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FEBS J. Author manuscript; available in PMC 2011 February 1.

Published in final edited form as:
*FEBS J.* 2010 February ; 277(4): 951–963. doi:10.1111/j.1742-4658.2009.07538.x.

# Functional and genetic characterization of the promoter region of apolipoprotein H (*β2*-glycoprotein-I)

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## Abstract

This study characterized the human apolipoprotein H (*APOH*, a.k.a. $\beta_2$-glycoprotein I) promoter and its variants by *in vitro* functional experiments and investigated their relation with human plasma $\beta_2$GPI levels. We examined the individual effects of 12 *APOH* promoter SNPs in the 5' flanking region of *APOH* (~1.4 kb) on luciferase activity in COS-1 cells and HepG2 cells and their impact on plasma $\beta_2$GPI levels in 799 U.S. Whites, the DNA-binding properties of *APOH* promoter using electrophoretic mobility shift assay (EMSA) in HepG2 cells, the effects of serial deletion analysis of *APOH* 5' flanking region in COS-1 and HepG2 cells, and cross-species conservation of the *APOH* promoter sequence. The variant alleles of three SNPs (−1219G>A, −643T>C and −32C>A) showed significantly lower luciferase expression (51%, 40% and 37%, respectively) as compared to the wild-type allele. EMSA demonstrated that these three variants specifically bind with protein(s) from HepG2 cell nuclear extracts. Three-site haplotype analysis (−1219G>A, −643T>C, and −32C>A) revealed one haplotype carrying −32A (allele frequency = 0.075) to be significantly associated with decreased plasma $\beta_2$GPI levels ($P < 0.001$). Deletion analysis localized the core *APOH* promoter to ~160 bp upstream of ATG codon with the presence of critical *cis*-acting elements between −166 and −65. Cross-species conservation analysis of the *APOH* promoters of 7 species indicated that basic promoter elements are highly conserved across species. In conclusion, we have characterized the functional promoter of *APOH* and identified functional variants that affect the transcriptional activity of the *APOH* promoter.

### Keywords

APOH; $\beta_2$-glycoprotein I; promoter; polymorphisms; association

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**Supporting information**

Table S1 List of liver-specific transcription factors for *APOH* promoter (Matinspector)

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## Introduction

Human apolipoprotein H (APOH), also known as $\beta_2$-glycoprotein I ($\beta_2$GPI) (in this study, we will use *APOH* to refer to the gene as used in human genome databases and $\beta_2$GPI to refer to the protein as commonly used in the rheumatology literature) is a major autoantigen recognized by predominant antiphopholipid antibodies (APA) found in sera of many autoimmune diseases such as primary antiphospholipid syndrome (PAPS) and systemic lupus erythematosus (SLE) [1,2]. *APOH* spans 18 kilobases (kb) on chromosome 17q23–24 [3] and encodes for a mature protein of 326 amino acid (aa) residues. $\beta_2$GPI is a 50-kDa single chain plasma glycoprotein exhibiting internal homology comprised of four contiguous homologous regions of about 60 aa residues, and an additional variable fifth C-terminal domain. The variable configuration of the fifth domain is essential for the binding of $\beta_2$GPI to anionic phospholipids [4–6]. Primer extensions determined alternate transcription start sites (TSSs) at 31 base pairs (bp) and 21 bp upstream of the *APOH* translation start codon [3]. TSS 31 bp upstream agreed completely with the consensus for an initiator element (*Inr*) known to sustain transcription initiation. Previously [7], an atypical TATA box and HNF-1α *cis*-elements have been identified to be critical for *APOH* cell type-specific transcriptional regulation leading to differential expression of *APOH* in humans.

$\beta_2$GPI is primarily expressed in the liver and sporadically in intestinal cell lines and tissues [8]. The plasma concentration of $\beta_2$GPI is approximately 20 mg/dL of which a small portion is bound to lipoproteins and the rest exists in lipid free form [9–11]. There is a wide range of interindividual variation in $\beta_2$GPI 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 African Americans [12], which may have clinical relevance in $\beta_2$GPI -related pathways. Family and heritability data have provided strong support for the genetic basis of $\beta_2$GPI plasma variation but the exact molecular basis of this variation remains largely unknown. $\beta_2$GPI is suggested to regulate thrombin inactivation by heparin cofactor II [13] and thus variation in plasma $\beta_2$GPI may affect prothrombic tendency in PAPs patients. Thus, it is important to determine the molecular basis of $\beta_2$GPI plasma variation. Previously we have shown that two SNPs in coding regions (Cys306Gly, Trp316Ser) [12,14] and one SNP in the promoter (−32 C > A) [15] region of *APOH* have significant impact on $\beta_2$GPI plasma variation. Since then we have characterized complete DNA sequence variation in *APOH* and identified ~ 150 SNPs, including 13 SNPs and 1 deletion (−742delT) in the 5´-region [16].

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. Given the importance of promoters in harboring functionally relevant SNPs that regulate gene expression and phenotypic variation, it is important to examine the role of promoter SNPs in relation to disease, gene expression and corresponding plasma levels. Recently we have reported associations of *APOH* promoter SNPs with SLE risk and carotid plaque formation in SLE patients [17].

