1219G>A and -643T>C SNPs with SLE risk after adjusting for age (P = 0.016 for each). However, the follow-up single-site analysis for the difference in genotype distribution and allele frequencies (Table 15) showed a significant association for only -643T>C; the genotype distribution of -643T>C in cases/controls was: 68.8%/74.6% for TT, 26.2%/23.2% for TC and 5.0%/2.2% for CC (age-adjusted P = 0.020); the -643C allele frequency was 18.1% in SLE cases *vs.* 13.8% in controls. The age-adjusted OR for the -643C allele under the additive model was 1.41 (95% CI: 1.07-1.86, P = 0.014). In order to replicate the -643 finding, we genotyped an independent sample from Chicago (109 SLE cases, 81 controls). No association/trend for association was observed in the Chicago (P = 0.919); the genotype distribution of -643T>C in cases/controls was: 73.3%/73.8% for TT and 26.7%/26.3% for TC). When the data from both cohorts was combined and analyzed by adjusting for the recruitment site, the association became less significant despite the increase in sample size and power (combined adjusted OR was 1.33 (95% CI: 1.04-1.70, P = 0.025). This suggests that the association of -643 with SLE risk may not be real. Analysis of genotype frequencies revealed no

significant difference between SLE patients and control subjects for the *APOH* promoter polymorphisms in the black population (Table 16). A caveat of this study is that the sample size of the black population is small and we did not have sufficient power in our study for the black population. Therefore, all further analyses were performed only in the white population.

Table 14. Multiple regression * analysis of *APOH* promoter SNPs for SLE disease status, nephritis and subclinical CVD (carotid plaque and IMT)

	P Value for **					
	SLE	**Nephritis**	**Carotid Plaque**	**Carotid IMT**	**APA**	
					Cases	*Controls*
rs8178819 (-1219G>A)	**0.016**	**0.014**	0.344	0.887	0.308	0.830
rs3760290 (-1190G>C)	0.982	0.790	0.070	0.322	0.105	0.374
rs8178820 (-759A>G)	0.182	0.185	0.884	0.903	0.469	0.686
rs3760292 (-643T>C)	**0.016**	0.944	**0.003**	**0.036**	0.703	0.302
(-38G>A)	0.295	0.200	**0.022**	0.836	**4.57×10^{-4}**	0.644
rs8178822 (-32C>A)	0.646	0.344	**0.033**	0.753	0.356	0.617

** SLE risk was analyzed under additive model while lupus nephritis and carotid plaque, carotid IMT and APA under dominant model.*
*** Adjusted for age for SLE and nephritis risk; adjusted for age, HDL-C, LDL-C, cholesterol and triglycerides for carotid plaque and IMT.*

Table 15. Distribution of *APOH* promoter SNPs between SLE white cases and controls

Whites							
SNP*	**Genotypes/ Alleles**	**Cases n (%)**	**Controls n (%)**	**SNP***	**Genotypes/ Alleles**	**Cases n (%)**	**Controls n (%)**
-1284C>G	Genotype			-700C>A	Genotype		
(rs8178818)	CC	343 (99.7)	452 (99.8)	(rs3760291)	CC	201 (58.8)	257 (57.0)
	CG	1 (0.3)	1 (0.2)		CA	120 (35.1)	168 (37.3)
	GG	0 (0.0)	0 (0.0)		AA	21 (6.1)	26 (5.8)
	total	344	453		total	342	451
		**P* = 1.000				**P* = 0.827	
	Allele				Allele		
	C/G	0.999/0.001	0.999/0.001		C/A	0.763/0.237	0.756/0.244
		***P* = 0.848				***P* = 0.744	
-1219G>A	Genotype			-643T>C	Genotype		
(rs8178819)	GG	295 (86.3)	367 (81.6)	(rs3760292)	TT	236 (68.8)	337 (74.6)
	GA	42 (12.3)	77 (17.1)		TC	90 (26.2)	105 (23.2)
	AA	5 (1.5)	6 (1.3)		CC	17 (5.0)	10 (2.2)
	total	342	450		total	343	452
		**P* = 0.169				****P* = 0.053**	
	Allele				Allele		
	G/A	0.924/0.076	0.901/0.099		T/C	0.819/0.181	0.862/0.138
		***P* = 0.107				****P* = 0.023**	
-1190G>C	Genotype			-38G>A	Genotype		
(rs3760290)	GG	126 (36.7)	168 (37.3)		GG	332 (96.8)	431 (95.4)
	GC	166 (48.4)	227 (50.3)		GA	11 (3.2)	19 (4.2)
	CC	51 (14.9)	56 (12.4)		AA	0 (0.0)	2 (0.4)
	total	343	451		total	343	452
		**P* = 0.599				**P* = 0.463	
	Allele				Allele		
	G/C	0.609/0.391	0.624/0.376		G/A	0.984/0.016	0.975/0.025
		***P* = 0.547				***P* = 0.185	
-759A>G	Genotype			-32C>A	Genotype		
(rs8178820)	AA	184 (53.8)	260 (57.5)	(rs8178822)	CC	300 (87.5)	397 (87.8)
	AG	129 (37.7)	166 (36.7)		CA	41 (12.0)	54 (11.9)
	GG	29 (8.5)	26 (5.8)		AA	2 (0.6)	1 (0.2)
	total	342	452		total	343	452
		**P* = 0.273				**P* = 0.765	
	Allele				Allele		
	A/G	0.727/0.273	0.759/0.241		C/A	0.934/0.066	0.938/0.062
		***P* = 0.146				***P* = 0.768	

SNPs are numbered according to their positions relative to the first base of the start codon (ATG)
***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 16. Distribution of *APOH* promoter SNPs between SLE black cases and controls

Blacks							
SNP*	**Genotypes/ Alleles**	**Cases n (%)**	**Controls n (%)**	**SNP***	**Genotypes/ Alleles**	**Cases n (%)**	**Controls n (%)**
-1284C>G	Genotype			-700C>A	Genotype		
(rs8178818)	CC	46 (100.0)	42 (100.0)	(rs3760291)	CC	35 (77.8)	35 (83.3)
	CG	0 (0.0)	0 (0.0)		CA	10 (22.2)	6 (14.3)
	GG	0 (0.0)	0 (0.0)		AA	0 (0.0)	1 (2.4)
	total	46	42		total	45	42
		**P = 1.000				**P = 0.413	
	Allele				Allele		
	C/G	1.000/0.000	1.000/0.000		C/A	0.889/0.111	0.905/0.095
						***P = 0.730	
-1219G>A	Genotype			-643T>C	Genotype		
(rs8178819)	GG	43 (93.5)	37 (88.1)	(rs3760292)	TT	38 (82.6)	34 (81.0)
	GA	3 (6.5)	5 (11.9)		TC	8 (17.4	8 (19.1)
	AA	0 (0.0)	0 (0.0)		CC	0 (0.0)	0 (0.0)
	total	46	42		total	46	42
		**P = 0.471				**P = 1.000	
	Allele				Allele		
	G/A	0.967/0.033	0.940/0.060		T/C	0.913/0.087	0.905/0.095
		***P = 0.397				***P = 0.849	
-1190G>C	Genotype			-38G>A	Genotype		
(rs3760290)	GG	22 (47.8)	18 (42.9)		GG	46 (100.0)	40 (95.2)
	GC	17 (37.0)	20 (47.6)		GA	0 (0.0)	2 (4.8)
	CC	7 (15.2)	4 (9.5)		AA	0 (0.0)	0 (0.0)
	total	46	42		total	46	42
		**P = 0.578				**P = 0.225	
	Allele				Allele		
	G/C	0.663/0.337	0.667/0.333		G/A	1.000/0.000	0.976/0.024
		***P = 0.959				***P = 0.152	
-759A>G	Genotype			-32C>A	Genotype		
(rs8178820)	AA	35 (76.1)	35 (83.3)	(rs8178822)	CC	38 (82.6)	38 (90.5)
	AG	11 (23.9)	6 (14.3)		CA	7 (15.2)	4 (9.5)
	GG	0 (0.0)	1 (2.4)		AA	1 (2.2)	0 (0.0)
	total	46	42		total	46	42
		**P = 0.290				**P = 0.526	
	Allele				Allele		
	A/G	0.880/0.120	0.905/0.095		C/A	0.902/0.098	0.952/0.048
		***P = 0.602				***P = 0.195	

SNPs are numbered according to their positions relative to the first base of the start codon (ATG)
***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Six-site haplotype analysis identified a total of nine haplotypes with a frequency of > 1% (Table 17). Using EH program, the overall haplotype distribution was found to differ significantly between cases and controls (P = 0.009). Using Haploview program, one particular haplotype (H7) was found to be present predominantly in cases (χ^2 = 19.151, P = 1.21 x 10^{-5}). This haplotype harbored the minor alleles for the -1190G>C, -759A>G and -643T>C SNPs. When we carried out a permutation test for haplotypes in Haploview (permutation number = 1000), none of the permutations exceeded the highest observed χ^2, confirming that the observed association of H7 was not just by chance.

Table 17. Six-site haplotype analysis* of *APOH* promoter SNPs in SLE cases and controls

							Haplotype frequencies		
	rs8178819	rs3760290	rs8178820	rs3760292		rs8178822	Total	Cases	Controls
Haplotype	(-1219G>A)	(-1190G>C)	(-759A>G)	(-643T>C)	(-38G>A)	(-32C>A)	(n=783)	(n=339)	(n=444)
H1	G	G	A	T	G	C	0.441	0.424	0.455
H2	G	C	G	T	G	C	0.156	0.138	0.168
H3	G	G	A	C	G	C	0.119	0.104	0.129
H4	G	C	A	T	G	C	0.083	0.099	0.071
H5	A	C	G	T	G	C	0.057	0.043	0.069
H6	G	C	A	T	G	A	0.053	0.054	0.052
H7	G	C	G	C	G	C	0.018	0.045	0.000
H8	G	G	A	T	A	C	0.017	0.014	0.019
H9	A	G	A	T	G	C	0.012	0.018	0.007
								P **= 0.009**	

** Using program EH version 1.2 (excluding individuals with missing genotypes).*
*** P value for overall haplotype distribution difference between cases and controls.*

Although the -643C allele appears to be functional, the two haplotypes (H3 and H7 in Table 2) that carry -643C, only H7 showed significant association with SLE risk. This suggests that other variants in the H7 haplotype might also be important in affecting *APOH* expression. To test this hypothesis, we mutated the defining alleles in H7 haplotype (-1190G>C, -759A>G, -

643T>C) and compared its gene expression with the wild-type and H3 haplotypes. As shown in Figure 51, the H7 haplotype had the lowest luciferase activity as compared to the H3 haplotype in duplicate experiments. The data values (mean ± SD) along with the significance of difference (*P* - values) between constructs are summarized in Table 18.

Table 18. Dual-luciferase of H3 and H7 haplotypes in COS 1 cells

Experiment I				
Constructs	**Average**	**SD**	**% Decrease**	***P* - values**
pGL3-Basic	1.00	0.13	--	--
Wild-type	6.99	0.68	--	--
H3 haplotype	3.94	0.05	43.63	0.016
H7 haplotype	3.33	0.14	52.36	0.001
Experiment II				
Constructs	**Average**	**SD**	**% Decrease**	***P* - values**
pGL3-Basic	1.00	0.09	--	--
Wild-type	6.84	0.95	--	--
H3 haplotype	3.94	0.07	42.40	0.033
H7 haplotype	3.17	0.13	53.65	0.003

% Decrease of each mutant is as compared with wild-type

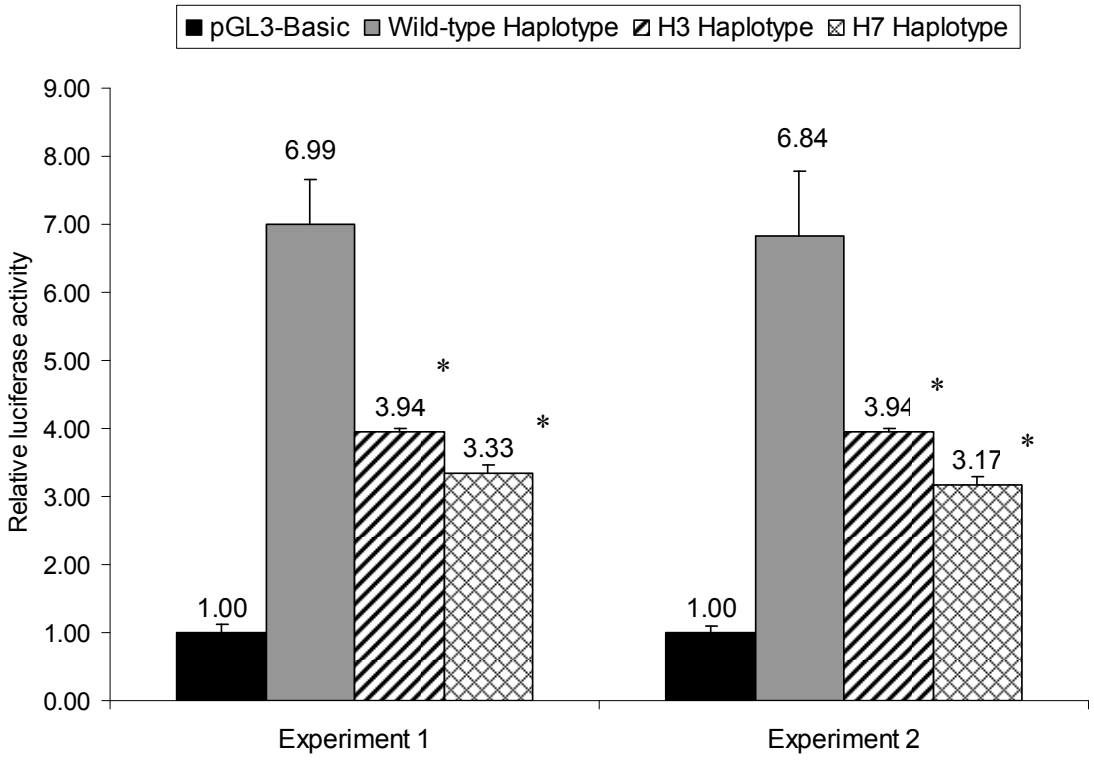


Figure 51. Dual-luciferase of *APOH* haplotypes

*Bars indicate the means of luciferase activity from two independent experiments (*P < 0.05). The effect of wild-type and mutants were measured as the mean of the firefly luciferase levels, which were normalized by the Renilla luciferase activity, which served as the reference for the transfection efficiency. The results presented are from one out of two independent experiments. The wild-type haplotype contains the major alleles at all sites. While the H3 haplotype is defined by minor allele at SNP -643T>C, the H7 haplotype is defined by minor alleles at SNPs -643T>C, -1190G>C and -759A>G.*

6.3.4 *APOH* promoter polymorphisms and lupus nephritis

Because we obtained significant evidence that *APOH* promoter SNPs influenced SLE risk, we next investigated whether these SNPs influenced specific clinical manifestations of SLE. Multiple regression analysis in lupus patients stratified by the presence (n = 103) or absence (n = 241) of renal disease showed significant association with -1219G>A (P = 0.014) after adjusting for age (Table 14). The -1219A allele frequency was 4.4% in SLE cases with nephritis *vs.* 9.0% in SLE cases without nephritis. The age-adjusted OR for the -1219A allele under dominant model was 0.36 (95% CI: 0.16-0.83, P = 0.016). Single-site genotype and allele carrier frequencies of the *APOH* promoter SNPs in SLE cases with/without nephritis is illustrated in Table 19. Although no significant associations were seen in the multiple regression analysis, two of the highly correlated promoter SNPs -759A>G and -700C>A SNPs were significantly associated with renal disease in SLE patients. The frequencies of the -759G (22.5% *vs*. 29.4%) and -700A (16.2% *vs.* 26.9%) alleles was lower in SLE nephritis subjects as compared to those without, respectively.

