



**HAL**  
open science

## The functional interaction on gene expression of SNPs, defining haplotype , and their paradoxical association with plasma triglyceride but not plasma apoAV levels

Jutta Palmen, Andrew J.P. Smith, Birgit Dorfmeister, Wendy Putt, Steve E. Humphries, Philippa J. Talmud

### ► To cite this version:

Jutta Palmen, Andrew J.P. Smith, Birgit Dorfmeister, Wendy Putt, Steve E. Humphries, et al.. The functional interaction on gene expression of SNPs, defining haplotype , and their paradoxical association with plasma triglyceride but not plasma apoAV levels. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2008, 1782 (7-8), pp.447. 10.1016/j.bbadis.2008.03.003 . hal-00501572

**HAL Id: hal-00501572**

**<https://hal.science/hal-00501572>**

Submitted on 12 Jul 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

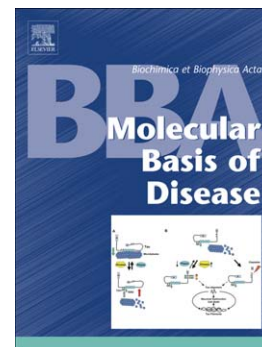
The functional interaction on *in vitro* gene expression of *APOA5* SNPs, defining haplotype *APOA5*\*2, and their paradoxical association with plasma triglyceride but not plasma apoAV levels

Jutta Palmen, Andrew J.P. Smith, Birgit Dorfmeister, Wendy Putt, Steve E. Humphries, Philippa J. Talmud

PII: S0925-4439(08)00074-4  
DOI: doi: [10.1016/j.bbadis.2008.03.003](https://doi.org/10.1016/j.bbadis.2008.03.003)  
Reference: BBADIS 62803

To appear in: *BBA - Molecular Basis of Disease*

Received date: 24 January 2008  
Revised date: 27 February 2008  
Accepted date: 12 March 2008



Please cite this article as: Jutta Palmen, Andrew J.P. Smith, Birgit Dorfmeister, Wendy Putt, Steve E. Humphries, Philippa J. Talmud, The functional interaction on *in vitro* gene expression of *APOA5* SNPs, defining haplotype *APOA5*\*2, and their paradoxical association with plasma triglyceride but not plasma apoAV levels, *BBA - Molecular Basis of Disease* (2008), doi: [10.1016/j.bbadis.2008.03.003](https://doi.org/10.1016/j.bbadis.2008.03.003)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**The functional interaction on *in vitro* gene expression of *APOA5* SNPs,  
defining haplotype *APOA5*\*2, and their paradoxical association with plasma triglyceride but  
not plasma apoAV levels**

Jutta Palmen, Andrew JP Smith, Birgit Dorfmeister, Wendy Putt, Steve E Humphries and  
Philippa J Talmud\*

Division of Cardiovascular Genetics, Department of Medicine, University College London, 5  
University Street, London WC1E 6JF, UK.

**\*Corresponding author**

Prof. Philippa Talmud

Division of Cardiovascular Genetics, Department of Medicine, Rayne Building,

University College London,

5 University Street, London WC1E 6JF, United Kingdom

Tel: +44 – 20-7679-6968, Fax: +44-20-7679-6212. Email: p.talmud@ucl.ac.uk

**Key words:** : *APOA5*\*2, plasma apoAV, -1131T>C, -3A>G, +1891T>C, Kozak sequence,  
plasma TG

**Short Title:** Functional interaction of *APOA5*\*2 SNPs on *APOA5* expression

**ABSTRACT**

Plasma triglyceride (TG) and apoAV levels are reported to be positively correlated, yet SNPs defining haplotype *APOA5*\*2 have consistently shown association with elevated plasma triglyceride (TG) but not plasma apoAV levels. We previously reported that individually -1131T>C, -3A>G and +1891T>C did not influence luciferase activity or *in vitro* translation efficiency. To investigate the combined effect of these SNPs additional constructs were examined. Compared to the wildtype -1131T/-3A/+1891T (TAT), the triple rare allele construct -1131C/-3G/+1891C (CGC) conferred 46% lower luciferase activity ( $p < 0.0001$ ), showing these SNPs are acting co-operatively. Although only these two combinations occur *in vivo*, we experimentally altered the TAT construct one site at a time; -3G (TGT) had the largest effect (94% lower luciferase), with lesser effects from CAT (-77%) and TAC (-70.3%) (all  $p < 0.0001$ ). Deletion constructs excluding one site at a time showed that -3G/1891C (-GC) in combination, compared to -AT, was having the largest effect on luciferase activity (-59%,  $p = 0.055$ ). Using sequence homology and EMSA analysis no transcription factor binding at -1131 or +1891 was identified, though +1891 lies within a putative mRNA stability motif. Taken together, these data identify -3A>G in the Kozak sequence as functional, affecting translation initiation and driving the haplotype effects, whilst showing interaction with +1891T>C and to a lesser extent -1131T>C. A paradox arises since these results predict that *APOA5*\*2 will lead to *reduced* apoAV with concomitant reduced LPL activation or lipoprotein-receptor interaction, resulting higher plasma TG levels. We conclude that *APOA5* expression, and not circulating plasma apoAV levels, are causatively associated with plasma TG levels.