The objective of this study was: 1) to characterize a ~ 1.4 kb (1,418 bp) genomic fragment in the 5-region of human *APOH* to identify the functional promoter; 2) to examine the impact of all 13 reported *APOH* promoter SNPs in Caucasians (−1284C>G, −1219G>A, −1190G>C, −759 A>G, −700C>A, −643T>C, −38G>A, and −32C>A) and African Americans (−1076G>A, −1055T>G, −627A>C, −581A>C and −363C>T) on *APOH* gene expression; 3) to determine the association of 8 promoter SNPs in Caucasians on $\beta_2$GPI levels among U.S. Whites, and (4) to determine the cross-species conservation of the *APOH* promoter sequence.

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## Results

### Identification and characterization of the *APOH* promoter region

In order to localize the active promoter region and to identify regions that are important for regulation of the human *APOH* expression, 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 (del) constructs containing 5'- truncated fragments of *APOH* promoter fused upstream to a promoterless firefly luciferase (*Luc*) gene of the pGL3-Basic reporter vector. The sequence of each construct was verified by sequencing (data not shown). Figure 1A shows expression of deletion mutants in COS-1 cells. 5′ deletions of the promoter sequence to −815 (Del mutant 1, −815/+43), and −575 (Del mutant 2, −575/+43) increased promoter activity slightly compared to the wild-type, but the difference was not significant (wild-type *vs*. Del mutant 1; $P = 0.260$, wild-type *vs.* Del mutant 2; $P = 0.135$). Successive removal of nucleotides from −575 (Del mutant 2, −575/+43), to −325 (Del mutant 3, −325/+43), enhanced promoter activity appreciably (wild-type *vs.* Del mutant 3; $P = 0.019$), suggesting the possibility of negative regulatory elements within the −575/−325 regions. The Del mutant 3 construct (−325/+43) conferred maximum luciferase activity in COS-1 cells. A slight decrease in promoter activity was observed after further deletion of sequence from −325 to −166 (Del mutant 4, −166/+43; $P = 0.04$). However, when the sequence from −166 to −65 was removed (Del 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 between −166 to −65. We replicated the deletion analysis using human HepG2 cell line, since liver is a major site of synthesis of $\beta_2$GPI and found an overall similar trend as seen in COS-1 cells with Del mutant 3 (−325/+43) showing the highest and Del mutant 5 (−65/+43) showing the lowest ($P < 0.001$) promoter activity (Figure 1B). 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. 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 human *APOH* containing key *cis*-acting elements that regulate *APOH* expression.

### Functional characterization of *APOH* promoter SNPs

In order to investigate the differential allele-specific effect on promoter activity, pGL3-Basic-*APOH* promoter constructs harboring individual point mutations for 12 out of 14 *APOH* promoter sequence variants identified earlier [16] (−1284C>G, −1219G>A, −1190G>C, −1076G>A, −1055T>G, −759A>G, −700C>A, −643T>C, −627A>C, −363C>T −38G>A, and −32C>A) were generated. The relative luciferase activity assessed in three independent experiments performed in triplicate for all the above *APOH* promoter SNPs is listed in Table 1. The insertion/deletion polymorphism (−742delT) could not be characterized due to repetitive sequences in the surrounding region. Similarly, the −581A>C mutant construct was not successful.

A total of three SNPs were found to be significantly associated with differential gene expression (36% or higher difference at $P<0.001$), including two previously reported −643T > C [17] and −32C > A [15]. An additional SNP, −1219G>A, showed a significant difference of ~ 51% in luciferase gene expression between wild-type and mutant alleles (Figure 2). EMSA was performed in order to determine whether the *APOH* promoter −1219G>A SNP affects the binding activity of nuclear factors. Upon incubation of radiolabeled oligonucleotides specific for wild-type (−1219G) and mutant (−1219A) alleles with HepG2 nuclear extracts, DNA-protein complexes were observed, indicating the presence of nuclear factor(s) (Figure 3). Competition assays using increasing amounts of unlabeled wild-type oligonucleotides confirmed the specificity of the binding.

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Potential liver-specific transcription factor-binding sites for the three promoter SNPs that showed differential gene expression (−1219 G > A, −643 T > C and −32 C > A) were sought by using MatInspector program from Genomatrix software (http://www.genomatix.de/index.html) [18], which matches by comparing DNA sequences with weighted matrix descriptions of functional binding sites, based on the TRANSFAC database (http://www.biobase.de). Figure 4 shows the locations of these three functional SNPs relative to potential binding sites along with all other SNPs detected in the 5' flanking region. The list of all the predicted transcription factors, including their consensus sequences and specific binding sites is given in the Supplementary table S1. The program identified binding sites for the −1219G > A and −643T > C SNPs (Figure 4). While the binding site for HNF1 was observed adjacent to the −1219G > A SNP site, the −643T > C SNP region showed binding to CLOX and CLOX homology CCAAT displacement protein (CDP) factors. EMSA results previously reported by us [15] 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, *in 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.