Table 19. Distribution of *APOH* promoter SNPs between SLE white cases with and without lupus nephritis

Whites							
SNP*	**Genotypes/ Alleles**	**Nephritis n (%)**	**No Nephritis n (%)**	**SNP***	**Genotypes/ Alleles**	**Nephritis n (%)**	**No Nephritis n (%)**
-1284C>G (rs8178818)	Genotype			-700C>A (rs3760291)	Genotype		
	CC	102 (99.0)	241 (100.0)		CC	73 (71.6)	128 (53.3)
	CG	1 (1.0)	0 (0.0)		CA	25 (24.5)	95 (39.6)
	GG	0 (0.0)	0 (0.0)		AA	4 (3.9)	17 (7.1)
	total	103	241		total	102	240
		**P = 0.299				****P = 0.007**	
	Allele				Allele		
	C/G	0.995/1.000	0.005/0.000		C/A	0.838/0.162	0.731/0.269
		***P = 0.316				****P = 0.001**	
-1219G>A (rs8178819)	Genotype			-643T>C (rs3760292)	Genotype		
	GG	94 (92.2)	201 (83.8)		TT	69 (67.7)	167 (69.3)
	GA	7 (6.9)	35 (14.6)		TC	27 (26.5)	63 (26.1)
	AA	1 (1.0)	4 (1.7)		CC	6 (5.9)	11 (4.6)
	total	102	240		total	102	241
		**P = 0.105				**P = 0.832	
	Allele				Allele		
	G/A	0.956/0.044	0.910/0.090		T/C	0.809/0.191	0.824/0.176
		****P = 0.019**				***P = 0.649	
-1190G>C (rs3760290)	Genotype			-38G>A	Genotype		
	GG	45 (44.1)	81 (33.6)		GG	97 (95.1)	235 (97.5)
	GC	43 (42.2)	123 (51.0)		GA	5 (4.9)	6 (2.5)
	CC	14 (13.7)	37 (15.4)		AA	0 (0.0)	0 (0.0)
	total	102	241		total	102	241
		**P = 0.183				**P = 0.314	
	Allele				Allele		
	G/C	0.652/0.348	0.591/0.409		G/A	0.975/0.025	0.988/0.012
		***P = 0.131				***P = 0.313	
-759A>G (rs8178820)	Genotype			-32C>A (rs8178822)	Genotype		
	AA	66 (64.7)	118 (49.2)		CC	90 (88.2)	210 (87.1)
	AG	26 (25.5)	103 (42.9)		CA	10 (9.8)	31 (12.9)
	GG	10 (9.8)	19 (7.9)		AA	1 (2.0)	0 (0.0)
	total	102	240		total	102	241
		****P = 0.008**				**P = 0.765	
	Allele				Allele		
	A/G	0.775/0.225	0.706/0.294		C/A	0.931/0.069	0.936/0.064
		****P = 0.057**				***P = 0.837	

SNPs are numbered according to their positions relative to the first base of the start codon (ATG)
***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

6.3.5 *APOH* promoter polymorphisms and subclinical cardiovascular disease (carotid plaque and carotid IMT)

Multiple regression analysis of 6 SNPs in lupus patients stratified by the presence (n = 81) or absence (n = 164) of carotid plaque revealed significant associations with -643T>C (P = 0.003), -38G>A (P = 0.022) and -32C>A (P = 0.033) SNPs after adjusting for age and lipid profile (Table 14). Subsequent single-site analyses (adjusted for age and lipid profile) revealed significant genotype differences between the two groups for -643T>C and -32C>A SNPs but not for -38G>A. Unadjusted single-site analysis revealed significance only for the -643T>C SNP (Table 20: P = 0.017 for the genotype distribution; P = 0.002 for the allele distribution). The -643C allele frequency was 8.6% in SLE cases with carotid plaque *vs.* 18.4% in patients without plaque; the age- and lipid profile-adjusted OR for the -643C allele carriers (TT *vs*. TC + CC genotypes) was 0.37 (95% CI: 0.17-0.81, P = 0.013). The -32A allele frequency was 8.6% in SLE cases with carotid plaque *vs*. 5.8% in patients without plaque; the adjusted OR for the -32A allele carriers (CC *vs*. AA+CA genotypes) was 2.63 (95% CI: 1.09-6.35, P = 0.031). Multiple linear regression analysis of carotid IMT using the same model as for plaque demonstrated a significant association for only -643T>C after adjusting for age and lipid profile (P = 0.036) (Table 14) which was not observed in the single-site analysis (Table 21).

Because -643T>C SNP was found to be associated with both carotid plaque and IMT in multiple regression analysis, we also genotyped this SNP in our Chicago SLE sample. The association of the -643T>C SNP with carotid plaque was confirmed in this independent sample of SLE patients from Chicago (37 with carotid plaque *vs.* 72 without carotid plaque). The age and lipid profile adjusted OR for the -643C allele carriers (TC + CC *vs*. TT genotypes) was 0.22 (95% CI: 0.06-0.78, P = 0.019). When the data from both Pittsburgh and Chicago cohorts were

combined and analyzed by adjusting for the recruitment site, the adjusted OR was 0.35 (95% CI: 0.18-0.67, P = 0.002) suggesting a protective effect of -643C allele against developing carotid plaque.

Table 20. Distribution of *APOH* promoter SNPs between SLE white cases with and without carotid plaque

Whites							
SNP*	**Genotypes/ Alleles**	**Plaque n (%)**	**No Plaque n (%)**	**SNP***	**Genotypes/ Alleles**	**Plaque n (%)**	**No Plaque n (%)**
-1284C>G (rs8178818)	Genotype			-700C>A (rs3760291)	Genotype		
	CC	81 (100.0)	164 (100.0)		CC	47 (58.0)	90 (55.6)
	CG	0 (0.0)	0 (0.0)		CA	26 (32.1)	63 (38.9)
	GG	0 (0.0)	0 (0.0)		AA	8 (9.9)	9 (5.6)
	total	81	164		total	81	162
		**P* = 1.000				**P* = 0.320	
	Allele				Allele		
	C/G	1.000/0.000	1.000/0.000		C/A	0.741/0.259	0.750/0.250
						***P* = 0.826	
-1219G>A (rs8178819)	Genotype			-643T>C (rs3760292)	Genotype		
	GG	74 (91.4)	139 (85.8)		TT	68 (84.0)	109 (66.9)
	GA	6 (7.4)	23 (14.2)		TC	12 (14.8)	48 (29.5)
	AA	1 (1.2)	0 (0.0)		CC	1 (1.2)	6 (3.7)
	total	81	162		total	81	163
		**P* = 0.088				***P* = 0.017**	
	Allele				Allele		
	G/A	0.951/0.049	0.929/0.071		T/C	0.914/0.086	0.816/0.184
		***P* = 0.331				****P* = 0.002**	
-1190G>C (rs3760290)	Genotype			-38G>A	Genotype		
	GG	29 (35.8)	56 (34.2)		GG	80 (98.8)	156 (95.1)
	GC	38 (46.9)	87 (53.1)		GA	1 (1.2)	8 (4.9)
	CC	14 (17.3)	21 (12.8)		AA	0 (0.0)	0 (0.0)
	total	81	164		total	81	164
		**P* = 0.537				**P* = 0.279	
	Allele				Allele		
	G/C	0.593/0.407	0.607/0.393		G/A	0.994/0.006	0.976/0.024
		***P* = 0.764				***P* = 0.083	
-759A>G (rs8178820)	Genotype			-32C>A (rs8178822)	Genotype		
	AA	46 (56.8)	86 (53.1)		CC	68 (84.0)	145 (88.4)
	AG	27 (33.3)	67 (41.4)		CA	12 (14.8)	19 (11.6)
	GG	8 (9.9)	9 (5.6)		AA	1 (1.2)	0 (0.0)
	total	81	162		total	81	164
		**P* = 0.278				**P* = 0.281	
	Allele				Allele		
	A/G	0.735/0.265	0.738/0.262		C/A	0.914/0.086	0.942/0.058
		***P* = 0.942				***P* = 0.265	

SNPs are numbered according to their positions relative to the first base of the start codon (ATG)
***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 21. Single-site analysis of *APOH* promoter SNPs with carotid IMT

SNP	***P*-value**
rs8178819 (-1219G>A)	0.113
rs3760290 (-1190G>C)	0.366
rs8178820 (-759A>G)	0.828
rs3760292 (-643T>C)	0.244
(-38G>A)	0.087
rs8178822 (-32C>A)	0.628
rs8178819 (-1219G>A)	0.634

**Adjusted for age, HDL-C, LDL-C, cholesterol and triglycerides*

6.3.6 *APOH* promoter polymorphisms and the occurrence of antiphospholipid antibodies

We categorized subjects into "APA-positive" and "APA-negative" groups. APA-positive group included participants positive for any of the following 3 antibodies; aCL (IgG and/or IgM), anti-β_2GPI (IgA and/or IgG and/or IgM) and LAC. APA-negative group contained individuals negative for all APA. None of the *APOH* promoter SNPs was found to have a significant association with the presence of APA in controls in multiple regression using dominant model (Table 14). However, in cases, the distribution of the -38G>A SNP differed significantly between APA-positive and APA-negative groups in both multiple regression ($P = 4.57 \times 10^{-4}$) and single-site analyses ($P = 0.004$). It is important to note that -38G>A is a relatively rare variant and was only detected in APA-positive patients (MAF = 0.027) but not in APA-negative patients. The significant difference in -38G>A genotype distribution was also observed when the comparison was subcategorized between aCL-positive *vs.* APA-negative ($P = 0.018$), LAC-positive *vs.* APA-negative ($P = 0.001$) and anti-β_2GPI-positive *vs.* APA-negative ($P = 0.002$)

cases using multiple regression under dominant model (data not shown). The genotype and allele frequency distribution of the seven *APOH* promoter SNPs (-1219G>A, -1190G>C, -759A>G, -700C>A, -643T>C, -38G>A, -32C>A) between APA-positive group (subdivided into aCL-positive, lac-positive, anti- β_2GPI-positive and any APA-positive) *vs.* APA-negative groups are shown in Tables (22-49) separately for white cases and controls. No significant differences in the immunologic (aCL, LAC, anti-β_2GPI and APA) parameters were observed for the *APOH* promoter -1219G>A, -1190G>C, -759A>G, -700C>A, -643T>C, and -32C>A SNPs in both groups.

Table 22. Distribution of -1219G>A SNP with anticardiolipin antibodies (aCL) in white cases and controls

-1219G>A (rs8178819) SNP									
White Cases					**White Controls**				
	aCL Positive		**aCL Negative**			**aCL Positive**		**aCL Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	74	(90.2)	188	(86.2)	GG	106	(86.2)	242	(80.1)
GA	7	(8.5)	30	(13.8)	GA	16	(13.0)	56	(18.5)
AA	1	(1.2)	0	(0.0)	AA	1	(0.8)	4	(1.3)
Total	82		218		Total	123		302	
		*******P* = 0.143					*******P* = 0.384		
G	0.945		0.931		G	0.927		0.894	
A	0.055		0.069		A	0.073		0.106	
		********P* = 0.518					********P* = 0.115		
White Cases					**White Controls**				
	aCL Positive		**APA Negative**			**aCL Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	74	(90.2)	121	(85.8)	GG	106	(86.2)	163	(81.5)
GA	7	(8.5)	20	(14.2)	GA	16	(13.0)	35	(17.5)
AA	1	(1.2)	0	(0.0)	AA	1	(0.8)	2	(1.0)
Total	82		141		Total	123		200	
		*******P* = 0.191					*******P* = 0.615		
G	0.945		0.929		G	0.927		0.903	
A	0.055		0.071		A	0.073		0.098	
		********P* = 0.494					********P* = 0.274		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 23. Distribution of -1219G>A SNP with lupus anticoagulant (lac) in white cases and controls

-1219G>A (rs8178819) SNP									
White Cases					**White Controls**				
	lac Positive		**lac Negative**			**lac Positive**		**lac Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	72	(87.8)	217	(86.1)	GG	16	(76.2)	302	(83.0)
GA	9	(11.0)	32	(12.7)	GA	5	(23.8)	58	(15.9)
AA	1	(1.2)	3	(1.2)	AA	0	(0.0)	4	(1.1)
Total	82		252		Total	21		364	
		***P* = 0.935**					***P* = 0.493**		
G	0.933		0.925		G	0.881		0.909	
A	0.067		0.075		A	0.119		0.091	
		****P* = 0.715**					****P* = 0.578**		
White Cases					**White Controls**				
	lac Positive		**APA Negative**			**lac Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	72	(87.8)	121	(85.8)	GG	16	(76.2)	163	(81.5)
GA	9	(11.0)	20	(14.2)	GA	5	(23.8)	35	(17.5)
AA	1	(1.2)	0	(0.0)	AA	0	(0.0)	2	(1.0)
Total	82		141		Total	21		200	
		***P* = 0.386**					***P* = 0.633**		
G	0.933		0.929		G	0.881		0.903	
A	0.067		0.071		A	0.119		0.098	
		****P* = 0.877**					****P* = 0.679**		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 24. Distribution of -1219G>A SNP with anti-β_2-glycoprotein I (anti- β_2GPI) in white cases and controls

-1219G>A (rs8178819) SNP									
White Cases					**White Controls**				
	ß$_2$GPI Positive		**ß$_2$GPI Negative**			**ß$_2$GPI Positive**		**ß$_2$GPI Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	90	(88.2)	172	(86.9)	GG	62	(81.6)	304	(81.5)
GA	12	(11.8)	25	(12.6)	GA	13	(17.1)	64	(17.2)
AA	0	(0.0)	1	(0.5)	AA	1	(1.3)	5	(1.3)
Total	102		198		Total	76		373	
		**P = 0.905					**P = 1.000		
G	0.941		0.932		G	0.901		0.901	
A	0.059		0.068		A	0.099		0.099	
		***P = 0.652					***P = 0.985		
White Cases					**White Controls**				
	ß$_2$GPI Positive		**APA Negative**			**ß$_2$GPI Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	90	(88.2)	121	(85.8)	GG	62	(81.6)	163	(81.5)
GA	12	(11.8)	20	(14.2)	GA	13	(17.1)	35	(17.5)
AA	0	(0.0)	0	(0.0)	AA	1	(1.3)	2	(1.0)
Total	102		141		Total	76		200	
		**P = 0.702					**P = 1.000		
G	0.941		0.929		G	0.901		0.903	
A	0.059		0.071		A	0.099		0.098	
		***P = 0.590					***P = 0.967		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 25. Distribution of -1219G>A SNP with APA-positive and APA-negative in white cases and controls