## INTRODUCTION

*APOA5* encodes a 366 amino acid protein, apolipoprotein (apo)AV, expressed in the liver [1] and present on chylomicrons (CM), VLDL and HDL, but not on IDL, LDL or lipoprotein particle-free plasma, suggesting that VLDL containing apoAV is cleared before conversion into these lipoproteins [2]. *APOA5* expression is tightly regulated by a number of transcription factors including member of the nuclear hormone receptor super-family. *APOA5* is highly up-regulated by PPAR $\alpha$  [3], the farnesoid X receptor (FXR) [4] and HNF-4 [5]. *APOA5* is also down-regulated by insulin via the PI3K signalling pathway, which modulates the binding of USF1 to the *APOA5* promoter [6].

Variation in *APOA5* has been associated with effects on plasma triglyceride and to a lesser extent HDL levels [reviewed in [7,8]]. Seven common *APOA5* SNPs define three common haplotypes; the common haplotype, *APOA5*\*1 is defined by all wildtype alleles, *APOA5*\*2 carries rare alleles of -1131T>C (rs rs662799), -3A>G (rs651821), 751A>G (rs2072560) and +1891T>C (rs2266788). Since there is 100% LD amongst these SNPs they only occur in the combinations -1131T/-3A /1891T or -1131C/-3G/1891C. *APOA5*\*3 carries the rare allele of c.56C>G (rs3135506) alone on a wildtype background [9]. Functional studies on c.56C>G showed that the amino substitution S19W in the signal sequence, resulting from this base change, affects signal peptide function with the W19 protein showing 50% less secretion in an *in vitro* assay [10]. By contrast, testing individually the effects of the *APOA5*\*2 SNPs on gene expression using a luciferase reporter gene assay, -1131C, compared to -1131T, showed 15% non-significant lower expression [10], supporting a previous report that this base change was not functional [4], while +1891T>C also showed no effect on *in vitro* expression[10]. The -3A>G occurs in the Kozak sequence of *APOA5*, a motif from -6 to -1 which affects translation [11], but this change also did not significantly affect functionality in an *in vitro*

reticulocyte transcription/translation assay of apoAV. In constructs incorporating sites -1131T>C and -3A>G, luciferase activity was only 16% lower in 1131C/-3G compared to 1131T/-3A (p=0.33) [10].

Alleles -1131C and -3G have consistently been associated with higher plasma TG levels, an effect seen in all ethnic groups [12-16]. While in Caucasians and Indian Asians there is strong linkage disequilibrium with *APOC3* SNPs, within the same gene cluster on chromosome 11, in Afro-Caribbeans, this LD is very low [17]. Thus in a study of Caucasians we proposed that the association of *APOA5* -1131T>C with plasma TG levels could reflect this LD with *APOC3* SNP as suggested by haplotype analysis across the cluster [13]. However, in re-analysing this data [13] using multiple regression analysis, both *APOA5* -1131T>C and *APOC3* 1100C>T remained in the model, suggesting significant and independent effects on TG levels (see supplementary table).

In transgenic and knockout animal studies apoAV levels are inversely related to plasma TG levels. Compared to wildtype mouse the homozygous *APOA5* transgenic has 70% lower plasma TG levels and the *apoA5* knockout 400% higher TG levels [1]. The same relationship was reported in a young boy homozygous for the rare *APOA5* Q148X truncation, with no detectable apoAV and TG levels >50mmol/l [18]. Recently ELISAs for measuring plasma levels of apoAV have been developed [19,20] and several studies reported a counter-intuitive *positive* correlation between plasma apoAV levels and plasma TG levels [21-24]. This is the paradox that we have tried to unravel by undertaking more in-depth functional analyses of *APOA5*\*2 SNPs. In this paper we report the functional interaction between the -1131T>C, -3A>G, and +1891T>C alleles in determining gene expression *in vitro*, and relate this to the relationship between apoAV and TG levels.

## MATERIAL AND METHODS

### Materials

Oligoneucleotides were from MWG-Biotech, UK. Vectors used in the experimentation, pGLbasic and the pGEM-T vector system (TM042), as well as the Dual luciferase assay system (TM040) were all obtained from Promega, UK. BigDye Terminator v3.1 sequencing kit was obtained from Applied Biosystems, UK. Restriction enzymes were obtained from New England Biolabs. Illustra, DNA and gel Band Purification kit, was supplied by GE Healthcare, UK. Lipofectamine 2000 was obtained from Invitrogen, UK. NE-PER Nuclear and Cytoplasmic Reagents Kit and the Chemiluminescent Nucleic Acid Detection Module were from Pierce, USA and the Complete Protease Inhibitor was supplied by Roche, UK.