In order to determine the cross-species conservation of the *APOH* promoter sequence, we used the ECR Browser (http://ecrbrowser.dcode.org/) to visualize the conservation profile of the 5'-region of *APOH* (1,418 bp; −1375/+43 nucleotides from the translation initiation codon ATG) to identify the Evolutionary Conserved Regions (ECRs). Figure 5 shows the graphical display of the pairwise alignments and comparisons of sequences from 6 other species (monkey, dog, cow, mouse, rat, opossum) to that of human (base genome). Consistent with our deletion analyses, which indicated the presence of critical promoter elements in the region spanning between −166 to −65, the ECR extending from 5'-end of the gene (exon+UTR) to immediately upstream region (~250 bp upstream of the ATG start codon) was highly conserved across all 7 species.

## *APOH* promoter SNPs and plasma $\beta_2$GPI levels

The distribution of plasma $\beta_2$GPI levels showed only a modest difference (17.90 ± 4.15 mg/dl vs. 18.72 ± 4.68; $P = 0.054$) in mean plasma $\beta_2$GPI levels between cases (n = 241) and controls (n = 206), therefore the association analyses were done using the combined case + control cohort data. Stepwise regression analysis revealed that age, BMI and ever smoking were the significant determinants of the plasma $\beta_2$GPI levels. Only −32C>A SNP showed significant associations with the adjusted mean plasma $\beta_2$GPI levels in both single-site ($P < 0.001$) and multiple regression ($P < 0.001$) analyses. Mean plasma $\beta_2$GPI levels were higher in homozygotes of the wild-type allele, CC (mean = 18.62 mg/dL) compared to both the heterozygotes, CA (mean = 16.24 mg/dL) and homozygotes of less common allele, AA (mean = 13.90 mg/dL). Eight-site haplotype analysis including 6 *APOH* promoter SNPs (present in Whites) and 2 coding SNPs identified a total of 11 haplotypes with a frequency of > 1% (Table 2). Since data for plasma $\beta_2$GPI levels was available only for the White population we excluded the five SNPs present in Blacks. Out of the eight SNPs present in Whites, −1284C>G SNP was excluded due to its rare presence (MAF< 0.01) and −700C>A SNP which is in high linkage disequilibrium to −759A>G as shown previously [17]. Three haplotypes (H5, H6, H10) showed significant association with plasma $\beta_2$GPI 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 β2GPI levels. Although the −32C>A SNP was significant in single-site analysis, the other haplotype (H7)

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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 significantly decrease gene expression *in vitro* showed significant impact on plasma $\beta_2$GPI levels. Three-site haplotype analysis (data not shown) with the functionally relevant (based on dual-luciferase and EMSA data) −1219G>A, −643T>C, and −32C>A SNPs were consistent with the individual SNP results. That is, only the haplotype carrying −32A was significantly associated with decreased plasma $\beta_2$GPI levels ($P < 0.001$).

## Discussion

The goals of this study were (i) to clone and characterize a 1,418 bp fragment of the 5´-region of *APOH*, (ii) to functionally characterize the *APOH* promoter SNPs present in the 1,418 bp fragment, (iii) to examine the effect of the *APOH* promoter SNPs on plasma $\beta_2$GPI levels, and (iv) to determine the cross-species conservation of the *APOH* promoter sequence.

To identify regions of the *APOH* promoter that affect its basal transcription, several 5' -promoter deletion mutants were linked to the luciferase reporter gene and assayed. Promoter constructs containing either −1375/+43 (wild-type) or −166/+43 (Del mutant 4) of upstream sequence had similar high levels of basal transcriptional activity when transfected into either COS-1 or HepG2 cell lines. These results indicates that all of the necessary machinery for driving basal *APOH* expression is localized in this −166/+43 sequence. Further deletion from −166 to −65 revealed regions within the *APOH* promoter that are important for its function. This deletion resulted in ~ 60% decrease in transcriptional activity in COS-1 cells and an even more pronounced (~98%) decrease in HepG2 cells, indicating the presence of an activator motif(s) within this sequence. These results are consistent with the previous deletion analysis [7] that identified the proximal promoter region necessary for hepatic-specific *APOH* expression. The smallest *APOH* 5´deletion mutant (−65/+43) used in this study differed from the prior study [7] as it lacked both the critical *cis*-elements (TATTA and *HNF*-1α) identified within this region, whereas the smallest deletion mutant used in the previous study [7] lacked only the TATTA element. Despite this difference, our study replicates the key findings in which the smallest 5´deletion mutant almost completely abolished luciferase activity by ~98% (present study) and ~91% (Wang and Chiang) [7] in HepG2 cells, emphasizing the vital role of the TATTA *cis*-element in *APOH* transcription. Our cross-species conservation analysis of *APOH* promoters from different species indicates that basic promoter elements are highly conserved across the 7 species examined.