-1219G>A (rs8178819) SNP									
White Cases					White Controls				
	APA Positive		APA Negative			APA Positive		APA Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	145	(88.4)	121	(85.8)	GG	154	(83.2)	163	(81.5)
GA	17	(10.4)	20	(14.2)	GA	29	(15.7)	35	(17.5)
AA	2	(1.2)	0	(0.0)	AA	2	(1.1)	2	(1.0)
Total	164		141		Total	185		200	
		**P = 0.293					**P = 0.880		
G	0.936		0.929		G	0.911		0.903	
A	0.064		0.071		A	0.089		0.098	
		***P = 0.735					***P = 0.692		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 26. Distribution of -1190G>C SNP with anticardiolipin antibodies (aCL) in white cases and controls

-1190G>C (rs3760290) SNP									
White Cases					**White Controls**				
	aCL Positive		**aCL Negative**			**aCL Positive**		**aCL Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	28	(34.2)	83	(37.7)	GG	57	(45.6)	105	(34.9)
GC	39	(47.6)	111	(50.5)	GC	55	(44.0)	162	(53.8)
CC	15	(18.3)	26	(11.8)	CC	13	(10.4)	34	(11.3)
Total	82		218		Total	125		302	
		***P* = 0.352**					***P* = 0.113**		
G	0.579		0.630		G	0.676		0.618	
C	0.421		0.370		C	0.324		0.382	
		****P* = 0.263**					****P* = 0.103**		
White Cases					**White Controls**				
	aCL Positive		**APA Negative**			**aCL Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	28	(34.2)	56	(39.7)	GG	57	(45.6)	66	(33.2)
GC	39	(47.6)	68	(48.2)	GC	55	(44.0)	107	(53.8)
CC	15	(18.3)	17	(12.1)	CC	13	(10.4)	26	(13.1)
Total	82		141		Total	125		200	
		***P* = 0.403**					***P* = 0.083**		
G	0.579		0.638		G	0.676		0.601	
C	0.421		0.362		C	0.324		0.399	
		****P* = 0.219**					****P* = 0.050**		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 27. Distribution of -1190G>C SNP with lupus anticoagulant (lac) in white cases and controls

-1190G>C (rs3760290) SNP									
White Cases					**White Controls**				
	lac Positive		**lac Negative**			**lac Positive**		**lac Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	33	(39.8)	89	(35.3)	GG	7	(31.8)	136	(37.4)
GC	39	(47.0)	124	(49.2)	GC	15	(68.2)	180	(49.5)
CC	11	(13.3)	39	(15.5)	CC	0	(0.0)	48	(13.2)
Total	83		252		Total	22		302	
		**P* = 0.744					**P* = 0.096		
G	0.633		0.599		G	0.659		0.621	
C	0.367		0.401		C	0.341		0.379	
		***P* = 0.442					***P* = 0.604		
White Cases					**White Controls**				
	lac Positive		**APA Negative**			**lac Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	33	(39.8)	56	(39.7)	GG	7	(31.8)	66	(33.2)
GC	39	(47.0)	68	(48.2)	GC	15	(68.2)	107	(53.8)
CC	11	(13.3)	17	(12.1)	CC	0	(0.0)	26	(13.1)
Total	83		141		Total	22		199	
		**P* = 0.962					**P* = 0.153		
G	0.633		0.638		G	0.659		0.601	
C	0.367		0.362		C	0.341		0.399	
		***P* = 0.903					***P* = 0.438		

***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 28. Distribution of -1190G>C SNP with anti-β_2-glycoprotein I (anti- β_2GPI) in white cases and controls

-1190G>C (rs3760290) SNP									
White Cases					**White Controls**				
	ß$_2$GPI Positive		**ß$_2$GPI Negative**			**ß$_2$GPI Positive**		**ß$_2$GPI Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	41	(39.8)	71	(35.7)	GG	30	(39.5)	138	(39.5)
GC	50	(48.5)	100	(50.3)	GC	43	(56.6)	43	(56.6)
CC	12	(11.7)	28	(14.1)	CC	3	(4.0)	3	(4.0)
Total	103		199		Total	76		374	
		**P = 0.753					**P = 0.031		
G	0.641		0.608		G	0.678		0.614	
C	0.359		0.392		C	0.322		0.386	
		***P = 0.429					***P = 0.127		
White Cases					**White Controls**				
	ß$_2$GPI Positive		**APA Negative**			**ß$_2$GPI Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	41	(39.8)	56	(39.7)	GG	30	(39.5)	66	(33.2)
GC	50	(48.5)	68	(48.2)	GC	43	(56.6)	107	(53.8)
CC	12	(11.7)	17	(12.1)	CC	3	(4.0)	26	(13.1)
Total	103		141		Total	76		199	
		**P = 1.000					**P = 0.067		
G	0.641		0.638		G	0.678		0.601	
C	0.359		0.362		C	0.322		0.399	
		***P = 0.955					***P = 0.088		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 29. Distribution of -1190G>C SNP with APA-positive and APA-negative in white cases and controls

-1190G>C (rs3760290) SNP									
White Cases					White Controls				
	APA Positive		APA Negative			APA Positive		APA Negative	
	n	(%)	*n*	(%)		*n*	(%)	*n*	(%)
GG	57	(34.3)	56	(39.7)	GG	78	(41.7)	66	(33.2)
GC	83	(50.0)	68	(48.2)	GC	93	(49.7)	107	(53.8)
CC	26	(15.7)	17	(12.1)	CC	16	(8.6)	26	(13.1)
Total	166		141		Total	187		199	
		**P = 0.521					**P = 0.138		
G	0.593		0.638		G	0.666		0.601	
C	0.407		0.362		C	0.334		0.399	
		***P = 0.253					***P = 0.059		

***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 30. Distribution of -759A>G SNP with anticardiolipin antibodies (aCL) in white cases and controls

-759A>G (rs8178820) SNP									
White Cases					White Controls				
	aCL Positive		aCL Negative			aCL Positive		aCL Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
AA	47	(58.0)	115	(52.5)	AA	81	(64.8)	171	(56.6)
AG	30	(37.0)	88	(40.2)	AG	39	(31.2)	115	(38.1)
GG	4	(4.9)	16	(7.3)	GG	5	(4.0)	16	(5.3)
Total	81		219		Total	125		302	
		**P* = 0.669					**P* = 0.311		
A	0.765		0.726		A	0.804		0.757	
G	0.235		0.274		G	0.196		0.243	
		***P* = 0.319					***P* = 0.121		
White Cases					White Controls				
	aCL Positive		APA Negative			aCL Positive		APA Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
AA	47	(58.0)	76	(53.9)	AA	81	(64.8)	111	(55.2)
AG	30	(37.0)	54	(38.3)	AG	39	(31.2)	78	(38.8)
GG	4	(4.9)	11	(7.8)	GG	5	(4.0)	12	(6.0)
Total	81		141		Total	125		201	
		**P* = 0.737					**P* = 0.249		
A	0.765		0.730		A	0.804		0.746	
G	0.235		0.270		G	0.196		0.254	
		***P* = 0.411					***P* = 0.082		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 31. Distribution of -759A>G SNP with lupus anticoagulant (lac) in white cases and controls

-759A>G (rs8178820) SNP									
White Cases					White Controls				
	lac Positive		lac Negative			lac Positive		lac Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
AA	48	(58.5)	133	(52.8)	AA	11	(50.0)	212	(57.9)
AG	27	(32.9)	98	(38.9)	AG	11	(50.0)	132	(36.1)
GG	7	(8.5)	21	(8.3)	GG	0	(0.0)	22	(6.0)
Total	82		252		Total	22		366	
		**P* = 0.626					**P* = 0.366		
A	0.750		0.722		A	0.750		0.760	
G	0.250		0.278		G	0.250		0.240	
		***P* = 0.479					***P* = 0.887		
White Cases					White Controls				
	lac Positive		APA Negative			lac Positive		APA Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
AA	48	(58.5)	76	(53.9)	AA	11	(50.0)	111	(55.2)
AG	27	(32.9)	54	(38.3)	AG	11	(50.0)	78	(38.8)
GG	7	(8.5)	11	(7.8)	GG	0	(0.0)	12	(6.0)
Total	82		141		Total	22		201	
		**P* = 0.720					**P* = 0.483		
A	0.750		0.730		A	0.750		0.746	
G	0.250		0.270		G	0.250		0.254	
		***P* = 0.649					***P* = 0.957		

***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 32. Distribution of -759A>G SNP with anti-β_2-glycoprotein I (anti- β_2GPI) in white cases and controls

-759A>G (rs8178820) SNP									
White Cases					White Controls				
	ß$_2$GPI Positive		ß$_2$GPI Negative			ß$_2$GPI Positive		ß$_2$GPI Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
AA	61	(60.4)	101	(50.8)	AA	51	(67.1)	209	(55.7)
AG	37	(36.6)	81	(40.7)	AG	23	(30.3)	142	(37.9)
GG	3	(3.0)	17	(8.5)	GG	2	(2.6)	24	(6.4)
Total	101		199		Total	76		374	
		**P = 0.098					**P = 0.158		
A	0.787		0.711		A	0.822		0.747	
G	0.213		0.289		G	0.178		0.253	
		***P = 0.038					***P = 0.030		
White Cases					White Controls				
	ß$_2$GPI Positive		APA Negative			ß$_2$GPI Positive		APA Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
AA	61	(60.4)	76	(53.9)	AA	51	(67.1)	111	(55.2)
AG	37	(36.6)	54	(38.3)	AG	23	(30.3)	78	(38.8)
GG	3	(3.0)	11	(7.8)	GG	2	(2.6)	12	(6.0)
Total	101		141		Total	76		201	
		**P = 0.242					**P = 0.180		
A	0.787		0.730		A	0.822		0.746	
G	0.213		0.270		G	0.178		0.254	
		***P = 0.147					***P = 0.044		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 33. Distribution of -759A>G SNP with APA-positive and APA-negative in white cases and controls

-759A>G (rs8178820) SNP									
White Cases					White Controls				
	APA Positive		APA Negative			APA Positive		APA Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
AA	88	(53.7)	76	(53.9)	AA	118	(63.1)	111	(55.2)
AG	65	(39.6)	54	(38.3)	AG	62	(33.2)	78	(38.8)
GG	11	(6.7)	11	(7.8)	GG	7	(3.7)	12	(6.0)
Total	164		141		Total	187		201	
		**P* = 0.935					**P* = 0.240		
A	0.735		0.730		A	0.797		0.746	
G	0.265		0.270		G	0.203		0.254	
		***P* = 0.906					***P* = 0.093		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 34. Distribution of -700C>A SNP with anticardiolipin antibodies (aCL) in white cases and controls

-700C>A (rs3760291)									
White Cases					**White Controls**				
	aCL Positive		**aCL Negative**			**aCL Positive**		**aCL Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	44	(54.3)	126	(57.5)	CC	79	(63.7)	170	(56.3)
CA	30	(37.0)	80	(36.5)	CA	40	(32.3)	116	(38.4)
AA	7	(8.6)	13	(5.9)	AA	5	(4.0)	16	(5.3)
Total	81		219		Total	124		302	
		***P* = 0.651**					***P* = 0.408**		
C	0.728		0.758		C	0.798		0.755	
A	0.272		0.242		A	0.202		0.245	
		****P* = 0.465**					****P* = 0.160**		
White Cases					**White Controls**				
	aCL Positive		**APA Negative**			**aCL Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	44	(54.3)	82	(58.2)	CC	79	(63.7)	111	(55.2)
CA	30	(37.0)	51	(36.2)	CA	40	(32.3)	78	(38.8)
AA	7	(8.6)	8	(5.7)	AA	5	(4.0)	12	(6.0)
Total	81		141		Total	124		201	
		***P* = 0.647**					***P* = 0.309**		
C	0.728		0.762		C	0.798		0.746	
A	0.272		0.238		A	0.202		0.254	
		****P* = 0.431**					****P* = 0.119**		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 35. Distribution of -700C>A SNP with lupus anticoagulant (lac) in white cases and controls

-700C>A (rs3760291)									
White Cases					**White Controls**				
	lac Positive		**lac Negative**			**lac Positive**		**lac Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	48	(58.5)	147	(58.3)	CC	11	(50.0)	211	(57.8)
CA	28	(34.2)	90	(35.7)	CA	11	(50.0)	132	(36.2)
AA	6	(7.3)	15	(6.0)	AA	0	(0.0)	22	(6.0)
Total	82		252		Total	22		365	
		**P* = 0.838					**P* = 0.366		
C	0.756		0.756		C	0.750		0.759	
A	0.244		0.244		A	0.250		0.241	
		***P* = 0.880					***P* = 0.895		
White Cases					**White Controls**				
	lac Positive		**APA Negative**			**lac Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	48	(58.5)	82	(58.2)	CC	11	(50.0)	111	(55.2)
CA	28	(34.2)	51	(36.2)	CA	11	(50.0)	78	(38.8)
AA	6	(7.3)	8	(5.7)	AA	0	(0.0)	12	(6.0)
Total	82		141		Total	22		201	
		**P* = 0.900					**P* = 0.483		
C	0.756		0.762		C	0.750		0.746	
A	0.244		0.238		A	0.250		0.254	
		***P* = 0.881					***P* = 0.957		

***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 36. Distribution of -700C>A SNP with anti-β_2-glycoprotein I (anti- β_2GPI) in white cases and controls

-700C>A (rs3760291)									
White Cases					**White Controls**				
	ß$_2$GPI Positive		**ß$_2$GPI Negative**			**ß$_2$GPI Positive**		**ß$_2$GPI Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	66	(65.4)	105	(52.8)	CC	50	(65.8)	207	(55.4)
CA	31	(30.7)	78	(39.2)	CA	24	(31.6)	143	(38.2)
AA	4	(4.0)	16	(8.0)	AA	2	(2.6)	24	(6.4)
Total	101		199		Total	76		374	
		*******P* = 0.092					*******P* = 0.202		
C	0.807		0.724		C	0.816		0.745	
A	0.193		0.276		A	0.184		0.255	
		********P* = 0.020					********P* = 0.044		
White Cases					**White Controls**				
	ß$_2$GPI Positive		**APA Negative**			**ß$_2$GPI Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	66	(65.4)	82	(58.2)	CC	50	(65.8)	111	(55.2)
CA	31	(30.7)	51	(36.2)	CA	24	(31.6)	78	(38.8)
AA	4	(4.0)	8	(5.7)	AA	2	(2.6)	12	(6.0)
Total	101		141		Total	76		201	
		*******P* = 0.562					*******P* = 0.258		
C	0.807		0.762		C	0.816		0.746	
A	0.193		0.238		A	0.184		0.254	
		********P* = 0.236					********P* = 0.069		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 37. Distribution of -700C>A SNP with APA-positive and APA-negative in white cases and controls

-700C>A (rs3760291)									
White Cases					White Controls				
	APA Positive		APA Negative			APA Positive		APA Negative	
	n	(%)	*n*	(%)		*n*	(%)	*n*	(%)
CC	92	(56.1)	82	(58.2)	CC	116	(62.4)	111	(55.2)
CA	59	(36.0)	51	(36.2)	CA	63	(33.9)	78	(38.8)
AA	13	(7.9)	8	(5.7)	AA	7	(3.8)	12	(6.0)
Total	164		141		Total	186		201	
		***P* = 0.757					***P* = 0.288		
C	0.741		0.762		C	0.793		0.746	
A	0.259		0.238		A	0.207		0.254	
		****P* = 0.538					****P* = 0.122		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 38. Distribution of -643T>C SNP with anticardiolipin antibodies (aCL) in white cases and controls