### Functional studies of variants compromising haplotype *APOA5*\*2

To study the functionality of the -1131T>C SNP in the promoter, the -3A>G SNP in the Kozak sequence as well as the +1891T>C in the 3'UTR, different constructs were made. For the -1131T>C variant, a 665bp fragment was amplified by PCR from DNA from individuals with known -1131TT or -1131CC genotype. The fragment ranged from position -1177 to -512 using the original numbering of Olivier et al. [9]. Oligonucleotides were designed with Acc65I at the 5' end of the forward oligonucleotide and BglII at the 5' end of the reverse oligonucleotide. In addition, CGG and GA tails were introduced at 5' ends of forward and reverse oligonucleotides.

Oligonucleotides were as follows: forward primer, 5' *CGG GGTACC*

*AGAGGCCCTGCGAGTGGAGTT* 3'; reverse primer, 5' *GA AGATCT*

*TGCTCACCTGCTCACGTCTGG* 3'.

The fragment which incorporated both, the -1131 and the -3 sites, ranged from position -1177 to -1 (fragment 1176bp). Oligonucleotides were designed with Acc65I sites at their 5' end of the

forward oligonucleotide and BglIII at the 5' end of the reverse oligonucleotide. Again, CGG and GA tails were introduced at the 5' ends of forward and reverse primers. Oligonucleotides were as follows: forward primer, 5' *CGG GGTACC* AGAGGCCCTGCGAGTGGAGTT 3' and reverse primer -1131T/-3A, 5' *GA AGATCT* TCTGCTCTGAGAAGACAGGTGG 3' and -1131C/-3G, 5' *GA AGATCT* CCTGCTCTGAGAAGACAGGTGG 3'.

For the +1891T>C in the 3' UTR, an 816bp fragment was amplified by PCR from DNA of individuals with known +1891TT or +1891CC genotype. The fragment ranged from +1752 to +256. Oligonucleotides were designed with XbaI sites at the 5' end of the forward oligonucleotide and HpaI at the 5' ends of the reverse oligonucleotide. In addition, GG and CG tails were introduced at the 5' end of forward and reverse oligonucleotides. Oligonucleotides used were as follows: forward primer, 5' - *GC TCTAGA* GGCCCATTCACAGCTCCTTGT 3'; reverse primer, 5' *CGG GTTAAC* TGTGCTTTGGGGATAGTGGTGAGG 3'. The amplification conditions were: 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 53°C for 1 min, 72 °C for 1 min, ending with 72°C for 5 min.

PCR products were TA-cloned into pGEM-T vector system (TM042) following manufacturer's conditions (Promega, UK) and subsequently cloned into pGL3 basic vector (Promega, UK). The constructs were confirmed by sequencing with Applied Biosystems BigDye Terminator v3.1 sequencing kit on an ABI377 automated sequencer. The combined constructs (referred to as TAT for the construct containing all common SNPs or CGC for the construct with all mutant SNPs) were created by cloning the fragments which incorporated the -1131 and the -3 sites into Acc65I and BglIII sites and the +1891 fragment into HpaI and XbaI sites of the pGL3 basic vector (Promega, UK).

For the different combinations (TGT, TAC, CAT, CAC, TGC and CGT ;mutant SNPs underlined) restriction sites were used to cut the desired fragments (see figure 1). The fragments were run on a



1.5 % agarose gels the bands excised and purified using Illustra, DNA and gel Band Purification kit, GE Healthcare, before re-ligation in the desired combination into pGL3 basic and confirmation by sequencing.

To examine further which one of the three SNPs was responsible for potential differences between constructs TAT and CGC, “deletion” constructs were created excluding one site at a time. For this purpose, different constructs were used as templates and one SNP at the time was removed by restriction enzyme digest (for the -1131 fragment, Acc65I and BglII; for the -1131 and -3 fragments, Acc65I and BglII; for the -3 fragment, AatII (naturally occurring site in sequence) and BglII and for the +1891 fragment HpaI and XbaI). The fragments were run on a 1.5 % agarose gels the bands excised and purified using Illustra, DNA and Gel Band Purification kit, GE Healthcare, before re-ligation in the desired combination into pGL3 basic and confirmation by sequencing. Constructs were created as followed: -GC, C-C, -AT and T-T. Results from the TA- and CG- were taken from the previous publication [10].