About one third of promoter variants exert a functional effect on gene expression [19]. The functional importance of the *APOH* promoter SNPs was predicted by allelic differences in expression of the luciferase reporter gene. 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 of the 14 sequence variants located within the 1,418 bp of the 5'-flanking region of *APOH* for allele-specific regulatory effects on expression of the dual-luciferase reporter gene and by EMSA for SNPs within transcription factor binding sites. Of the 12 SNPs examined, three SNPs at positions −1219G>A, −643T>C and −32C>A showed a significant decrease in luciferase expression (~50%, ~40% and ~36%, respectively) in COS-1 cells. 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) [15]. Although 5´- serial deletion of *APOH* promoter identified the basal transcriptional activity restricted to the region ~160 bp upstream of ATG codon, it does not eliminate the possibility of the functional roles of the −643T>C and −1219G>A SNPs as

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part of the extended *APOH* promoter transcriptional machinery. To further substantiate the functional relevance of the three *APOH* promoter SNPs (−1219G>A, −643T>C, and −32C>A), EMSAs revealed strong *in vitro* protein binding for both wild-type and mutant type oligos for each SNP using nuclear extracts of HepG2 cells. However, no significant differential binding was observed for the two alleles for all SNPs. *In silico* analysis using MatInspector program for the prediction of liver-specific transcription factor binding sites revealed potential binding sites for the −1219G>A and −643T>C SNPs (Fig. 4). Binding of an important liver-enriched transcription factor, *HNF*1, was observed adjacent to the −1219 G > A polymorphic site, which could explain for the functional relevance of this SNP. *HNF*1 plays a prominent role in regulating genes that are expressed in hepatocytes [20]. The −643T>C SNP region binds to CLOX and CLOX homology CCAAT displacement protein (CDP) factors, that have been previously reported as transcriptional repressors [21]. This could probably explain the decrease in reporter gene expression observed by the mutant allele.

In addition to characterizing the basal *APOH* promoter and its functional variants, the effect of the *APOH* promoter SNPs on plasma $\beta_2$GPI levels were examined for a subgroup of the Pittsburgh white population (SLE cases, n = 241; and controls, n = 206). In univariate analysis, only the previously reported −32C>A 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 $\beta_2$GPI levels. Our previous report [17] suggested a role for the −643T>C polymorphism protecting against carotid plaque formation in autoimmune-mediated atherosclerosis in SLE patients and the −1219G>A SNP showed a moderate effect on lupus nephritis. Functional role for the two SNPs was established using promoter gene assays and EMSA. Despite the functional effects of the −1219 G > A and −643 T > C SNPs on gene expression, their lack of association with plasma $\beta_2$GPI levels is interesting. 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, where the preinitiation complex forms and the general transcription machinery assembles [22]. 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 $\beta_2$GPI, all of which may have an effect on the quantitative measure of plasma $\beta_2$GPI levels. Alternatively, it is also possible that a change in promoter activity does not necessarily result in a quantitative change at the 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 $\beta_2$GPI levels may be obscured by the strong effects of other factors (undefined promoter elements 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 possible binding to transcription factors, there is clearly a functional effect of the two polymorphisms on *APOH* regulation that are worthy of further investigation. However, haplotype analysis including *APOH* promoter SNPs alone or in conjunction with previously known coding SNPs affecting plasma $\beta_2$GPI levels (Cys306Gly and Trp316Ser, Table 1)

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gave us no new insights into determining the genetic basis of plasma $\beta_2$GPI levels. The significant haplotypes were defined predominantly by the minor alleles at the coding SNPs, which are already known to have a major effect on $\beta_2$GPI levels. 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 defined by the −32A allele and also a moderate effect for the −1219A allele. Another questionable mechanism for the lack of association of *APOH* promoter SNPs on plasma $\beta_2$GPI levels in this study is the modified capture-ELISA method that was used to determine the plasma $\beta_2$GPI levels, wherein, the analyzed antibodies could have been targeted against only a small number of the antigenic sites in $\beta_2$GPI. Therefore, given both the method and also the small sample size, further studies are warranted in larger cohorts using improvised methods (antibody titers measured against other/additional $\beta_2$GPI sites) that will help better to delineate the molecular basis of plasma $\beta_2$GPI levels.

## Materials and Methods

### Construction of *APOH* promoter luciferase reporter gene vector (wild-type and individual mutant constructs)

A 1,418 bp fragment of the human *APOH* 5´- region (−1375/+43 nucleotides from the translation initiation codon ATG) containing the promoter and the first untranslated exon was polymerase chain reaction (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 13 SNPs (−1284C>G, −1219G>A, −1076G>A, −1055T>G, −1190G>C, −759 A>G, −700C>A, −643T>C, −627A>C, −581A>C, −363C>T, −38G>A, −32C>A) and no deletion at −742 site. The PCR condition consisted of denaturation 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 and transformed into top 10 chemically competent cells (Invitrogen Corporation, Carlsbad, CA). Following transformation, the positive clones were confirmed by sequencing.

Constructs bearing mutant/minor alleles for each *APOH* promoter SNP 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 using the QuickChange II Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

### Construction of *APOH* promoter deletion mutants

A series of 5´- deletion mutants of the ~ 1.4 bp *APOH* promoter fragment were subcloned into a new *Luc* reporter vector (pGL3-Basic). For this purpose, the original wild-type construct carrying the 1,418 bp *APOH* promoter fragment served as a parental template for

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designing PCR primers to amplify several truncated *APOH* promoter fragments. We designed five *APOH* deletion mutant constructs differing in ~ 200 bp between each fragment as follows:

*APOH* Deletion fragment 1 (*APOH* del FR #1): It is the largest (858 bp) of all 5 fragments. 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.