-643T>C (rs3760292) SNP									
White Cases					White Controls				
	aCL Positive		aCL Negative			aCL Positive		aCL Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
TT	61	(75.3)	149	(67.7)	TT	93	(74.4)	221	(73.2)
TC	17	(21.0)	65	(29.6)	TC	28	(22.4)	75	(24.8)
CC	3	(3.7)	6	(2.7)	CC	4	(3.2)	6	(2.0)
Total	81		220		Total	125		302	
		**P = 0.297					**P = 0.645		
T	0.858		0.825		T	0.856		0.856	
C	0.142		0.175		C	0.144		0.144	
		***P = 0.315					***P = 0.999		
White Cases					White Controls				
	aCL Positive		APA Negative			aCL Positive		APA Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
TT	61	(75.3)	98	(69.5)	TT	93	(74.4)	154	(76.6)
TC	17	(21.0)	38	(27.0)	TC	28	(22.4)	44	(21.9)
CC	3	(3.7)	5	(3.6)	CC	4	(3.2)	3	(1.5)
Total	81		141		Total	125		201	
		**P = 0.591					**P = 0.587		
T	0.858		0.830		T	0.856		0.876	
C	0.142		0.170		C	0.144		0.124	
		***P = 0.425					***P = 0.478		

***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 39. Distribution of -643T>C SNP with lupus anticoagulant (lac) in white cases and controls

-643T>C (rs3760292) SNP									
White Cases					**White Controls**				
	lac Positive		**lac Negative**			**lac Positive**		**lac Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
TT	56	(68.3)	177	(70.0)	TT	15	(68.2)	277	(75.7)
TC	23	(28.1)	64	(25.3)	TC	7	(31.8)	81	(22.1)
CC	3	(3.7)	12	(4.7)	CC	0	(0.0)	8	(2.2)
Total	82		252		Total	22		366	
		***P* = 0.844**					***P* = 0.568**		
T	0.823		0.826		T	0.841		0.867	
C	0.177		0.174		C	0.159		0.133	
		****P* = 0.932**					****P* = 0.638**		
White Cases					**White Controls**				
	lac Positive		**APA Negative**			**lac Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
TT	56	(68.3)	98	(69.5)	TT	15	(68.2)	154	(76.6)
TC	23	(28.1)	38	(27.0)	TC	7	(31.8)	44	(21.9)
CC	3	(3.7)	5	(3.6)	CC	0	(0.0)	3	(1.5)
Total	82		141		Total	22		201	
		***P* = 0.965**					***P* = 0.487**		
T	0.823		0.830		T	0.841		0.876	
C	0.177		0.170		C	0.159		0.124	
		****P* = 0.859**					****P* = 0.546**		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 40. Distribution of -643T>C SNP with anti-β_2-glycoprotein I (anti- β_2GPI) in white cases and controls

-643T>C (rs3760292) SNP									
White Cases					**White Controls**				
	ß$_2$GPI Positive		**ß$_2$GPI Negative**			**ß$_2$GPI Positive**		**ß$_2$GPI Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
TT	70	(68.6)	140	(70.4)	TT	54	(71.1)	282	(75.2)
TC	28	(27.5)	54	(27.1)	TC	19	(25.0)	86	(22.9)
CC	4	(3.9)	5	(2.5)	CC	3	(4.0)	7	(1.9)
Total	102		199		Total	76		375	
		**P = 0.763					**P = 0.401		
T	0.824		0.839		T	0.836		0.867	
C	0.176		0.161		C	0.164		0.133	
		***P = 0.629					***P = 0.338		
White Cases					**White Controls**				
	ß$_2$GPI Positive		**APA Negative**			**ß$_2$GPI Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
TT	70	(68.6)	98	(69.5)	TT	54	(71.1)	154	(76.6)
TC	28	(27.5)	38	(27.0)	TC	19	(25.0)	44	(21.9)
CC	4	(3.9)	5	(3.6)	CC	3	(4.0)	3	(1.5)
Total	102		141		Total	76		201	
		**P = 1.000					**P = 0.319		
T	0.824		0.830		T	0.836		0.876	
C	0.176		0.170		C	0.164		0.124	
		***P = 0.857					***P = 0.242		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 41. Distribution of -643T>C SNP with APA-positive and APA-negative in white cases and controls

-643T>C (rs3760292) SNP									
White Cases					White Controls				
	APA Positive		APA Negative			APA Positive		APA Negative	
	n	(%)	*n*	(%)		*n*	(%)	*n*	(%)
TT	117	(70.9)	98	(69.5)	TT	134	(71.7)	154	(76.6)
TC	43	(26.1)	38	(27.0)	TC	47	(25.1)	44	(21.9)
CC	6	(3.0)	5	(3.6)	CC	6	(3.2)	3	(1.5)
Total	165		141		Total	187		201	
		**P = 0.975					**P = 0.379		
T	0.839		0.830		T	0.842		0.876	
C	0.161		0.170		C	0.158		0.124	
		***P = 0.750					***P = 0.182		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 42. Distribution of -38G>A SNP with anticardiolipin antibodies (aCL) in white cases and controls

-38G>A SNP									
White Cases					**White Controls**				
	aCL Positive		**aCL Negative**			**aCL Positive**		**aCL Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	79	(96.3)	214	(97.3)	GG	120	(96.0)	289	(95.7)
GA	3	(3.7)	6	(2.7)	GA	4	(3.2)	12	(4.0)
AA	0	(0.0)	0	(0.0)	AA	1	(0.8)	1	(0.3)
Total	82		220		Total	125		302	
		***P* = 0.708**					***P* = 0.788**		
G	0.982		0.986		G	0.976		0.977	
A	0.018		0.014		A	0.024		0.023	
		****P* = 0.694**					****P* = 0.943**		
White Cases					**White Controls**				
	aCL Positive		**APA Negative**			**aCL Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	79	(96.3)	141	(100.0)	GG	120	(96.0)	193	(76.6)
GA	3	(3.7)	0	(0.0)	GA	4	(3.2)	6	(21.9)
AA	0	(30.0)	0	(0.0)	AA	1	(0.8)	1	(1.5)
Total	82		141		Total	125		200	
		****P* = 0.049**					***P* = 1.000**		
G	0.982		1.000		G	0.976		0.980	
A	0.018		0.000		A	0.024		0.020	
		****P* = 0.080**					****P* = 0.738**		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 43. Distribution of -38G>A SNP with lupus anticoagulant (lac) in white cases and controls

-38G>A SNP								
White Cases					**White Controls**				
	lac Positive		**lac Negative**			**lac Positive**		**lac Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	78	(94.0)	247	(98.0)	GG	20	(90.9)	351	(96.2)
GA	5	(6.0)	5	(2.0)	GA	2	(9.1)	12	(3.3)
AA	0	(0.0)	0	(0.0)	AA	0	(0.0)	2	(0.6)
Total	83		252		Total	22		365	
		**P = 0.072					**P = 0.277		
G	0.970		0.990		G	0.955		0.978	
A	0.030		0.010		A	0.045		0.022	
		***P = 0.149					***P = 0.460		
White Cases					**White Controls**				
	lac Positive		**APA Negative**			**lac Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	78	(94.0)	141	(100.0)	GG	20	(90.9)	193	(76.6)
GA	5	(6.0)	0	(0.0)	GA	2	(9.1)	6	(21.9)
AA	0	(0.0)	0	(0.0)	AA	0	(0.0)	1	(1.5)
Total	83		141		Total	22		200	
		****P = 0.006**					**P = 0.264		
G	0.970		1.000		G	0.955		0.980	
A	0.030		0.000		A	0.045		0.020	
		*****P = 0.023**					***P = 0.429		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 44. Distribution of -38G>A SNP with anti-β_2-glycoprotein I (anti- β_2GPI) in white cases and controls

-38G>A SNP									
White Cases					**White Controls**				
	ß$_2$GPI Positive		**ß$_2$GPI Negative**			**ß$_2$GPI Positive**		**ß$_2$GPI Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	98	(95.2)	195	(98.0)	GG	70	(92.1)	360	(96.0)
GA	5	(4.9)	4	(2.0)	GA	6	(7.9)	13	(3.5)
AA	0	(0.0)	0	(0.0)	AA	0	(0.0)	2	(10.5)
Total	103		199		Total	76		375	
		**P* = 0.282					**P* = 0.200		
G	0.976		0.990		G	0.961		0.867	
A	0.024		0.010		A	0.039		0.133	
		***P* = 0.229					***P* = 0.314		
White Cases					**White Controls**				
	ß$_2$GPI Positive		**APA Negative**			**ß$_2$GPI Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	98	(95.2)	141	(100.0)	GG	70	(92.1)	193	(76.6)
GA	5	(4.9)	0	(0.0)	GA	6	(7.9)	6	(21.9)
AA	0	(0.0)	0	(0.0)	AA	0	(0.0)	1	(1.5)
Total	103		141		Total	76		200	
		***P* = 0.013**					**P* = 0.159		
G	0.976		1.000		G	0.961		0.980	
A	0.024		0.000		A	0.039		0.020	
		****P* = 0.024**					***P* = 0.260		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 45. Distribution of -38G>A SNP with APA-positive and APA-negative in white cases and controls

-38G>A SNP									
White Cases					**White Controls**				
	APA Positive		**APA Negative**			**APA Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
GG	157	(94.6)	141	(100.0)	GG	178	(95.2)	193	(76.6)
GA	9	(5.4)	0	(0.0)	GA	48	(4.3)	6	(21.9)
AA	0	(0.0)	0	(0.0)	AA	1	(0.5)	1	(1.5)
Total	166		141		Total	187		200	
		****P* = 0.004**					***P* = 0.795**		
G	0.973		1.000		G	0.973		0.980	
A	0.027		0.000		A	0.027		0.020	
		*****P* = 0.002**					****P* = 0.536**		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 46. Distribution of -32C>A SNP with anticardiolipin antibodies (aCL) in white cases and controls

-32C>A (rs8178822) SNP									
White Cases					**White Controls**				
	aCL Positive		**aCL Negative**			**aCL Positive**		**aCL Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	76	(92.7)	188	(85.5)	CC	110	(88.0)	264	(87.4)
CA	6	(7.3)	30	(13.6)	CA	15	(12.0)	37	(12.3)
AA	0	(0.0)	2	(0.9)	AA	0	(0.0)	1	(0.3)
Total	82		220		Total	125		302	
		**P* = 0.250					**P* = 1.000		
C	0.963		0.923		C	0.940		0.935	
A	0.037		0.077		A	0.060		0.065	
		***P* = 0.036					***P* = 0.800		
White Cases					**White Controls**				
	aCL Positive		**APA Negative**			**aCL Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	76	(92.7)	122	(86.5)	CC	110	(88.0)	176	(88.0)
CA	6	(7.3)	18	(12.8)	CA	15	(12.0)	24	(12.0)
AA	0	(0.0)	1	(0.7)	AA	0	(0.0)	0	(0.0)
Total	82		141		Total	125		200	
		**P* = 0.353					**P* = 1.000		
C	0.963		0.929		C	0.940		0.940	
A	0.037		0.071		A	0.060		0.060	
		***P* = 0.105					***P* = 1.000		

***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 47. Distribution of -32C>A SNP with lupus anticoagulant (lac) in white cases and controls

-32C>A (rs8178822) SNP									
White Cases					**White Controls**				
	lac Positive		**lac Negative**			**lac Positive**		**lac Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	78	(94.0)	216	(85.7)	CC	18	(81.8)	320	(87.7)
CA	5	(6.0)	34	(13.5)	CA	4	(18.2)	44	(12.1)
AA	0	(0.0)	2	(0.8)	AA	0	(0.0)	1	(0.3)
Total	83		252		Total	22		365	
		***P* = 0.145**					***P* = 0.374**		
C	0.970		0.925		C	0.909		0.937	
A	0.030		0.075		A	0.091		0.063	
		****P* = 0.011**					****P* = 0.529**		
White Cases					**White Controls**				
	lac Positive		**APA Negative**			**lac Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	78	(94.0)	122	(86.5)	CC	18	(81.8)	176	(88.0)
CA	5	(6.0)	18	(12.8)	CA	4	(18.2)	24	(12.0)
AA	0	(0.0)	1	(0.7)	AA	0	(0.0)	0	(0.0)
Total	83		141		Total	22		200	
		***P* = 0.136**					***P* = 0.494**		
C	0.970		0.929		C	0.909		0.940	
A	0.030		0.071		A	0.091		0.060	
		****P* = 0.044**					****P* = 0.492**		

***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

Table 48. Distribution of -32C>A SNP with anti-β_2-glycoprotein I (anti- β_2GPI) in white cases and controls

-32C>A (rs8178822) SNP									
White Cases					**White Controls**				
	ß$_2$GPI Positive		**ß$_2$GPI Negative**			**ß$_2$GPI Positive**		**ß$_2$GPI Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	85	(82.5)	179	(90.0)	CC	67	(88.2)	329	(87.7)
CA	17	(16.5)	19	(9.6)	CA	8	(10.5)	46	(12.3)
AA	1	(1.0)	1	(0.5)	AA	1	(1.3)	0	(0.0)
Total	103		199		Total	76		375	
		**P = 0.133					**P = 0.208		
C	0.908		0.947		C	0.934		0.939	
A	0.092		0.053		A	0.066		0.061	
		***P = 0.087					***P = 0.839		
White Cases					**White Controls**				
	ß$_2$GPI Positive		**APA Negative**			**ß$_2$GPI Positive**		**APA Negative**	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	85	(82.5)	122	(86.5)	CC	67	(88.2)	176	(88.0)
CA	17	(16.5)	18	(12.8)	CA	8	(10.5)	24	(12.0)
AA	1	(1.0)	1	(0.7)	AA	1	(1.3)	0	(0.0)
Total	103		141		Total	76		200	
		**P = 0.736					**P = 0.332		
C	0.908		0.929		C	0.934		0.940	
A	0.092		0.071		A	0.066		0.060	
		***P = 0.400					***P = 0.804		

***Genotype frequencies were determined by fisher's exact test*
****Allele frequencies were determined by standard Z test of binomial proportion*

Table 49. Distribution of -32C>A SNP with APA-positive and APA-negative in white cases and controls

-32C>A (rs8178822) SNP									
White Cases					White Controls				
	APA Positive		APA Negative			APA Positive		APA Negative	
	n	**(%)**	***n***	**(%)**		***n***	**(%)**	***n***	**(%)**
CC	146	(88.0)	122	(86.5)	CC	162	(86.6)	176	(88.0)
CA	19	(11.5)	18	(12.8)	CA	24	(412.8)	24	(12.0)
AA	1	(0.6)	1	(0.7)	AA	1	(0.5)	0	(0.0)
Total	166		141		Total	187		200	
		**P* = 0.865					**P* = 0.757		
C	0.937		0.929		C	0.930		0.940	
A	0.063		0.071		A	0.070		0.060	
		***P* = 0.706					***P* = 0.591		

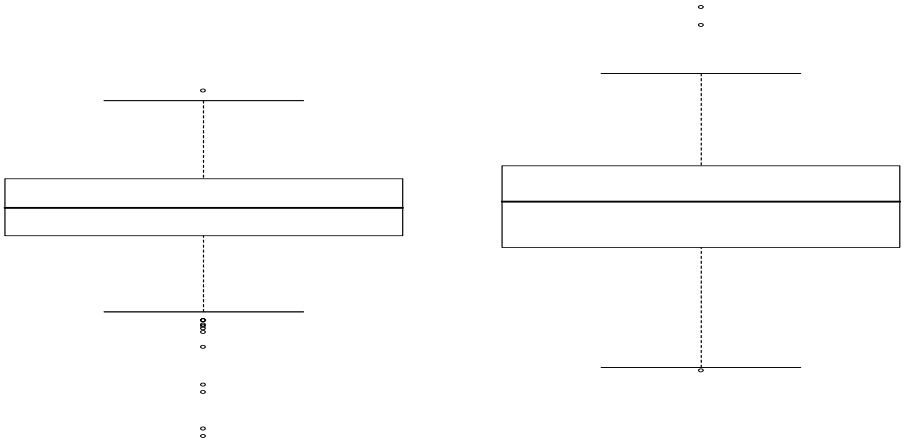
***Genotype frequencies were determined by fisher's exact test*