Luciferase activity was assayed using the Dual luciferase assay system (TM040, Promega, UK). Briefly, Huh7 cells, from a human hepatoma cell line, were used for transient transfection for all variants. Huh7 cells in 96-well format ( $4 \times 10^4$  cells/well) were transiently transfected with 200 ng of the appropriate plasmid or control vector, 1ng of pRL-TK co-transfector, and 0.5  $\mu$ l of Lipofectamine 2000, and the cells lysed 48 h after transfection. For the TAT and CGC constructs, results represent the mean of 30 experiments each performed in sets of 8 repeats per well. The different combinations (TGT, TAC, CAT, CAC, TGC, CGT) represent the mean of 5 experiments, each replicated 8 times per well and the “deletion constructs” represent the mean of 3 experiments, each performed in sets of 8 repeats per well. Statistical analysis was performed using SPSS version 12.0.1 (SPSS, Chicago, IL) and an independent sample T-test.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts were obtained from unstimulated and IL-6-stimulated Huh7 cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, USA) as described by the manufacturer, with the addition of Complete Protease Inhibitor (Roche, UK) to buffers CER I and NER I. EMSA probes for the *APOA5* genomic sequences:

-1131: 5'-GCAAATCT[C/T]ACTTTCGC-3'

-3: 5'-CTCAGAGCAG[G/A]TAATGGCAAG-3'

+1891: 5'-GTCTCCTGCA[C/T]ATCCAGCCTC-3'

Probes were labelled using the Biotin 3'-End DNA Labelling Kit (Pierce, USA) as described by the manufacturer, and annealed to the complementary labelled oligo. Each binding reaction consisted of 2 µl 10x binding buffer (100mM Tris, 500 mM KCl; pH 7.5), 1 µg p[dI-dC], 200 fmol biotin-labelled DNA, made to a total of 20 µl with H<sub>2</sub>O, and incubated at 25°C for 30 min, followed by the addition of 5x loading buffer. Competition reactions were carried out with 30 min incubation on ice, prior to addition of labelled probes, using 100X unlabelled competitor DNA. . Samples were loaded on to a 6% polyacrylamide gel, and electrophoresed for 150 min at 120 V and + 4°C. Gel products were transferred to nylon membrane by Southern blot analysis using the Chemiluminescent Nucleic Acid Detection Module (Pierce, USA).

## RESULTS

### **751A>G (c.172 -43A>G) does not alter function by *in silico* analysis**

The *APOA5*\*2 haplotype is defined by four common SNPs in complete LD, -1131T>C (rs662799), -3A>G (rs651821), +751A>G (rs2072560) and +1891T>C (rs2266788). However, the functional potential of the +751A>G was never previously examined. We used several computer programmes to analyse if this SNP intron 2 (c.172-43) had the potential to affect splicing; [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html) identifies canonical splice sites by neural networking while <http://rulai.cshl.edu/cgiin/tools/ESE3/esefinder.cgi?process=home> identifies potential exonic splicing enhancers (ESE) – SR protein potential binding sites. There were no potential canonical splice sites or ESEs at position c.172 - 43. The possibility of the presence of a transcription factor binding site was also examined using MatInspector (<http://www.genomatix.de/products/MatInspector/index.html>), a transcription factor search engine, but none were identified. For this reason no further experimentation was carried out on this SNP.

### **The combined effect of -1131T>C, -3A>G and +1891T>C on luciferase activity.**

To test whether these SNPs which occur together as a haplotype were acting co-operatively, constructs were made for transient transfection into Huh7 cells, including a 1176bp *APOA5* promoter fragment incorporating positions -1131 and -3, introduced 5' of the luciferase gene, and 816 bp of the *APOA5* 3'UTR, positioned 3' of luciferase, which included site +1891 (Figure 1). As shown in Figure 2a the relative luciferase activity of the rare allele combination -1131C/-3G/+1891C (**CGC**) was 54% lower compared to wildtype -1131T/-3A/+1891T (TAT) ( $p < 0.0001$ ), confirming that the three rare alleles were acting in a co-operative manner.

To identify the relative contribution of the three sites to luciferase activity, constructs corresponding to the six other combinations of the three sites were used to transiently transfect

Huh7 cells. None of these combinations occur *in vivo*. One out of three wildtype sites (TAT) was converted to a rare allele, i.e. leaving two wildtype sites in place, -1131C/ -3A/ +1891T (CAT), -1131T/ -3G/ +1891T (TGT) and -1131T/ -3A/ +1891C (TAC). As shown in Figure 2a, all constructs gave significantly lower luciferase levels than wildtype TAT, but TGT had the largest effect; CAT (-76.7%); TGT (-97%); TAC (-70.3%) (all  $p < 0.0001$ ) and significantly lower levels than the triple mutant CGC itself (CAT  $p = 0.014$ , TGT  $p < 0.0001$ ; TAC  $p = 0.035$ ). When two sites of TAT were changed at a time to rare alleles, leaving only one wildtype site, luciferase levels were also highly significantly different from wildtype TAT (all  $p < 0.0001$ ), (CGT (-69.2%), TGC (-70.7%) and CAC (-53%), but none were significantly different from CGC (all  $p > 0.05$ ). Thus one or two changes from TAT lowered luciferase activity significantly to the level of the mutant CGC or below, with the -3A>G having the greatest lowering effect. This is more clearly seen in Figure 2b.