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. The PCR products were gel purified (Qiagen, Valencia, CA) and then digested 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. The ligated product was then transformed into competent *E. coli* followed by screening of recombinant plasmids by colony PCR technique. The positive clones were further confirmed by restriction digestion and DNA sequencing.

## Cell culture, transient transfection and dual-luciferase reporter gene assay

Dual-Luciferase reporter gene assays (Promega, Madison, WI) were performed to measure the *in vitro* promoter activity between wild-type and mutant constructs carrying minor allele at individual SNP sites for each of the *APOH* promoter SNPs. The wild-type and mutant *APOH* promoter constructs along with along the *Renilla* luciferase control vector (pRL-TK) (Promega, Madison, WI) were used to transiently co-transfect COS-1 (African green monkey kidney) from the American Type Culture Collection (ATCC CRL-1650, Rockville, MD) and HepG2 cells (Human hepatocellular liver carcinoma; ATCC HB-8065). COS-1 cells were cultured at 37°C under 5% CO2 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. HepG2 cells were grown in Eagle's minimal essential medium (EMEM, ATCC) supplemented with 10% fetal bovine serum, and penicillin/streptomycin. A day prior to transfection, $1.6 \times 10^5$ cells were seeded in each well of a 12-well plate with 1mL of antibiotic-free DMEM/EMEM media. Transfection was performed by Lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA) as per manufacturer's instructions. After 48 hrs, the cells were lysed and assayed for light outputs using the dual-luciferase reporter system (Promega Corporation, Madison, WI). Firefly and *Renilla* luciferase 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 normalized to the average activity of the promoter-less empty vector to yield data reflecting

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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).

### Electrophoretic mobility shift assay (EMSA)

EMSA was performed for the *APOH* promoter SNPs to analyze the binding of nuclear proteins from HepG2 nuclear extracts. To make double-stranded probes and competitors, equal amounts of complementary oligos (Sigma Genosys, TX and Operon Biotechnologies, AL) corresponding to the wild-type or mutant alleles for each *APOH* SNP 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 [$\gamma$-$^{32}$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 1×, 5×, 20×, 50×, and 100× 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. For setting up of EMSA experimental procedures, an earlier published positive shift assay for the *APOH* promoter SNP −32C>A was reproduced and used as a positive control.

### Subjects

For genetic association of *APOH* promoter SNPs with plasma $\beta_2$GPI levels, we genotyped 345 Caucasian women with SLE from the Pittsburgh Lupus Registry and 454 Caucasian healthy control women from the Central Blood Bank of Pittsburgh by Pyrosequencing. Details regarding the phenotypic characteristics of this lupus case-control cohort along with genetic screening have been published elsewhere [17]. Plasma $\beta_2$GPI levels were determined by the modified capture-ELISA method as described previously [12]. Data for plasma $\beta_2$GPI levels were available only for a subgroup of Caucasian SLE cases (n = 241) and controls (n = 206). This study was approved by the University of Pittsburgh Institutional Review Board and all participants provided written informed consent.

### Statistical analysis

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 Haploview programs to check for individual haplotype associations with plasma $\beta_2$GPI levels. Age, BMI, ever smoking and case-control status were used as covariates. A *P*- value of less than 0.05 was considered as suggestive evidence of association. Student's *t*-test was used to determine the significance of reporter gene expression difference between the wild-type and mutant constructs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This study was supported by the National Heart, Lung, and Blood Institute Grant HL 54900.

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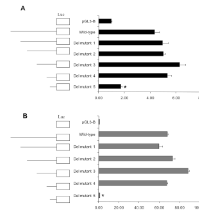


**Figure 1.**

**A**. Dual-luciferase reporter gene expression of *APOH* promoter deletion mutants in COS – 1 cells. Left panel, schematic representation of 5'-deleted fragments of the *APOH* promoter in conjunction with the luciferase gene in pGL3-basic vector. The nucleotides are numbered from the translation start site (ATG). 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 three independent experiments. pGL3-B indicates the promoterless vector. Asterisk (*) indicates that Del mutant 5 has significantly lower luciferase activity than the wild type (P<0.001).

**B**. Dual-luciferase reporter gene expression of *APOH* promoter deletion mutants in HepG2 cells. Left panel, schematic representation of 5'-deleted fragments of the *APOH* promoter in conjunction with the luciferase gene in pGL3-basic vector. The nucleotides are numbered from the translation start site. 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. pGL3-B indicates the promoterless vector. Asterisk (*) indicates that Del mutant 5 has significantly lower luciferase activity than the wild type (P<0.001).