****Allele frequencies were determined by standard Z test of binomial proportion*

6.4 GENETIC ASSOCIATION OF APOH PROMOTER SNPs WITH PLASMA β_2GPI LEVELS

The distribution of plasma β_2GPI levels among the white SLE cases and controls is presented in Figure 52. Cases had slightly lower levels than controls (17.90 ± 4.15 *vs.* 18.72 ± 4.68; $P = 0.05$) Hence all further analyses were done combining the subgroup of white SLE cases (n = 241) and controls (n = 206) that had information for plasma β_2GPI levels. Stepwise regression analysis showed that age, BMI and ever smoking were the significant determinants of the plasma β_2GPI levels. Adjusted mean plasma β_2GPI levels ± SD among *APOH* promoter SNP genotypes are presented in Table 50. Only the -32C>A SNP was significant in both single-site (Table 51; $P < 0.001$) and multiple regression analysis ($P < 0.001$) adjusted for the disease, age, BMI, ever smoking covariates. Mean plasma β_2GPI levels were higher in homozygotes of the wild-type allele (18.62 ± 4.19) compared to the heterozygotes (16.24 ± 5.18) and homozygotes of less common allele (13.90 ± 0.00). Two coding polymorphisms, Cys306Gly and Trp316Ser, previously reported to be major regulators of plasma β_2GPI levels [113], were included in both multiple regression and haplotype analyses along with all six *APOH* promoter SNPs. Our initial studies demonstrated that the -32C>A SNP is in moderately high LD with the codon 316 mutation ($D' = 0.74$, $r^2 = 0.63$) [116]; therefore the Trp316Ser was excluded from multiple regression analysis. When all six *APOH* promoter SNPs and the coding Cys306Gly SNP were included in a multiple linear regression model adjusted for covariates (Table 52), the significant effect of -32C>A SNP remained ($P < 0.001$), consistent with single-site analysis and similarly also for the known coding Cys306Gly polymorphism ($P < 0.001$). The -32C>A and Cys306Gly SNPs accounted for 5.4% and 21.5% variation, respectively in plasma β_2GPI levels (Table 52). Eight-site haplotype analysis identified a total of 11 haplotypes with a frequency of > 1% (Table 53) using the haplo.glm

function in the haplo.stats package in R program. Three haplotypes showed significant association with plasma β_2GPI levels ($P < 0.001$). The haplotype (H5) harbored minor alleles for the -1190G>C, -32C>A, and Trp316Ser SNPs. The other two significant haplotypes were predominantly defined by the minor alleles of the two coding polymorphisms, (H6: Cys306Gly; H10:Trp316Ser; respectively) that are already known to be major determinants of plasma β_2GPI levels. Although the -32C>A SNP was significant in single-site analysis, the other haplotype (H7) defined by minor alleles only at -1190G>C and -32C>A SNPs and not for Trp316Ser did not show significant association, suggesting that the effect of the 1190G>C and -32C>A SNPs is dependent upon Trp316Ser polymorphism. None of the individual haplotypes harboring less common alleles for the -643T>C (H2 and H9) and -1219G>A SNPs (H4) that are found to be significantly decrease gene expression *in vitro* showed significant impact on plasma β_2GPI levels. Three-site haplotype analysis (Table 54) with only the functionally relevant SNPs based on luciferase and EMSA data (-1219G>A, -643T>C, and -32C>A) revealed one haplotype carrying -32A (freq: 0.075) to be significantly associated with decreased plasma β_2GPI levels ($P < 0.001$). Another haplotype harboring the minor allele for -1219A (freq: 0.080) showed a significant albeit less pronounced association with plasma β_2GPI levels ($P = 0.046$).



	White Cases	White Controls
Mean (mg/dL) ± SD	17.90 ± 4.15	18.72 ± 4.68

Figure 52. Box plot of plasma β_2GPI levels in white cases and controls

In this box-plot diagram, the horizontal line within each box represents the median. Horizontal lines outside the box represent the 10th and the 90th percentiles.

Table 50. Mean ± SD of plasma β_2GPI levels of *APOH* promoter polymorphisms

SNP	White Cases + Controls		
-1284C>G Mean ± SD	CC (*n*) 18.26 ± 4.41 (446)	CG (*n*) 24.19 ± NA (1)	GG (*n*) NA ± NA (0)
	P * = 0.218		
-1219G>A Mean ± SD	GG (*n*) 18.11 ± 4.43 (358)	GA (*n*) 19.04 ± 4.45 (81)	AA (*n*) 20.48 ± 2.80 (3)
	P * = 0.263		
-1190G>C Mean ± SD	GG (*n*) 18.75 ± 4.18 (141)	GC (*n*) NA ± NA (242)	CC (*n*) 17.83 ± 4.31 (62)
	P * = 0.345		
-759A>G Mean ± SD	AA (*n*) 18.22 ± 4.62 (241)	AG (*n*) 16.28 ± 4.08 (174)	GG (*n*) 18.49 ± 4.56 (30)
	P * = 0.830		
-700C>A Mean ± SD	CC (*n*) 18.00 ± 4.69 (245)	CA (*n*) 18.60 ± 4.01 (169)	AA (*n*) 19.09 ± 2.90 (30)
	P * = 0.380		
-643T>C Mean ± SD	TT (*n*) 18.05 ± 4.33 (332)	TC (*n*) 18.92 ± 4.30 (102)	CC (*n*) 17.96 ± 6.52 (10)
	P * = 0.2331		
-38G>A Mean ± SD	GG (*n*) 18.20 ± 4.41 (426)	GA (*n*) 19.77 ± 4.39 (20)	AA (*n*) NA ± NA (0)
	P * = 0.296		
-32C>A Mean ± SD	CC (*n*) 18.62 ± 4.19 (381)	CA (*n*) 16.24 ± 5.18 (64)	AA (*n*) 13.90 ± NA (1)
	***P* * < 0.001**		
Cys306Gly Mean ± SD	TT (*n*) 18.86 ± 3.80 (408)	TG (*n*) 12.87 ± 5.18 (37)	GG (*n*) 0.50 ± 0.42 (2)
	***P* * < 0.001**		
Trp316Ser Mean ± SD	GG (*n*) 18.81 ± 4.03 (397)	GC (*n*) 14.16 ± 4.21 (47)	CC (*n*) 9.40 ± NA (1)
	***P* * < 0.001**		

P - Adjusted for disease, age , bmi, ever smoking*

Table 51. Single-site analysis of plasma β_2GPI levels of *APOH* promoter SNPs

SNP	*P** - value
Disease	---
Age	0.232
BMI	0.051
Smokever	0.991
rs8178818 (-1284C>G)	0.218
rs8178819 (-1219G>A)	0.263
rs3760290 (-1190G>C)	0.345
rs8178820 (-759A>G)	0.830
rs3760291 (-700C>A)	0.380
rs3760292 (-643T>C)	0.331
(-38G>A)	0.296
rs8178822 (-32C>A)	**<0.001**

P - Adjusted for disease, age , bmi, ever smoking*

Table 52. Multiple regression * analysis of *APOH* SNPs for plasma β_2GPI levels

SNP	*P* - value	Adjusted R^2 **
Disease	0.176	0.003
Age	0.129	0.004
BMI	0.144	0.005
Smokever	0.371	-0.006
rs8178819 (-1219G>A)	0.441	---
rs3760290 (-1190G>C)	0.498	---
rs8178820 (-759A>G)	0.957	---
rs3760292 (-643T>C)	0.633	---
(-38G>A)	0.437	---
rs8178822 (-32C>A)	**<0.001**	0.054
rs1801689 (306T>G)	**<0.001**	0.215

**Adjusted for disease, age, BMI and ever smoking.*
***R^2 was calculated only for covariates and significant P-values.*

Table 53. Haplotype analysis of *APOH* SNPs for plasma β_2GPI levels

HAPLOTYPE**	-1219G>A	-1190G>C	-759A>G	-643T>C	-38G>A	-32C>A	Cys306Gly	Trp316Ser	CASES + CONTROLS			
									freq	coef	se	*P*
base haplotype	G	G	A	T	G	C	T	G	0.384	-	-	-
H1	G	C	G	T	G	C	T	G	0.156	-0.218	0.408	0.593
H2	G	G	A	C	G	C	T	G	0.098	0.791	0.534	0.139
H3	G	C	A	T	G	C	T	G	0.081	0.301	0.538	0.576
H4	A	C	G	T	G	C	T	G	0.062	-0.046	0.675	0.946
H5	**G**	**C**	**A**	**T**	**G**	**A**	**T**	**C**	**0.042**	**-4.632**	**0.737**	**< 0.001**
H6	**G**	**G**	**A**	**T**	**G**	**C**	**G**	**G**	**0.038**	**-5.439**	**0.739**	**< 0.001**
H7	G	C	A	T	G	A	T	G	0.023	-1.271	1.031	0.218
H8	G	G	A	T	A	C	T	G	0.017	0.280	1.128	0.804
H9	G	G	G	C	G	C	T	G	0.013	-0.097	1.255	0.938
H10	**G**	**C**	**A**	**T**	**G**	**C**	**T**	**C**	**0.013**	**-4.748**	**1.247**	**< 0.001**
H11	G	G	G	T	G	C	T	G	0.011	-0.727	1.453	0.617
rare haplotype	*	*	*	*	*	*	*	*	0.062	-1.604	-	0.024

**R software (haplostats package) for ß$_2$GPI plasma levels, regression model included disease, age, BMI, smokever.*

***Only the haplotypes with more than 0.01 total frequencies are shown*

Alleles found to decrease gene expression in vitro

Alleles found to be significantly associated with low plasma ß$_2$GPI levels in univariate analysis

Alleles found to decrease gene expression in vitro and also associated with low plasma ß$_2$GPI levels in univariate analysis

Table 54. Three-site haplotype analysis of *APOH* promoter SNPs for plasma β_2GPI levels

Haplotype**	-1219G>A (rs8178819)	-643T>C (rs3760292)	-32 C>A (rs8178822)	Cases + Controls			
				freq	coef	se	P
Base haplo	G	T	C	0.712	---	---	---
H1	G	C	C	0.120	0.576	0.455	0.206
H 2	A	T	C	0.080	1.170	0.585	0.046
H 3	G	T	A	0.075	-2.307	0.574	< 0.001
H 4	A	C	C	0.013	-1.562	1.472	0.289

- *software (haplostats package) for β_2GPI plasma levels, BMI*
- *** Only the haplotypes with more than 0.01 total frequencies are shown*

Alleles found to decrease gene expression in vitro

7.0 DISCUSSION

The goals of this study were to i) clone and characterize 1,418 bp of the putative *APOH* promoter and to elucidate molecular mechanisms underlying the regulation of its expression, (ii) functionally characterize the SNPs as well as haplotypes present in the 1,418 bp fragment of the 5´-flanking region of the *APOH*, (iii) understand the role of the *APOH* promoter SNPs in determining the genetic risk of SLE and its related phenotypes in a SLE-case control cohort, and (iv) examine the influence of *APOH* promoter genetic polymorphisms on gene expression and plasma β_2GPI levels.

7.1 CHARACTERIZATION OF THE *APOH* PROMOTER

In order to assess basal, proximal promoter activity in the 5′-flanking region, a series of 5' deletions fused to the luciferase gene were constructed (see Figure 23) and transfected into COS-1 and HepG2 cells. The ability of the 5′-flanking region of *APOH* to function as a promoter was assessed by its capacity to drive the expression of a luciferase reporter gene. The wild-type (-1,375/+43) construct was defined by a 1,418 bp genomic DNA fragment of *APOH* 5′-flanking region fused to a promoterless firefly luciferase gene of the pGL3-basic vector (see Figure 21). A series of five truncated 5′-flanking *APOH* deletion mutant constructs, differing in approximately 200 bp, were generated with the 3′ end always terminating +43bp from the translation start site.

The 5′ ends began at bases -815/+43 (deletion mutant 1), -575/+43 (deletion mutant 2), -325/+43 (deletion mutant 3), -166/+43 (deletion mutant 4), -65/+43 (deletion mutant 5).

To provide insights into mechanisms that control basal *APOH* expression, our 5′- serial deletion analysis results suggest that 166 bp region upstream to the translation start site (ATG) is the minimum proximal promoter that consists of functional domains critical for basal *APOH* transcriptional activity. Starting with the wild-type (-1,375 bp upstream ATG), successive deletions extending to nt -325 (deletion mutants 1-3) enhanced promoter activity in COS-1 cells while a slightly different pattern, deletion mutant 1 was lower in expression as compared to wild-type, deletion mutants 2 and 3 were observed in HepG2 cell line. This slight difference in expression can be explained based on tissue-specific factors that play a key role in driving *APOH* expression. For both cell lines, deletion mutant 3 (-325/+43) was associated with highest promoter activity. No statistical significant differences were seen between wild-type and deletion mutants 1-3, that was consistent for both cell types. A deletion extending to nt -166 resulted in a slight to moderate reduction in activity while deletion of another 100 nt (deletion mutant 5 -66/+43) decreased promoter activity by more than ~60% in COS-1 cells and even more pronounced in HepG2 cells (~98%). Therefore, functional characterization of the *APOH* 5′ – flanking region revealed that sequences between nt -166 and -66 are likely to contain elements that participate in recruiting components of the core *APOH* transcription machinery.

Consistent with the earlier report by Wang and Chiang (2004) [83], our results demonstrate that ~160 bp upstream of the translation start site contains all the necessary elements to confer basal hepatic-specific expression of *APOH*. In the previous study of Wang and Chiang (2004) [83], a 4.1-kb genomic fragment of the 5′-flanking untranslated region of *APOH* was cloned and functionally characterized by transient transfection assays in the human hepatoma

cell lines (HepG2 and Huh7 cells). Serial deletion analyses revealed the region between -197 to +7 to show strong basal promoter activity in hepatoma cells and identified two critical *cis*-acting elements, an atypical TATA box (TATTA) located between nt -97 and -92 and HNF-1α at position between nt -65 and -51 for cell-specific regulation of *APOH* expression. Neither of the two elements was able to initiate transcription by itself indicating that both TATTA sequence and the HNF-1 element are necessary for *APOH* transcription. The nucleotide numbering used in the prior study (Wang and Chiang, 2004) [83] starts at the transcription start site 21 bp upstream of the ATG codon; the thymidine residue at this precise position is designated +1. This differs from the current study, wherein, the *APOH* fragments are numbered relative to the translation start codon ATG. Therefore, in order to do a comparative analysis between the present study *vs*. the previous one, the *APOH* deletion mutants used in the present study were numbered based on the method used in the Wang and Chiang paper, (Table 55 and Figure 53).