#### **Pair-wise effect of -1131T>C, -3A>G and +1891T>C on luciferase activity**

To investigate further the effects of the three SNPs, changes were made to wildtype TAT or mutant CGC, deleting one site at a time. The -1131C/-3G (CG-) combination showed 16% lower luciferase activity compared to -1131T/-3A (TA-) ( $p = 0.37$ ) (see Figure 3) [10]. It was on the basis of these results that we concluded that in combination -1131T>C/ -3A>G were not having a strong effect on promoter activity. As shown in Figure 3, since construct lengths were different, comparison was made for each pair, thus for the -1131T>C and +1891T>C construct, C-C (-1131C/+1891C) compared to T-T (-1131T/+1891T), showed 23% lower relative luciferase activity which was not statistically significant ( $p = 0.36$ ), suggesting that this combination, which excluded the -3 site, showed little functional difference in expression. By comparison, the combined effect of -3A>G together with +1891T>C, -GC had 59% lower luciferase activity relative to -AT ( $p < 0.055$ ). These results suggest that the combination of -3G and +1891C is

driving the haplotype effect, but cannot explain it fully. Thus all three sites are necessary to achieve the overall difference between TAT and CGC.

### **Transcription binding sites at positions -1131, -3 and +1891.**

To investigate if there were any transcription factor binding sites in the vicinity of -1131, -3 or +1891 which could explain this promoter effect, MatInspector (<http://www.genomatix.de/products/MatInspector/index.html>), a transcription factor search engine, was used. A putative Oct-1 transcription factor binding site was identified *in silico*, spanning position +1891. No obvious transcription factors mapped at the other sites. However, using an EMSA, competing labeled -1131T and -1131C probes, -3A or -3G probes and +1891T and +1891C probes with excess unlabelled competitor probe no novel bands were seen. To test if the assay worked, Sp1 was used as a positive control (Figure 4). Furthermore, an Oct-1 monoclonal antibody used as a specific competitor produced no supershift (data not shown).

## DISCUSSION

Although the association of *APOA5* -1131T>C and/or -3A>G SNPs, with plasma TG levels has been consistent [7,8], functional assays of the haplotype *APOA5*\*2 SNPs individually could not provide an explanation for this association [10]. This current set of experiments suggests that the functionality of *APOA5*\*2 relies on the interaction of three of the four SNPs, which define the haplotype and which show 100% LD. This results in a 54% lower luciferase activity by the rare allele construct CGC compared to the wildtype construct TAT. Because of this complete LD, no other combinations of these SNPs occur *in vivo*. This means that it is not possible to examine their individual association with plasma TG levels or plasma apoAV levels, and hence infer function separately.

By creating various combinations of the three variant sites, we have managed to obtain a better understanding of their effects. We previously showed that *individually* these SNPs do not have a statistically significant effect on gene expression [10]. Using pair-wise constructs it is clear that taking any two of the rare alleles in combination does not significantly affect gene expression, and indeed a large effect is only seen in a three allele construct. However, compared to the three common allele construct, TAT, the level of gene expression was even lower than CGC when a single rare site was present, as in CAT, TGT and TAC, with TGT having the most dramatic lowering effect. Thus we conclude that while all three variant sites are contributing to the large overall difference, their contribution is not equal with the major effect due to the combination of the -3A>G and the +1891T>C, in addition to some contribution from -1131T>C. However, since the luciferase assay is an artificial system, and these variants are not within their normal *APOA5* or chromatin environment, extrapolating to the endogenous milieu must be made with caution.

The -3G>A lies in the Kozak sequence, preceding the initiating AUG codon, which represents a highly conserved motif in eukaryotes regulating ribosomal translation [11]. The -3A>G SNP was tested previously in a reticulocyte transcription/translation assay which measured the relative amount of apoAV translated from -3A construct compared to a -3G construct [10]. However, although this assay specifically examined apoAV translation, it was not sensitive, and we reported no difference in translation of apoAV in the presence of the -3A compared to the -3G Kozak sequence. Also in combination with -1131T>C (TA- vs. CG-) no significant differences were seen [10]. However, by changing the wildtype sites TAT, singly or in pairs, all changes significantly affected luciferase levels, suggesting all three sites contributed to the difference in TAT vs. CGC, but only in the context of the whole fragment.