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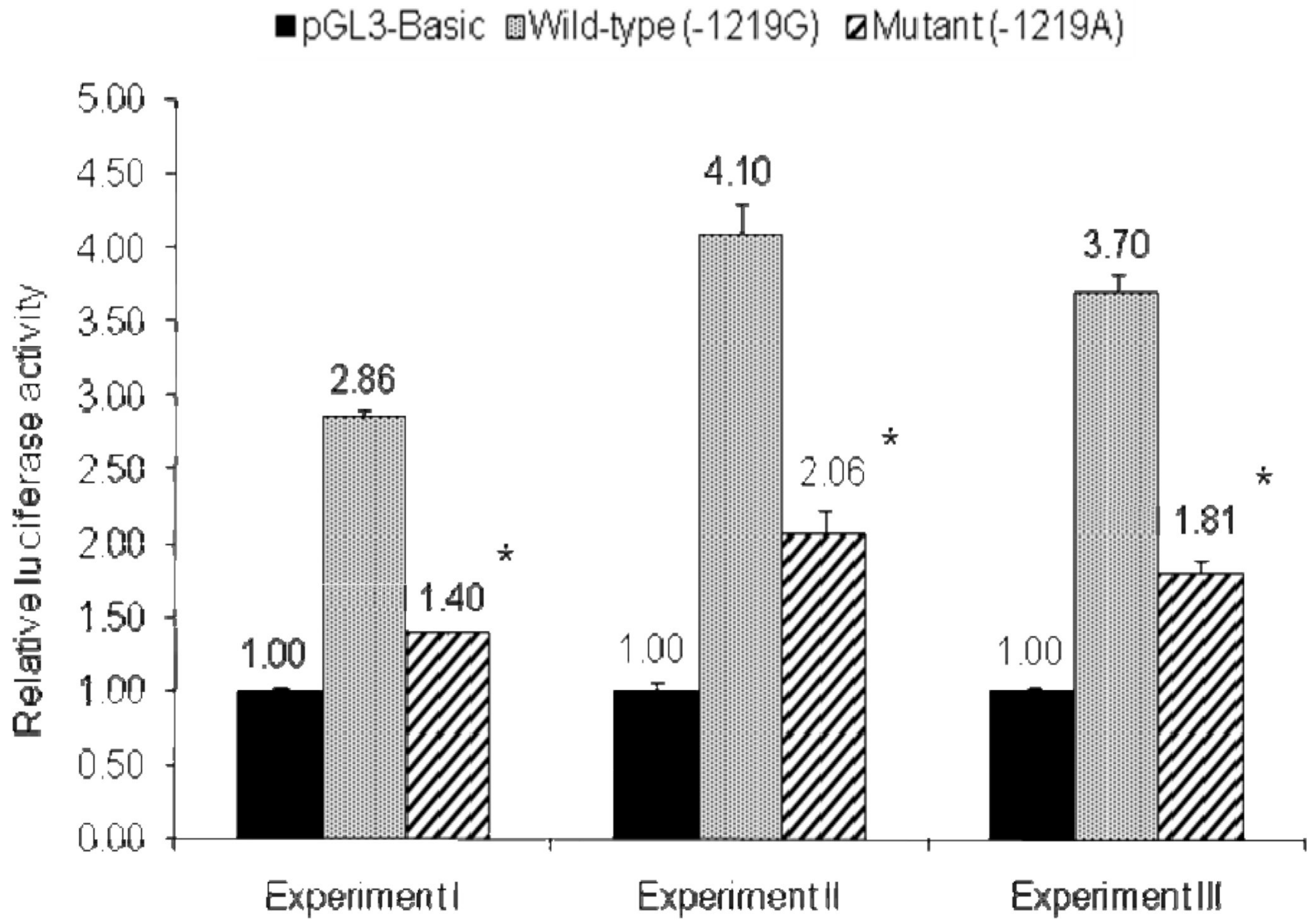


**Figure 2.**
Dual-luciferase reporter gene expression of *APOH* promoter −1219G>A SNP. (* $P < 0.0001$). Results are shown for three independent experiments.