Table 55. Numbering of *APOH 5´*deletion fragments for comparative analysis

Numbering based on translation start site (ATG codon) (This study)	Numbering based on transcription start site 21 bp upstream of ATG codon (Wang and Chiang, 2004)
APOH deletion mutant 1 (-815/+43)	*APOH* deletion mutant 1 (-794/+64)
APOH deletion mutant 2 (-575/+43)	*APOH* deletion mutant 2 (-554/+64)
APOH deletion mutant 3 (-325/+43)	*APOH* deletion mutant 3 (-304/+64)
APOH deletion mutant 4 (-166/+43)	*APOH* deletion mutant 4 (-145/+64)
APOH deletion mutant 5 (-65/+43)	*APOH* deletion mutant 5 (-44/+64)

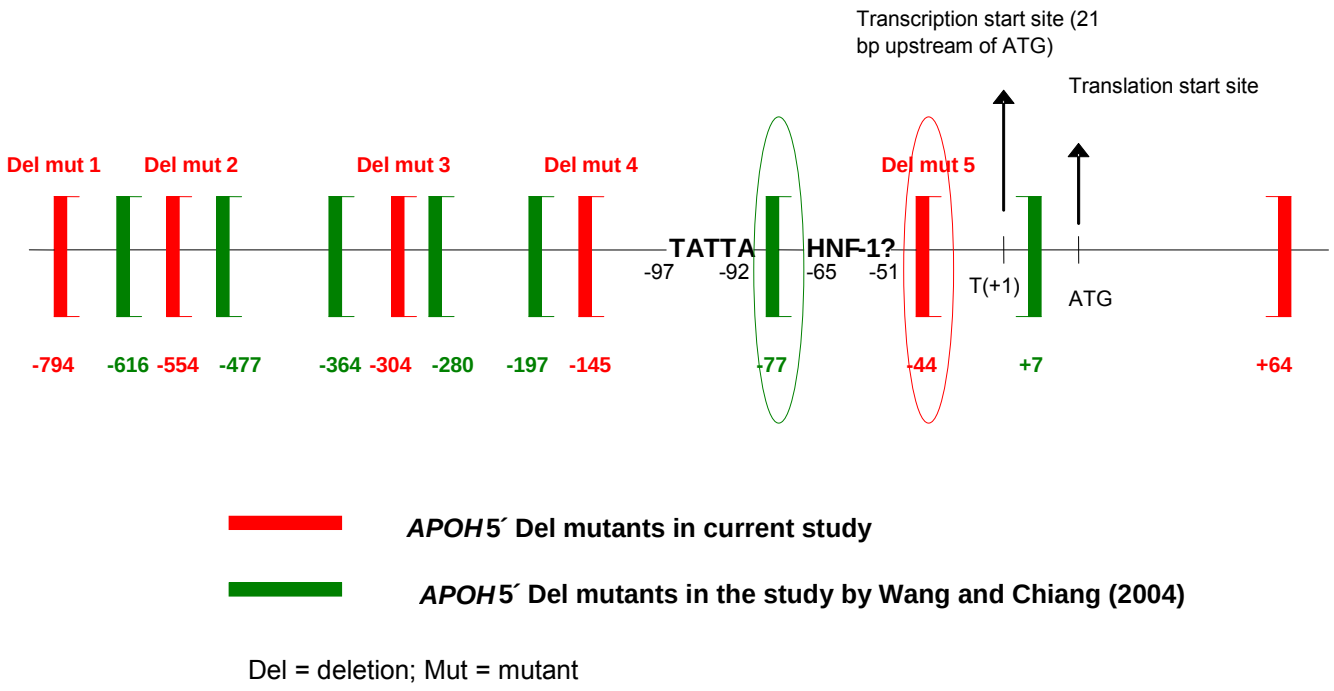


Figure 53. Comparison analysis of *APOH* 5´deletion fragments

Fragments are numbered from the thymidine (T) residue positioned at transcription start site 21 bp upstream of the translaton start site (ATG).

The smallest *APOH* 5´deletion mutant used in this study lacked both the *cis*-elements (TATTA and HNF-1α; red circle, Figure 53), whereas, the smallest deletion mutant used in the previous study (Wang and Chiang, 2004) [83] lacked only the TATTA element (green circle, Figure 53). Despite this difference between fragments, our study replicates the key findings from the prior study (Figure 54) in which the smallest 5´deletion mutant almost completely abolished luciferase activity by ~98% (present study) and ~91% (Wang and Chiang, 2004) [83] in HepG2 cells, emphasizing the vital role of the TATTA *cis*-element in *APOH* transcription.

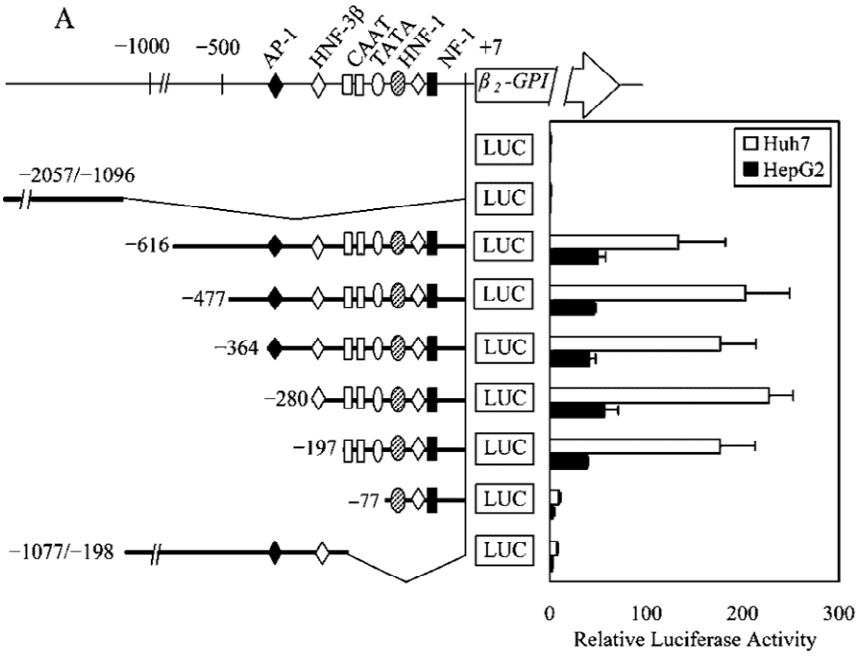


Figure 54. *APOH* 5′deletion analysis
*From Wang and Chiang, 2004, [83]. Luciferase (LUC) reporter gene driven by the human APOH promoter and the relative luciferase activities. The 5′-ends of these constructs were numbered in base pairs relative to the β2 -GPI transcription start site, and the putative cis-elements are indicated at the top. (**A**) The reporter constructs containing sequentially deleted 5′-flanking fragments were cloned and transiently transfected into Huh7 and HepG2 cells. Results are expressed as the means ± S.D. for at least two independent experiments performed in triplicate.*

Potential liver-specific transcription factor–binding sites within the 5'-flanking DNA sequence of the human *APOH* were identified by using MatInspector program from Genomatrix software (http://www.genomatix.de/index.html); [184] which locates matches by comparing DNA sequences with weighted matrix descriptions of functional binding sites, based on the TRANSFAC database (http://www.biobase.de). The results of these searches have been compiled, and the putative sites for transcription factors are displayed in Figures 55 and 56 and Table 56.

```
AGTGGCAGCA CACTCTTCTT ATCATGGAAC ACAATGTTCA TTAGGATCGT 50
                                V$CEBP
                             79---------------93   99-
AATTTAAGAA TCATCTGCCT GACAGATGGA GATTTCTCAA GCTGATGTGT 100 (-1284C>G)
   V$NR2F, V$NF1F,  V$FKHD,  O$VTBP           O$INRE
-------------------------------------137 139-------149
CTCAGGGAAT TTTAGACCTC TGGATATAAA CCAAGATCAC CCAAATGAGA 150
                                  V$CLOX
              V$HNF1 165-----------------------------
      159-----------------175
GCAAGCGGAG GTTATTTATT CAGAGCTTGC TATAGGAAGG GAGGCAGCCA 200 (-1219G>A; 1190G>C)
V$HNF1, V$CLOX, V$NF1F, V$HNF6
-----------------------------------------238     250-
TTAACAGTTG TGTTTGGCAG AGACTCAAAG TCAAAGTCAG ACAGAGGAGT 250
    O$VTBP
----------------266                 286---------------
GGGAAAGCTT TATAATAGAA AAAAGGAAAG GCTTCAGGTG TGCTCCAACG 300 (-1076G>A)
V$CLOX
------308                                        345----
GATTGTTGGT ATGGGGAGGA TGGAAGGAAA CTAACTAGAA GTGAGGTGTC 350 (-1055T>G)

       V$CLOX
------------------367                              398---
CATGTGATTG GTTAGAGAAA TATGTTTGGA TTTCTCTGGT TGGCCTTAAG 400
     V$CLOX
-----------------420

TTGGGCCAAC TGCTATAGAG GTTGTAGGTT TGGCTTTCTA GACCTGTTGC 450
        V$RXRF, V$PERO, V$NR2F, V$FKHD, O$VTBP, O$VTBP,
453-------------------------------------------------
TACAGATGTG TGGGTCAGAG TTCTGTTTTC ACATATGGCC TGGCTATTGT 500
  O$PTBP, O$PTBP
---------------514

CCATTTATAT ATCCTTTTTT TTTTTTTTAG AGCAAATGAT GTAGAAGAGA 550
                  V$EKLF
           564----------------580

ACTAGGGAAT CAAAGACAAC CCCCCTTCCG AACCCTCTCA AGCAACAACA 600

TCAGCACTGG CCCATTATCT TATCCTACTC AAGTTTTTTT TTTCTTCTTC 650 (-759A>G; -742delT)
               V$CLOX, V$HNF6, V$CLOX
           662--------------------------689

GAGTCCCAAG TTGTTAATTT CAAATCAATT TTCAAATTCC TGAAACAGAT 700 (-700C>A)
                                V$CLOX, V$NR2F, V$CLOX,
                             727----------------------
CTAGAAACCT GTCTAGACAG ATCCAAGACA TATTAAGAAT GGATGAGGAG 750 (-643T>C; -627A>C)
V$RXRF, V$CLOX, V$PARF, V$CEBP                 V$RXRF
--------------------------775           788----------

GACTTTGTAT TGATCTGACG TAAGAGAAGA TAGAGAATTC AAGGATAGCT 800 (-581A>C)
    V$NR2F
-------------814

CTAAGGTCCT AACTGGAGCT ATAGGAGCTT GCAAGAGAGG ATGTTGAGCT 850

CAGTTTGTAG GGAATTAAAG TTGTAAGTGC CTCCTGGAAG ACATTCTTTG 900
                               O$VTBP,
```

Figure 55. (Continued Below)

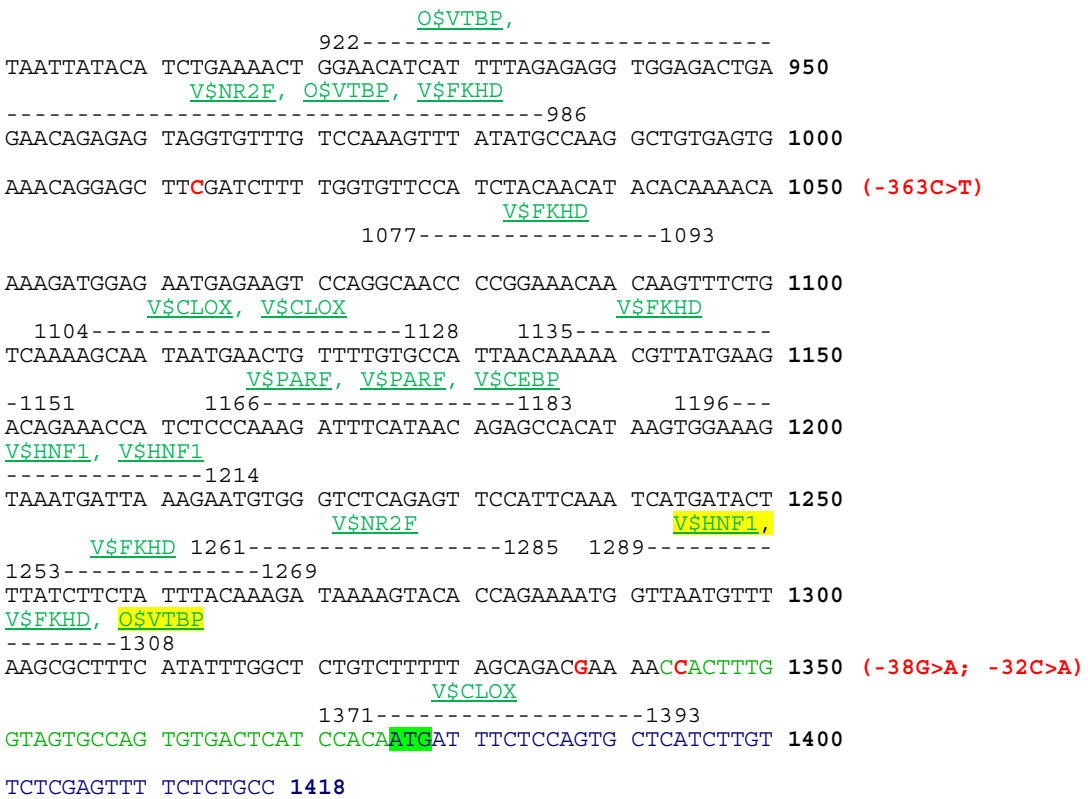


Figure 55. Liver-specific transcription factor binding sites for the *APOH* promoter

As determined by the TRANSFAC and Matinspector databases. The start of the coding region (ATG) is highlighted in green.