#### **How can we explain the interaction of the -3A>G and +1891T>C?**

The assay used here assesses overall effects on luciferase gene expression by combining transcription and translation effects. This indirect but sensitive assay, confirms that the -3A>G in the Kozak sequence does influence the amount of luciferase protein produced and that it is likely to be doing so by affecting translation. Neither *in silico* nor *in vitro* data supported the presence of a nuclear transcription factor binding to this region of DNA. This was not surprising since the translation machinery at the AUG is likely to block access to a transcription factor [25]. The construct -GC, containing the -3G together with the +1891C, showed the largest effect on luciferase activity lowering, suggesting an interaction between these two sites. While *in silico* the search engine identified a putative Oct-1 site spanning position +1891, *in vitro* data suggested this was not functional. An alternative possibility is that changes within the 3'UTR of the gene may affect mRNA stability. There are several reported SNPs in the 3'UTR of genes that affect mRNA stability and are associated with disease phenotypes [26-29]. *In vitro* studies of *PAI2* confirmed that a sequence motif GATAAATCC in the 3' UTR affected mRNA stability [27]. This motif

shares 5/10 sites with the sequence flanking +1891T>C, CTGCAT/CATCC, thus it might provide an underlying mechanism for the +1891T>C effect seen in the whole construct. Future work is needed to confirm this.

There is a precedent set by the *CETP* promoter SNPs for functional interaction [30]. The *CETP* TaqIB SNP in intron 1 has consistently been associated with *CETP* and HDL levels [31-33] yet was considered to be unlikely to be functional. Three promoter SNPs, -629C>A (rs1800775), -971G>A (rs4783961) and -1337C>T (rs17368435), in LD with TaqIB, were identified, each showing varying association with *CETP* and HDL levels, yet each alone could not explain the TaqIB association. Using luciferase reporter constructs, the interaction of these SNPs was demonstrated. The proposed basis for this was suggested to be a three dimensional conformational change in the promoter, enabling Sp1/Sp3: DNA interactions and leading to chromatin remodelling and transcription repression [30]. We can only speculate on the basis of the *APOA5*\*2 interaction. Our data suggest that the interaction is at the level of reduced transcription/translation (-3A>G) together with a reduction in mRNA stability(+1891T>C). The mechanism governing the contribution of the -1131T>C in the reduction of gene expression remains to be determined. A functional HNF4 transcription factor binding site lies close to -1131 [5] and this might have a small effect on transcription in an allele-specific manner in the context of the whole haplotype.

### **The paradox arising from the relationship between apoAV levels, plasma TG and *APOA5* SNPs**

The exact function of apoAV remains unclear. Although expressed primarily in the liver [1], there are very few studies that support a role for apoAV in the production of TG-rich lipoproteins from the liver [34]. However, apoAV promotes LPL hydrolysis of TG-rich lipoproteins by stabilising



the LPL dimer [35,36]. In addition, apoAV has been shown to interact with members of the LDL-receptor family and can thus 'bridge' TG-rich particles to the receptors for catabolism [37,38]. Transgenic and knockout mouse models suggest an inverse relationship between plasma apoAV and TG levels [1]. Surprisingly, several studies have reported a *positive* correlation between plasma apoAV and plasma TG levels [21-24]. This correlation may be more complex than at first appears and it has been suggested that, after correction for apoCIII levels, the relationship between apoAV and plasma TG is no longer significant [20].

For S19W (c.56C>G) both molecular modelling and functional studies confirmed that this substitution alters signal peptide function, with the W19 showing reduced secretion potential which should lead to less apoAV secretion from hepatocytes [10]. Again surprisingly W19 has consistently been reported to be associated with *higher* plasma apoAV levels [21,22,24], leading Henneman et al to question the validity of the functional studies predicting reduced secretion of apoAV [24]. However, all the functional studies on apoAV suggest an *inverse* relationship with plasma TG levels [35-38]. Our current studies suggest that the functional effect of *APOA5*\*2 would lead to a 50% lower apoAV expression, which is in good agreement, for example, with the 42% higher TG levels seen in -1131CC homozygotes compared to -1131TT individuals [13]. However, paradoxically -1131T>C is not associated with difference in plasma apoAV levels in at least 4 studies [21-24].

Before the direct effects on gene expression of these *APOA5* SNPs was demonstrated, their association with TG levels could be explained by the LD with *APOC3* SNPs, since the functionality of these SNPs is well documented [39,40]. This LD effect would also explain the lack of association of TG-raising SNPs with detectable effects on plasma apoAV levels [22]. However these current experiments, together with our re-analysis of the original haplotype data

[13]showing *APOA5*-113T>C and *APOC3* 1100C>T have independent effects on plasma TG levels in multiple regression analysis, make this less likely. Olano-Martin et al have used a similar approach to demonstrate that the effect of the *APOA5*\*2 haplotype, as represented by -1131T>C, on fasting TG levels and postprandial response, was independent of *APOC3* variants (in this case the *APOC3* 3238C>G) using linear regression analysis. Only the -1131T>C remained in the model, exhibiting an independent association [41].