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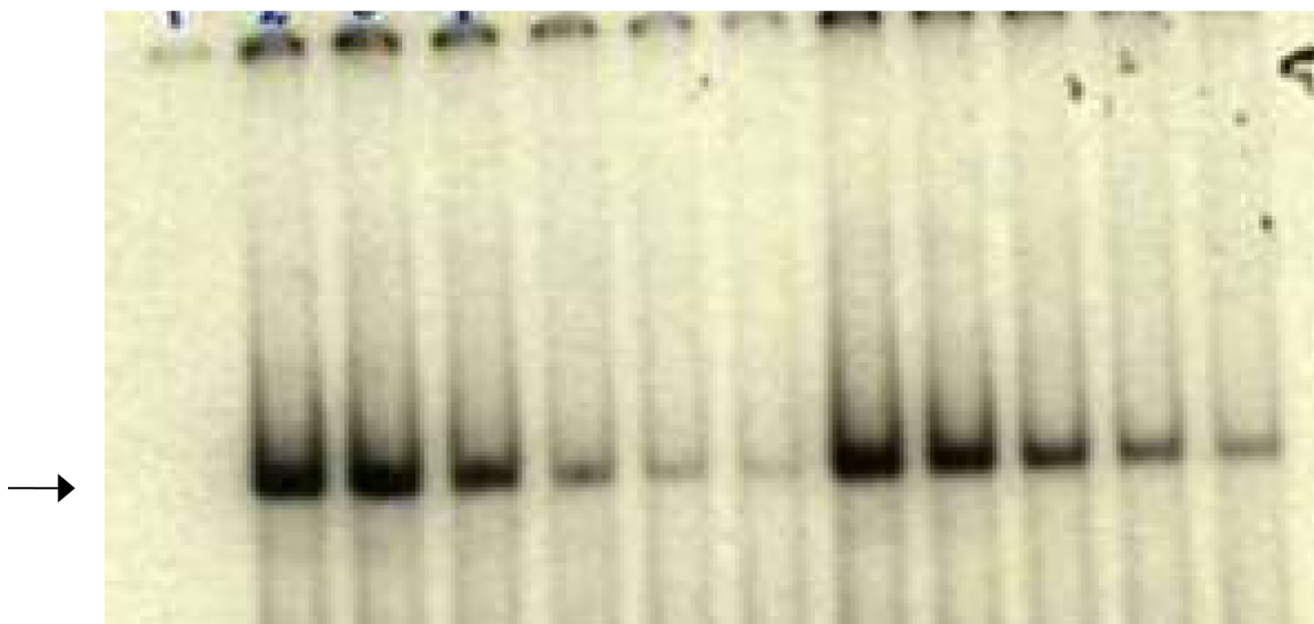


**Figure 3.**

EMSA result for −1219G>A polymorphism. Each sample contains a mixture of 5 μg of nuclear extract derived from human HepG2 cell nuclear extract and 30×mer $^{32}P$-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 (1×, 5×, 20×, 50×, 100×, respectively); lanes 8 to 12 have increasing amounts of A oligo competitor (1×, 5×, 20×, 50×, 100×, respectively).

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

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

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

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

ACTAGGGAAT CAAAGACAAC CCCCCTTCCG AACCCTCTCA AGCAACAACA 600

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

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

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

CTAAGGTCCT AACTGGAGCT ATAGGAGCTT GCAAGAGAGG ATGTTGAGCT 850

CAGTTTGTAG GGAATTAAAG TTGTAAGTGC CTCCTGGAAG ACATTCTTTG 900
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                               VTBP,
                      922-----------------------------
TAATTATACA TCTGAAAACT GGAACATCAT TTTAGAGAGG TGGAGACTGA 950
               NR2F, VTBP, FKHD
--------------------------------------986
GAACAGAGAG TAGGTGTTTG TCCAAAGTTT ATATGCCAAG GCTGTGAGTG 1000

AAACAGGAGC TTCGATCTTT TGGTGTTCCA TCTACAACAT ACACAAAACA 1050 (-363C>T)
                                    FKHD
                         1077-----------------1093

AAAGATGGAG AATGAGAAGT CCAGGCAACC CCGGAAACAA CAAGTTTCTG 1100
            CLOX, CLOX                      FKHD
  1104----------------------1128    1135--------------
TCAAAAGCAA TAATGAACTG TTTTGTGCCA TTAACAAAAA CGTTATGAAG 1150
                   PARF, PARF, CEBP
-1151         1166------------------1183       1196---
ACAGAAACCA TCTCCCAAAG ATTTCATAAC AGAGCCACAT AAGTGGAAAG 1200
  HNF1, HNF1
--------------1214
TAAATGATTA AAGAATGTGG GTCTCAGAGT TCCATTCAAA TCATGATACT 1250
                       V$NR2F                   HNF1,
      V$FKHD 1261------------------1285  1289---------
1253--------------1269
TTATCTTCTA TTTACAAAGA TAAAAGTACA CCAGAAAATG GTTAATGTTT 1300
 FKHD, VTBP
--------1308
AAGCGCTTTC ATATTTGGCT CTGTCTTTTT AGCAGACGAA AACCACTTTG 1350 (-38G>A; -32C>A)
                                CLOX
                      1371-------------------1393
GTAGTGCCAG TGTGACTCAT CCACAATGAT TTCTCCAGTG CTCATCTTGT 1400