Two critical *cis*-factors for *APOH* basal transcription [hepatic nuclear factor1 (HNF1) and vertebrate TATA binding factor (VTBP)]

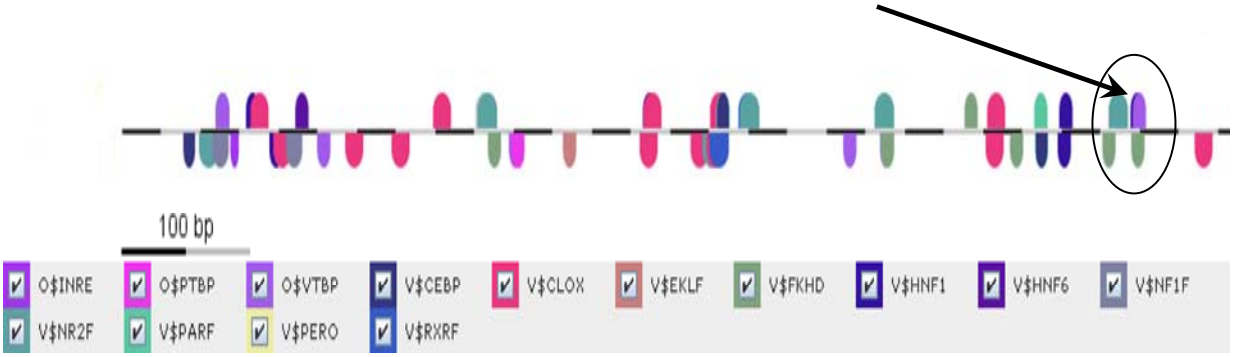


Figure 56. Schematic graphical representation of liver-specific transcription factors
From MatInspecter, Genomatix

Table 56. MatInspector output for liver-specific transcription factors of *APOH* promoter

Family	Further Information	Start pos.	End pos.	Strand	Sequence
V$CEBP	Ccaat/Enhancer Binding Protein	79	93	-	cagcttgaGAAAtct
V$NR2F	Nuclear receptor subfamily 2 factors	99	123	-	tccagaggtctAAAAttccctgaga
V$NF1F	Nuclear factor 1	116	136	-	atcTTGGtttatatccagagg
V$FKHD	Fork head domain factors	120	136	+	tggataTAAAccaagat
O$VTBP	Vertebrate TATA binding protein factor	121	137	+	ggataTAAAccaagatc
O$INRE	Core promoter initiator elements	139	149	-	tcTCATttggg
V$HNF1	Hepatic Nuclear Factor 1	159	175	+	gGTTAtttattcagagc
V$CLOX	CLOX and CLOX homology (CDP) factors	165	187	+	ttattcagagcttgctATAGgaa
V$HNF1	Hepatic Nuclear Factor 1	189	205	-	tGTTAatggctgcctcc
V$CLOX	CLOX and CLOX homology (CDP) factors	194	216	-	ccaaacacaactgttaATGGctg
V$NF1F	Nuclear factor 1	210	230	-	actttgagtctctGCCAaaca
V$HNF6	Onecut homeodomain factor HNF6	222	238	+	actcaaagTCAAagtca
O$VTBP	Vertebrate TATA binding protein factor	250	266	-	tattaTAAAgctttccc
V$CLOX	CLOX and CLOX homology (CDP) factors	286	308	-	ccaaCAATccgttggagcacacc
V$CLOX	CLOX and CLOX homology (CDP) factors	345	367	-	tctctaacCAATcacatggacac
V$CLOX	CLOX and CLOX homology (CDP) factors	398	420	+	agttgggccaactgctATAGagg
V$RXRF	RXR heterodimer binding sites	453	477	+	agatgtgtgggtcagaGTTCtgttt
V$PERO	Peroxisome proliferator-activated receptor	454	476	+	gatgtgtgggtcagAGTTctgtt
V$NR2F	Nuclear receptor subfamily 2 factors	455	479	+	atgtgtgggtcagAGTTctgttttc
V$FKHD	Fork head domain factors	468	484	-	tatgtgaaAACAgaact
O$VTBP	Vertebrate TATA binding protein factor	495	511	-	atataTAAAtggacaat
O$VTBP	Vertebrate TATA binding protein factor	497	513	-	ggataTATAaatggaca
O$PTBP	Plant TATA binding protein factor	498	512	-	gataTATAaatggac
O$PTBP	Plant TATA binding protein factor	500	514	-	aggaTATAtaaatgg
V$EKLF	Basic and erythroid krueppel like factors	564	580	-	tcggaaGGGGggttgtc
V$CLOX	CLOX and CLOX homology (CDP) factors	662	684	-	ttgaaaattGATTtgaaattaac
V$HNF6	Onecut homeodomain factor HNF6	666	682	+	atttcaaaTCAAttttc
V$CLOX	CLOX and CLOX homology (CDP) factors	667	689	+	tttcaaATCAattttcaaattcc
V$CLOX	CLOX and CLOX homology (CDP) factors	727	749	-	ctcctcATCCattcttaatatgt
V$NR2F	Nuclear receptor subfamily 2 factors	742	766	-	cagatcaatacAAAGtcctcctcat
V$CLOX	CLOX and CLOX homology (CDP) factors	747	769	-	cgtcagATCAatacaaagtcctc
V$RXRF	RXR heterodimer binding sites	751	775	-	ctcttaCGTCagatcaatacaaagt
V$CLOX	CLOX and CLOX homology (CDP) factors	752	774	+	ctttgtattGATCtgacgtaaga
V$PARF	PAR/bZIP family	760	776	+	tgatctgacGTAAgaga
V$CEBP	Ccaat/Enhancer Binding Protein	761	775	+	gatctgacGTAAgag
V$RXRF	RXR heterodimer binding sites	788	812	+	tcaaggataGCTCtaaggtcctaac
V$NR2F	Nuclear receptor subfamily 2 factors	790	814	+	aaggatagctctaAGGTcctaactg
O$VTBP	Vertebrate TATA binding protein factor	922	938	-	ctctcTAAAatgatgtt
V$NR2F	Nuclear receptor subfamily 2 factors	962	986	+	ggtgtttgtcCAAAgtttatatgcc
O$VTBP	Vertebrate TATA binding protein factor	969	985	-	gcataTAAActttggac
V$FKHD	Fork head domain factors	970	986	-	ggcataTAAActttgga
V$FKHD	Fork head domain factors	1077	1093	+	accccggaAACAacaag
V$CLOX	CLOX and CLOX homology (CDP) factors	1104	1126	-	cacaaaacagttcattATTGctt
V$CLOX	CLOX and CLOX homology (CDP) factors	1106	1128	+	gcaaTAATgaactgttttgtgcc
V$FKHD	Fork head domain factors	1135	1151	-	gtcttcATAAcgttttt
V$PARF	PAR/bZIP family	1166	1182	+	aaagaTTTCataacaga
V$PARF	PAR/bZIP family	1167	1183	-	ctctgTTATgaaatctt
V$CEBP	Ccaat/Enhancer Binding Protein	1168	1182	-	tctgttatGAAAtct
V$HNF1	Hepatic Nuclear Factor 1	1196	1212	-	cTTTAatcatttacttt
V$HNF1	Hepatic Nuclear Factor 1	1198	1214	+	agtAAATgattaaagaa
V$FKHD	Fork head domain factors	1253	1269	-	tctttgtaAATAgaaga
V$NR2F	Nuclear receptor subfamily 2 factors	1261	1285	+	ttacaaagataAAAGtacaccagaa
V$HNF1	Hepatic Nuclear Factor 1	1289	1305	+	gGTTAatgtttaagcgc
V$FKHD	Fork head domain factors	1290	1306	-	agcgctTAAAcattaac
O$VTBP	Vertebrate TATA binding protein factor	1292	1308	+	taatgttTAAGcgcttt
V$CLOX	CLOX and CLOX homology (CDP) factors	1371	1393	-	tgagcactggagaaATCAttgtg

7.2 FUNCTIONAL CHARACTERIZATION OF *APOH* PROMOTER SNPS

SNPs that are located within coding regions and result in a change in the peptide sequence may be classified as 'damaging' or 'altering' if predicted to be in structurally or functionally important sites of the three dimensional structure of the protein. However, it is less straightforward to predict the functional importance of SNPs within regulatory regions (regulatory SNPs or rSNPs), such as promoters, enhancers, and silencers [185]. Allelic differences in expression of genes due to common polymorphisms in 5' regulatory regions could equally elucidate gene function as the examination of non-synonymous SNPs in coding regions [186]. As many as 35% of promoter SNPs may be of functional significance [163]. There are numerous examples of rSNPs associated with disease susceptibility, including hypercholesterolemia, hyperbilirubinemia, myocardial infarction, acute lung injury , and asthma [185].

In this study we “functionally” validated SNPs in the *APOH* promoter based on two experimental approaches (reporter assays and EMSA). For this purpose, we tested 12 SNPs located within the 1,418 bp of the 5'-flanking region of *APOH* for allele-specific regulatory effects on expression of the luciferase reporter gene and by EMSA for SNPs within TFBS, which could potentially influence the kinetics of binding affinity.

Potential regulatory activities (promoter, enhancer or silencer) of a putative regulatory sequence, and the effect of a particular change caused by alleles of the *APOH* promoter SNPs, were studied using dual-luciferase reporter gene assays (Promega). Fragments of DNA carrying alleles of the *APOH* promoter SNPs were cloned into a promoter-less pGL-3 basic reporter gene vector carrying a weak promoter. Different allelic constructs, together with a control plasmid (pRL_TK; *Renilla* luciferase) used for normalization of the efficiency of transfection, were transiently transfected into COS-1 cell lines. The level of reporter gene expression was evaluated

for both alleles of the SNPs and compared with the expression of the empty promoterless pGL3-basic vector. An increase or decrease in reporter gene expression caused by one of the alleles will help demonstrate that the allele is affecting the activity of the regulatory elements in the promoter region. Out of the 12 SNPs examined three SNPs at positions −1219A, −643C and -32A showed a significantly stronger reduction of luciferase expression (~50%, ~40% and ~36%, respectively) than the wildtype -1219G, -643T, and -32C alleles in COS-1 cells. Although, the 5′- serial deletion of *APOH* promoter identified the basal transcriptional activity restricted to the regions ~160 bp upstream of ATG codon, one cannot exclude the possibility of the functional roles of the -643T>C and -1219G>A SNPs as part of the extended *APOH* promoter transcriptional machinery. The -32C>A SNP is a part of the core *APOH* promoter region (-166 bp upstream from ATG) identified in this study and has been previously shown to play a key role in the transcription initiation process by serving as a site for the binding of transcription factor II D TFIID factor [115].

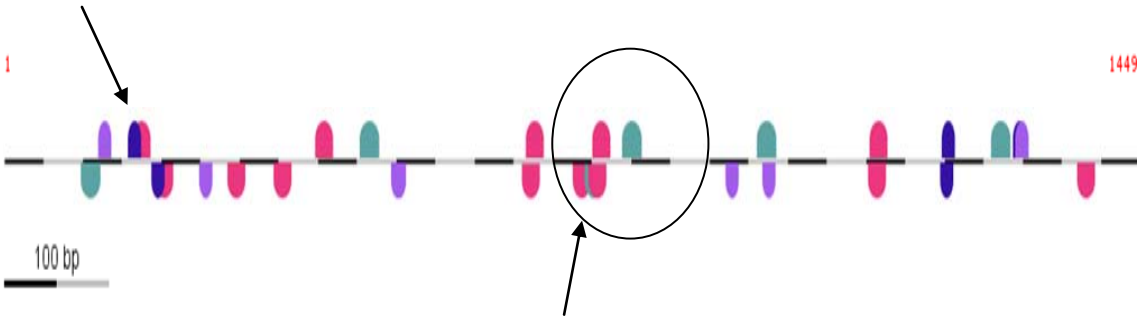
To confirm our hypothesis that the *APOH* promoter SNPs are involved in binding with a transcription factor, we constructed wild type and mutant type oligos for each SNP and examined their binding with nuclear extracts of HepG2 cells. Our EMSA results for five of the SNPs examined (-1284C>G, -1219G>A, -1190G>C, -643T>C and -1076G>A) showed binding of transcription factor(s) for both wild-type and mutant-type oligos for all SNPs with no differential binding for the two alleles.

In silico analysis (Figure 57) using MatInspector program from genomatrix for the prediction of liver-specific TFBS was done for two of the potentially “functional” SNPs, -1219G>A and -643T>C SNPs that showed both a significant effect in the dual-luciferase assay, as well as positive binding to TFs in EMSA using HepG2 nuclear cell extracts. For the -

1219G>A SNP, the results indicated the binding of an important liver-enriched transcription factor, HNF1, adjacent to the polymorphic site which could explain for the functional relevance of this SNP. HNF1 plays a prominent role in regulating genes that are expressed in hepatocytes [187]. The -643T>C SNP region binds to CLOX and CLOX homology CCAAT displacement protein (CDP) factors, that have been previously reported as transcriptional repressors [188]. This could probably explain the decrease in reporter gene expression seen by variant allele at this SNP.

EMSA results previously reported by Mehdi et al. (2003) [116] have revealed that the -32C>A SNP disrupts the binding of crude mouse hepatic nuclear extracts and purified TFIID, which is part of the RNA polymerase II preinitiation complex, indicating its functional role in the transcriptional regulation of *APOH* promoter. However, i*n silico* analysis using MatInspector program for liver-specific factors did not identify any liver-specific transcription factor to bind to the region including the -32C>A SNP; which is also consistent to what is reported in the previous study by Wang and Chiang (2004) [83].

-1219G>A (HNF1-hepatic nuclear factor1)



-643T>C (CLOX and CLOX homology CCAAT displacement protein (CDP) factors; CDP cut-like homeodomain protein, transcriptional repressor)

Figure 57. MatInspector predictions for the potentially "functional" *APOH* promoter SNPs

- Hepatic nuclear factor1
- CLOX and CLOX homology CCAAT displacement protein (CDP) factors
- Vertebrate TATA binding factor
- Fork head domain factors

By combining *in silico* and experimental approaches, this study provides evidence that the polymorphic sites at positions -1219G>A and -643T>C in addition to the previously reported -32C>A SNP [116] in the *APOH* promoter may be functionally important.

7.3 ASSOCIATION OF *APOH* PROMOTER SNPS WITH SLE AND SLE-RELATED PHENOTYPES

Because of the important role of β_2GPI in the production of APA and the observation that β_2GPI-mediated immune response in patients with autoimmune diseases may lead to atherosclerosis [127], it is important to understand the role of *APOH* genetic variation in relation to autoimmune diseases and associated premature CVD. A priori, one might expect that genetic variation in elements which control *APOH* expression (promoter region) can be associated with the disease risk. Promoter sequences are potential sources of polymorphisms affecting gene expression and phenotypic variation [163]. Promoter variants may potentially alter the affinities of existing protein-DNA interactions or recruit new proteins to bind to the DNA, altering the specificity and kinetics of the transcription process. To our knowledge, this is the first study to evaluate the role of *APOH* promoter SNPs in relation to SLE and related phenotypes, including lupus nephritis and subclinical CVD.

Numerous epidemiological studies have implicated differences in gender, race, ethnic/national origin on SLE incidence and prevalence [35]. Studies of racial tendencies showed that SLE is more frequent in more frequent in African Americans than in Caucasians in USA [32]. SLE occurrence is three to four times higher among African-American women compared to Caucasian women. Given the ethnic differences, this study examined the *APOH* promoter SNPs for racial differences between the white (n =799) and black (n = 90) population-based registry of SLE patients in Pittsburgh area. The genotypes of two *APOH* SNPs (-759A>G and -700C>A), in strong LD, significantly differed for both genotype and allele frequency distribution between the two ancestry groups.

Our study revealed a significant association for the -643T>C SNP with SLE risk in the Pittsburgh sample ($P = 0.014$). However, this association was not confirmed in a second relatively small sample from Chicago ($P = 0.919$). The significance level decreased in the combined Pittsburgh + Chicago sample ($P = 0.025$) compared to the Pittsburgh sample despite increase in the sample size and power. This suggests that either the effect of the -643T>C SNP on SLE risk is small, which is difficult to reproduce in all samples or this association is due to chance. The latter assumption was confirmed in the haplotype analysis where one haplotype that carried the defining -643C allele (H3 haplotype in Table 16) was not associated with SLE risk. On the other hand, our six-site haplotype analysis yielded a significant difference in overall haplotype distribution between SLE cases and controls ($P = 0.009$). The haplotype (H7 haplotype) with the most striking difference between cases and controls ($P = 1.21 \times 10^{-5}$) had a frequency of less than 2% in the total sample suggesting that only a small number of individuals were predicted to carry this haplotype. Notably, while no example of this haplotype was observed in 888 control chromosomes based on EH program, 4.5% of the patients carried this haplotype. H7 haplotype was not defined by a particular allele, although it carried the minor alleles for three *APOH* promoter SNPs (-643T>C, -1190G>C and -759A>G). The minor alleles at these three SNPs do not appear to be causative, as the presence of the -643C allele in H3 haplotype alone or the presence of the -1190C and -759G alleles together in H2 and H5 haplotypes did not demonstrate significant association. It appears that the simultaneous presence of these three alleles affect the risk of SLE in an additive fashion as demonstrated by the H7 haplotype. The apparently additive effects of these alleles were also obvious in the gene expression assay where the H7 haplotype was associated with greatest effect on luciferase activity. Alternatively, the unique H7 haplotype may be a marker for a functional variant present

in *APOH* or a nearby gene. Sequencing of the individuals who carry the haplotype H7 may help to identify putative functional variant(s).