Recently, the *in vivo* characterisation, by targeted introduction of either *APOA5*\*2 or *APOA5*\*3 into the mouse genome, was undertaken to test their functional effects *in vivo* [42]. Interestingly, while *APOA5*\*3 (S19W) mice had three-fold lower human apoAV plasma levels than wildtype *APOA5*\*1 mice, *APOA5*\*2 mice had plasma levels that were no different from *APOA5*\*1. The conclusions drawn from these experiments were that S19W is a functional SNP while the SNPs defining *APOA5*\*2 are not, at least in the milieu of the mouse. These experiments, however, do not explain why in humans S19W is associated with higher plasma apoAV levels, and thus do not shed additional light on the relationship between plasma measures of apoAV and TG in humans. The underlying paradox is that observational studies find a positive correlation between plasma apoAV and TG levels but all the functional studies of apoAV protein and *APOA5* variants support an *inverse* relationship between apoAV and plasma TG. This inverse relationship is supported by the *APOA5* truncation homozygote with extremely high plasma TG levels [18]. Thus, in conclusion, taken together, these functional studies support an inverse relationship between apoAV and plasma TG levels and raise questions about the relevance of circulating plasma apoAV as a marker of hypertriglyceridemia.

#### **ACKNOWLEDGEMENTS**

This work was supported by the British Heart Foundation (RG2005/014, FS/04/012, PG 04/110/17827).

**References**

1. L.A. Pennacchio, M. Olivier, J.A. Hubacek, J.C. Cohen, D.R. Cox, J.C. Fruchart, R.M. Krauss, E.M. Rubin, An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing, *Science* 294 (2001) 169-173.
2. P.J. O'Brien, W.E. Alborn, J.H. Sloan, M. Ulmer, A. Boodhoo, M.D. Knierman, A.E. Schultze, R.J. Konrad, The novel apolipoprotein A5 is present in human serum, is associated with VLDL, HDL, and chylomicrons, and circulates at very low concentrations compared with other apolipoproteins, *Clin.Chem.* 51 (2005) 351-359.
3. N. Vu-Dac, P. Gervois, H. Jakel, M. Nowak, E. Bauge, H. Dehondt, B. Staels, L.A. Pennacchio, E.M. Rubin, J. Fruchart-Najib, J.C. Fruchart, Apolipoprotein A5, a crucial determinant of plasma triglyceride levels, is highly responsive to peroxisome proliferator-activated receptor alpha activators, *J.Biol.Chem.* 278 (2003) 17982-17985.
4. X. Prieur, H. Coste, J.C. Rodriguez, The human apolipoprotein AV gene is regulated by peroxisome proliferator-activated receptor-alpha and contains a novel farnesoid X-activated receptor response element, *J.Biol.Chem.* 278 (2003) 25468-25480.
5. X. Prieur, F.G. Schaap, H. Coste, J.C. Rodriguez, Hepatocyte nuclear factor-4alpha regulates the human apolipoprotein AV gene: identification of a novel response element and involvement in the control by peroxisome proliferator-activated receptor-gamma coactivator-1alpha, AMP-activated protein kinase, and mitogen-activated protein kinase pathway, *Mol.Endocrinol.* 19 (2005) 3107-3125.
6. M. Nowak, A. Helleboid-Chapman, H. Jakel, G. Martin, D. Duran-Sandoval, B. Staels, E.M. Rubin, L.A. Pennacchio, M.R. Taskinen, J. Fruchart-Najib, J.C. Fruchart, Insulin-mediated down-regulation of apolipoprotein A5 gene expression through

- the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor, *Mol.Cell Biol.* 25 (2005) 1537-1548.
7. K.W. van Dijk, P.C. Rensen, P.J. Voshol, L.M. Havekes, The role and mode of action of apolipoproteins CIII and AV: synergistic actors in triglyceride metabolism?, *Curr.Opin.Lipidol.* 15 (2004) 239-246.
  8. C.Q. Lai, L.D. Parnell, J.M. Ordovas, The APOA1/C3/A4/A5 gene cluster, lipid metabolism and cardiovascular disease risk, *Curr.Opin.Lipidol.* 16 (2005) 153-166.
  9. M. Olivier, X. Wang, R. Cole, B. Gau, J. Kim, E.M. Rubin, L.A. Pennacchio, Haplotype analysis of the apolipoprotein gene cluster on human chromosome 11, *Genomics* 83 (2004) 912-923.
  10. P.J. Talmud, J. Palmen, W. Putt, L. Lins, S.E. Humphries, Determination of the functionality of common APOA5 polymorphisms, *J.Biol.Chem.* 280 (2005) 28215-28220.
  11. M. Kozak, At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells, *J.Mol.Biol.* 196 (1987) 947-950.
  12. L.A. Pennacchio, M. Olivier, J. Hubacek, R.M. Krauss, E.M. Rubin, J.C. Cohen, Two Independent Apolipoprotein A5 Haplotypes Influence Human Plasma Triglyceride Levels., *Hum.Mol.Gen.* 11 (2002) 3031-3038.
  13. P.J. Talmud, E. Hawe, S. Martin, M. Olivier, G.J. Miller, E.M. Rubin, L.A. Pennacchio, S.E. Humphries, Relative contribution of variation within the APOC3-A4-A5 gene cluster in determining plasma triglycerides, *Hum.Mol.Gen.* 11 (2002) 3039-3046.