TCTCGAGTTT TCTCTGCC 1418
```

**Figure 4.**

MatInspector results for the liver-specific transcription factor binding sites of the *APOH* promoter. The transcription factors are shown in green along with the exact binding position marked by a dotted line and the *APOH* promoter SNPs are in red. The ATG start codon is highlighted in grey.

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**Figure 5.**

ECR Browser conservation profile of the 5'-region of *APOH* (1,418 bp; −1375/+43 nucleotides from the translation initiation codon ATG). Sequence elements of significant length (≥ 100 nucleotides) that are conserved above a certain level of sequence identity (≥ 65%) between the two compared genomes are highlighted as ECRs (pink rectangles at the top of the graphs). The horizontal axis represents positions in the base genome (human) and the vertical axis represents % identity between the base and aligned genomes (monkey, dog, cow, mouse, rat and opossum). The color-coding used by ECR Browser is: blue for coding exons, yellow for UTRs, red for intergenic regions, and green for transposable elements and simple repeats.

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## Table 1

Dual luciferase results of each *APOH* promoter construct in COS−1 cells

| SNPs | Wildtype Allele (Mean ± SD) | Variant Allele (Mean ± SD) | % Decrease | *P* −value |
|---|---|---|---|---|
| −1284C>G | C<br>5.06 ± 0.10<br>5.27 ± 0.06<br>5.55 ± 0.46 | G<br>4.16 ± 0.36<br>4.56 ± 0.34<br>4.64 ± 0.46 | <br>17.79<br>13.47<br>16.40 | <br>0.014<br>0.023<br>0.075 |
| −1219G>A | G<br>2.86 ± 0.05<br>4.10 ± 0.21<br>3.70 ± 0.12 | A<br>1.40 ± 0.01<br>2.06 ± 0.16<br>1.81 ± 0.08 | <br>51.05<br>49.76<br>51.08 | <br>< 0.001<br>< 0.001<br>< 0.001 |
| −1190G>C | G<br>3.01 ± 0.19<br>2.79 ± 0.19<br>3.93 ± 0.50 | C<br>2.16 ± 0.03<br>1.98 ± 0.23<br>2.84 ± 0.08 | <br>28.24<br>29.03<br>27.74 | <br>< 0.01<br>< 0.01<br>< 0.01 |
| −1076G>A | G<br>10.01 ± 0.38<br>10.86 ± 0.53<br>8.40 ± 0.47 | A<br>9.13 ± 0.86<br>9.98 ± 0.60<br>7.74 ± 0.07 | <br>8.79<br>8.10<br>7.86 | <br>0.178<br>0.129<br>0.075 |
| −1055T>G | T<br>4.66 ± 0.18<br>7.66 ± 0.53<br>3.49 ± 0.09 | G<br>3.44 ± 0.17<br>6.13 ± 0.04<br>2.53 ± 0.14 | <br>26.18<br>19.97<br>27.51 | <br>< 0.01<br>< 0.01<br>< 0.01 |
| −759A>G | A<br>5.28 ± 0.29<br>4.82 ± 0.27<br>4.90 ± 0.12 | G<br>4.57 ± 0.11<br>4.27 ± 0.18<br>4.38 ± 0.50 | <br>13.45<br>11.41<br>10.61 | <br>0.017<br>0.042<br>0.155 |
| −700C>A | C<br>4.65 ± 0.05<br>4.90 ± 0.17<br>4.27 ± 1.32 | A<br>4.31 ± 0.10<br>4.58 ± 0.33<br>3.99 ± 0.51 | <br>7.31<br>6.53<br>6.56 | <br>< 0.01<br>0.214<br>0.745 |
| −643T>C | T<br>19.91 ± 1.68<br>5.73 ± 0.07<br>10.79 ± 0.88 | C<br>11.94 ± 0.15<br>3.20 ± 0.24<br>6.26 ± 0.39 | <br>40.03<br>44.15<br>41.98 | <br>0.001<br>< 0.001<br>0.002 |
| −627A>C | A<br>3.09 ± 0.15<br>6.72 ± 0.31<br>5.75 ± 0.23 | C<br>2.85 ± 0.11<br>6.18 ± 0.12<br>5.12 ± 0.01 | <br>7.77<br>8.04<br>10.96 | <br>0.086<br>0.049<br>0.009 |
| −363 C>T | C<br>3.82 ± 0.34<br>2.96 ± 0.49<br>2.88 ± 0.16 | T<br>3.34 ± 0.25<br>2.42 ± 0.40<br>2.44 ± 0.26 | <br>12.57<br>18.24<br>15.28 | <br>0.117<br>0.212<br>0.065 |
| −38G>A | G<br>4.56 ± 0.15<br>3.95 ± 0.20<br>3.81 ± 0.09 | A<br>3.62 ± 0.15<br>3.21 ± 0.17<br>3.16 ± 0.03 | <br>20.61<br>18.73<br>17.06 | <br>0.002<br>0.009<br>< 0.001 |
| −32C>A | C<br>18.91 ± 0.38<br>15.79 ± 1.03<br>16.71 ± 0.92 | A<br>11.92 ± 0.39<br>10.32 ± 0.17<br>10.56 ± 0.06 | <br>36.96<br>34.64<br>36.8 | <br>< 0.001<br>< 0.001<br>< 0.001 |

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## Table 2

Haplotype analysis* of *APOH* SNPs for plasma $\beta_2$GPI levels

| HAPLOTYPE^ | rs8178819 (−1219G>A) | rs3760290 (−1190G>C) | rs817820 (−759A>G) | rs3760292 (−643T>C) | (−38G>A) | rs8178822 (−32C>A) | rs1801689 (Cys306Gly) | rs1801690 (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 | 0.539 |
| 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 (haplo.stats package) for $\beta_2$GPI plasma levels; p-values were calculated from coefficients (coef) and standard errors (se) Regression model included disease, age, BMI, ever smoking

^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$\beta_2$GPI levels in univariate analysis

****Alleles found to decrease gene expression *in vitro* and also associated with low plasma$\beta_2$GPI levels in univariate analysis