The -643T>C SNP showed convincing association with the presence of carotid plaque among SLE patients as it was found to be associated in two independent samples from Pittsburgh and Chicago. In the combined Pittsburgh + Chicago SLE sample the association was more significant than observed in individual samples from each site (adjusted OR = 0.35, $P = 0.002$). The accumulation of oxLDL is believed to initiate the process of atherosclerosis and β_2GPI is known to inhibit the uptake of oxLDL by macrophages *in vitro* while it promotes the influx of oxLDL in the presence of APA. Several lines of evidence suggest that autoimmune vascular inflammation and oxidative stress may promote the formation of oxLDL/ β_2GPI complexes in the arterial wall as seen in SLE and APS patients (Matsuura et al. 2006). Since many SLE patients are positive for APA, it is likely that low β_2GPI expression associated with the -643C allele may retard the influx of oxLDL to macrophages in the presence of APA and thus provide protection against plaque formation (Figure 58). Alternatively, there may be another mechanism underlying the effect of -643C allele on carotid plaque formation or it may simply be in strong LD with another variant, especially given that another *APOH* promoter variant (-32A allele), which is also associated with low *APOH* expression, was associated with increased risk for carotid plaque after adjusting for covariates (OR = 2.63, $P = 0.031$). Additional studies in large data sets may help to delineate the role of *APOH* promoter SNPs in relation to carotid plaque formation.

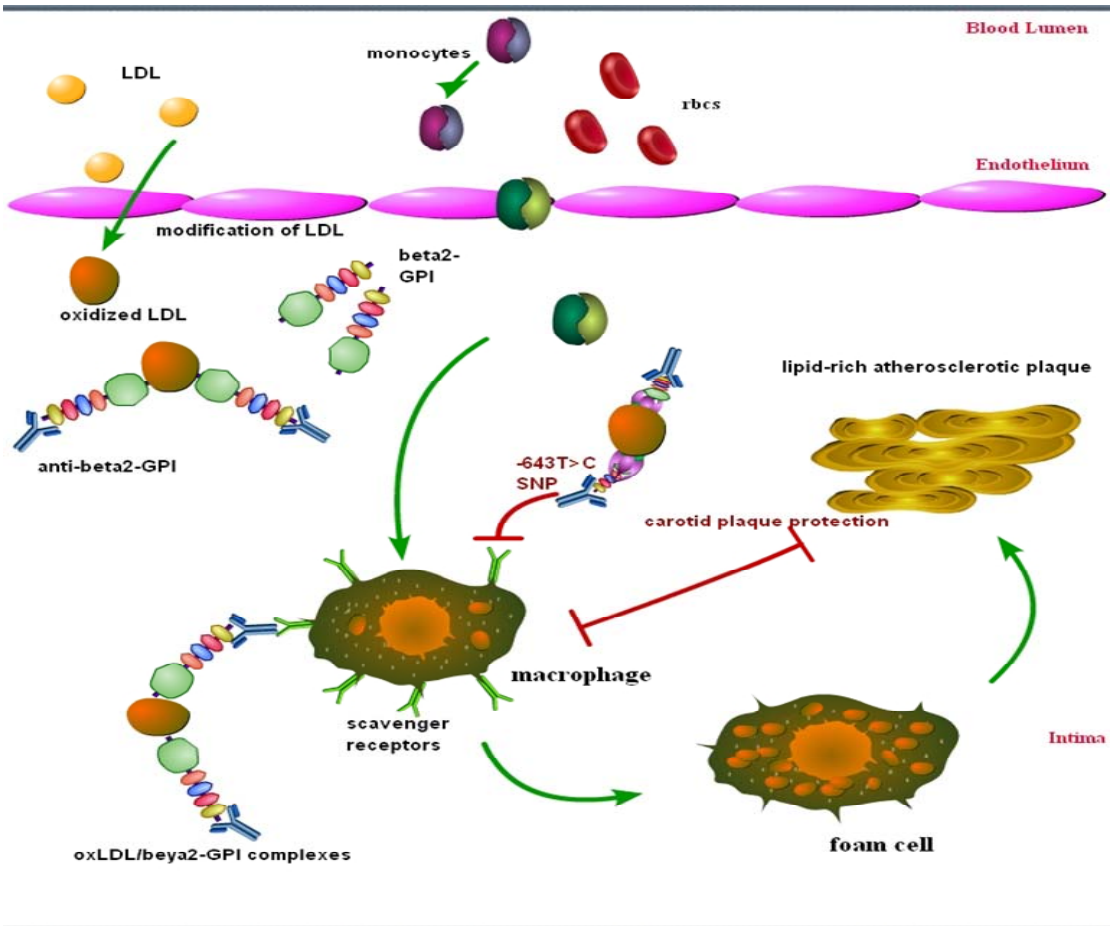


Figure 58. Proposed mechanism of *APOH* -643T>C SNP and carotid plaque protection

Renal disease is a major cause of morbidity in SLE patients. Some studies reported an increased production of APA in patients with Lupus nephritis, including anti-β_2GPI and anti-oxLDL [157, 189, 190]. We found that -1219G>A SNP may potentially affect the lupus nephritis risk both in single-site (P = 0.019) and in multiple regression (P = 0.014) with a age-adjusted OR = 0.36, P = 0.016 and this effect seems to be independent from APA occurrence given that this SNP did not show significant association with any of the APA examined in our sample. Rather, we found significant association of the -38G>A SNP with the occurrence of APA ($P = 4.57 \times 10^{-4}$), aCL (P = 0.018), LAC (P = 0.001) and anti-β_2GPI (P = 0.002) in SLE cases but not in controls using multiple regression under dominant model. It is possible that the -38G>A SNP

may be interacting with other factors that are involved in SLE etiopathogenesis to show its effect on APA in an autoimmune background. It is reasonable to expect that some factors would specifically show their effects on APA in association with disease status, thus contributing to the increased prevalence of APA in patients with autoimmune diseases as compared to the general population. Alternatively, the *APOH* variants may have modest effects on the occurrence of APA which may be difficult to reproduce in all samples. Finally, given the very low MAF of -38G>A SNP, we cannot exclude the possibility of its spurious association.

The power for most of the examined SNPs in this study was adequate to detect SLE disease- related risk. For four SNPs (-1190G>C, -759 A>G, - 700C>A, -643T>C), we had 80% power to detect ORs between 1.50 and 1.55. For the -1219G>A and -32C>A SNPs, we had 80% power to detect an OR of 1.61 and 1.75, respectively. The power for the less common SNPs (-1284C>G and -38G>A) with MAF < 0.05 was low.

7.4 ASSOCIATION OF *APOH* PROMOTER SNPS WITH PLASMA β_2GPI LEVELS

The regulatory promoter and expression of *APOH* at both the transcriptional and translational levels have not been fully characterized. The purpose of this study, in addition to, characterizing the basal *APOH* promoter and its functional variants, was also to establish the links between *APOH* promoter activity, gene expression and plasma β_2GPI levels that were available for a subgroup of the Pittsburgh white population (SLE cases, n = 241; and controls, n = 206). Since there was no observed difference between SLE patients and control subjects concerning both the mean and the distribution of the plasma β_2GPI levels, all analyses were done using the pooled

data combining both SLE patients and control subjects adjusted for disease status, age, BMI, smoking covariates. In univariate analysis, only the previously reported -32C>A *APOH* promoter SNP showed a significant effect after adjustment for covariates. None of the other *APOH* promoter SNPs used in this study had a significant effect on plasma β_2GPI levels. Using genetic association analysis and conventional genotype-phenotype analysis this study suggests a role for the -643T>C polymorphism in protection from formation of carotid plaque and therefore autoimmune-mediated atherosclerosis in SLE patients and also for the -1219G>A SNP having a moderate effect on lupus nephritis. Functional role for the two SNPs was established using promoter reporter technology and EMSA. Having demonstrated that the two polymorphisms do have a functional effect on gene expression, there are legitimate grounds to question whether these polymorphisms could have an effect on plasma β_2GPI levels. Although *in vitro* luciferase assays measuring promoter activity suggest that the two polymorphisms show an effect on gene expression, this may not entirely be a true reflection of the complexity of regulation that occurs *in vivo*. The regulation of human gene expression is a critical, highly coordinated, and complex process. The core promoter is generally within 50 bp of the transcription start site (TSS), where the preinitiation complex forms and the general transcription machinery assembles. The extended promoter can contain specific regulatory sequences that control spatial and temporal expression of the downstream gene. The transcription machinery, which consists of interconnected co-regulatory protein complexes in a regulatory network, is responsible for mRNA synthesis from a given promoter. Control of gene regulation could occur at various stages, including level of transcription, post-transcriptional regulation, alternative splicing, translation, post-translational modifications and secretion of β_2GPI, all of which may have an effect on the quantitative measure of plasma β_2GPI levels. Alternatively, it is also possible that a change in promoter

activity does not necessarily results in a quantitative change at protein level. Whether the *APOH* promoter SNPs (-643T>C and -1219G>A) could influence the promoter activity by either the former or latter methods is beyond the scope of *in vitro* experiments. Further studies will be needed to explore the mechanism for these associations. *APOH* promoter SNPs explain a small proportion of the variance in *APOH* expression, thus the ability of these SNPs to influence plasma β_2GPI levels may be obscured by the strong effects of other factors (other genetic loci which are in strong LD with the promoter SNPs and other regulatory factors that affect *in vivo* gene expression) in aggregate. However, given the reporter gene expression data on promoter activity and EMSA results indicating the binding to transcription factors , there is clearly a functional effect of the two polymorphisms on *APOH* regulation that are worthy of further investigation.

Haplotype analysis including *APOH* promoter SNPs and previously known coding SNPs affecting plasma β_2GPI levels (Cys306Gly and Trp316Ser) gave us no new insights into determining the genetic basis of plasma β_2GPI levels. The significant haplotypes were defined predominantly by the minor alleles at the coding SNPs, which are already known to have an effect. Consistent with the univariate data, none of the haplotypes defined by the minor alleles at *APOH* promoter SNPs reached significance. Although the -32C>A SNP was significant in the univariate analysis, the individual haplotype (H7) harboring the minor allele -32A was not significant, indicating that the effect of the -32C>A SNP is dependent upon the presence of the Trp316Ser coding SNP, which is in strong LD with the -32C>A SNP as shown in haplotype (H5). Three-site haplotype analysis with only the *APOH* promoter functionally relevant SNPs (-643T>C, -1219G>A and -32C>A) showed a highly significant effect for haplotype (H3) defined by the -32A allele and also a moderate effect for the -1219A allele (H2). Another questionable

mechanism for the lack of association of *APOH* promoter SNPs on plasma β_2GPI levels in this study is the modified capture-ELISA method that was used to determine the plasma β_2GPI levels, wherein, the analyzed antibodies could have been targeted against only a small number of the antigenic sites in β_2GPI.

Therefore, given both the limitation of the method and also the small sample size, further studies are warranted in larger cohorts using improvised methods (antibody titers are measured against other/additional β_2GPI) that will help better to delineate the molecular basis of plasma β_2GPI levels.

8.0 CONCLUSIONS

The present study has shown that the *APOH* promoter is characterized by the presence of two key *cis*-elements (atypical TATTA box and hepatic nuclear factor 1 (HNF-1). Although further studies are required to better define the molecular mechanisms controlling the specific expression of the *APOH*, our findings are in agreement with a previous study (Wang and Chiang, 2004) [83]suggesting that the region encompassing ~160 bp upstream of the ATG codon contain all the elements necessary to confer the basal tissue-specific transcriptional regulation of the *APOH*.

In vitro evidence revealed functional relevance for three of the *APOH* promoter SNPs at positions -1219G>A, -643T>C and -32C>A that resulted in lower promoter activity (51%, 40% and 37%, respectively) as compared to the respective wild-type alleles. This conclusion was further supported by using EMSA that demonstrated HepG2 nuclear protein(s) bind to the elements located in the regions of the three SNPs (-1219G>A, -643T>C and -32C>A) of the *APOH* promoter. Competition assays reflected on the relative stability and specificity of the formed DNA-protein complex.

We present evidence that individual *APOH* promoter SNPs as well as haplotypes may be involved in the etiology of SLE and especially the risk for autoimmune-mediated CVD. Our results merit further investigation of *APOH* in independent large SLE case-control samples with subclinical phenotype information.

Except the -32C>A SNP, none of the *APOH* promoter SNPs showed a significant effect on plasma β_2GPI levels in univariate analysis. Three-site haplotype analysis (-643T>C, -1219G>A and -32C>A SNPs) showed a highly significant effect for haplotype defined by the -32A allele and also a moderate effect for the -1219A allele. Molecular characterization of the *APOH* promoter SNP haplotypes will help to further delineate the molecular basis of plasma β_2GPI levels and to understand the functional role of *APOH* in various pathological pathways, thereby, contributing to future therapeutic strategies. Further confirmation, in additional studies and in-depth molecular experiments, are needed to determine the functional basis for these effects.

9.0 PUBLIC HEALTH SIGNIFICANCE

With cardiovascular-related disorders accounting for the highest mortality rates in the world, affecting the quantity and quality of life of patients and creating an economic burden of prolonged therapeutic intervention, there is great significance in understanding the cellular and molecular alterations that influence the progression of these pathologies. The turn of the 21st century is considered in medicine to mark the beginning of a "Golden Age" of American cardiovascular medicine and health. Despite the pharmacological and technological advances, CVD is still by far the leading cause of death and disability in the United States. There still remain several challenges in understanding the CVD epidemic burden.

Given the strong evidence for genetic factors in affecting SLE susceptibility and high mortality rates of CVD seen in SLE patients, identifying genetic variants will benefit patients through improved clinical management and help develop better preventive strategies.

This is an important study that provides evidence for individual *APOH* promoter SNPs as well as haplotypes may be involved in the etiology of SLE and especially the risk for autoimmune-mediated CVD. Systemic inflammation serves as a pivotal link between cardiovascular events and autoimmune diseases such as SLE and APS. One of the highlights of this research is the significant association of an *APOH* promoter variant -643T>C with autoimmune-mediated atherosclerosis. This study provides a mechanistic framework to better understand the pathogenesis involved in autoimmune-mediated atherosclerosis that will

aid in substantiating the current existing knowledge about the CVD epidemic. The clinical benefits of this knowledge will help in formulating newer therapeutic strategies using anti-inflammatory drugs that will allow for a better control of inflammation, individual risk assessment and treatment that may decrease the adverse consequences of accelerated atherosclerosis in this SLE patient population.

To gain a better understanding in the molecular mechanisms controlling the transcriptional regulation of human *APOH* expression that will help to delineate the molecular and functional role of β_2GPI implicated in several physiologic pathways, including lipid metabolism, coagulation, and the production of antiphospholipid antibodies, this study focuses on the functional characterization of *APOH* promoter variants and its putative *cis*-regulatory elements present in the cloned 1.4-kb genomic fragment of the 5′-flanking region of human *APOH.*

The primary impetus of this research project is to stimulate more and better research directed towards the illumination of the molecular and functional role of β_2GPI in normal health and in the pathogenesis of autoimmune diseases such as SLE.

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