14. B.E. Aouizerat, M. Kulkarni, D. Heilbron, D. Drown, S. Raskin, C.R. Pullinger, M.J. Malloy, J.P. Kane, Genetic analysis of a polymorphism in the human apoA-V gene: effect on plasma lipids, *J.Lipid Res.* 44 (2003) 1167-1173.
15. M.A. Austin, P.J. Talmud, F.M. Farin, D.A. Nickerson, K.L. Edwards, D. Leonetti, M.J. McNeely, H.M. Viernes, S.E. Humphries, W.Y. Fujimoto, Association of apolipoprotein A5 variants with LDL particle size and triglyceride in Japanese Americans, *Biochim.Biophys.Acta* 1688 (2004) 1-9.
16. Y. Jang, J.Y. Kim, O.Y. Kim, J.E. Lee, H. Cho, J.M. Ordovas, J.H. Lee, The -1131T-->C polymorphism in the apolipoprotein A5 gene is associated with postprandial hypertriglycerolemia; elevated small, dense LDL concentrations; and oxidative stress in nonobese Korean men, *Am.J.Clin.Nutr.* 80 (2004) 832-840.
17. B. Dorfmeister, J.A. Cooper, J.W. Stephens, H. Ireland, S.J. Hurel, S.E. Humphries, P.J. Talmud, The effect of APOA5 and APOC3 variants on lipid parameters in European Whites, Indian Asians and Afro-Caribbeans with type 2 diabetes, *Biochim.Biophys.Acta* 1772 (2007) 355-363.
18. C.P. Oliva, L. Pisciotta, V.G. Li, M.P. Sambataro, A. Cantafora, A. Bellocchio, A. Catapano, P. Tarugi, S. Bertolini, S. Calandra, Inherited Apolipoprotein A-V Deficiency in Severe Hypertriglyceridemia, *Arterioscler.Thromb.Vasc.Biol.* 25 (2005) 411-417.
19. M. Ishihara, T. Kujiraoka, T. Iwasaki, M. Nagano, M. Takano, J. Ishii, M. Tsuji, H. Ide, I.P. Miller, N.E. Miller, H. Hattori, A sandwich enzyme-linked immunosorbent assay for human plasma apolipoprotein A-V concentration, *J.Lipid Res.* 46 (2005) 2015-2022.
20. F.G. Schaap, M.C. Nierman, J.F. Berbee, H. Hattori, P.J. Talmud, S.F. Vaessen, P.C. Rensen, R.A. Chamuleau, J.A. Kuivenhoven, A.K. Groen, Evidence for a

- complex relationship between apoAV and apoCIII in patients with severe hypertriglyceridemia, *J.Lipid Res.* 47 (2006) 2333-2339.
21. G.M. Dallinga-Thie, A. van Tol, H. Hattori, van Vark-van der Zee LC, H. Jansen, E.J. Sijbrands, Plasma apolipoprotein A5 and triglycerides in type 2 diabetes, *Diabetologia* 49 (2006) 1505-1511.
  22. P.J. Talmud, J.A. Cooper, H. Hattori, I.P. Miller, G.J. Miller, S.E. Humphries, *APOA5* genotype, plasma apolipoprotein AV and triglyceride levels and the prospective risk of type 2 diabetes: results from the Northwick Park Heart Study II, *Diabetologia* 49 (2006) 2337-2340.
  23. S.F. Vaessen, F.G. Schaap, J.A. Kuivenhoven, A.K. Groen, B.A. Hutten, S.M. Boekholdt, H. Hattori, M.S. Sandhu, S.A. Bingham, R. Luben, J.A. Palmen, N.J. Wareham, S.E. Humphries, J.J. Kastelein, P.J. Talmud, K.T. Khaw, Apolipoprotein A-V, triglycerides and risk of coronary artery disease: the prospective Epic-Norfolk Population Study, *J.Lipid Res.* 47 (2006) 2064-2070.
  24. P. Henneman, F.G. Schaap, L.M. Havekes, P.C. Rensen, R.R. Frants, A. van Tol, H. Hattori, A.H. Smelt, K.W. van Dijk, Plasma apoAV levels are markedly elevated in severe hypertriglyceridemia and positively correlated with the *APOA5* S19W polymorphism, *Atherosclerosis* 193 (2007) 129-134.
  25. M. Kozak, A second look at cellular mRNA sequences said to function as internal ribosome entry sites, *Nucleic Acids Res.* 33 (2005) 6593-6602.
  26. F. Capon, M.H. Allen, M. Ameen, A.D. Burden, D. Tillman, J.N. Barker, R.C. Trembath, A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups, *Hum.Mol.Gen.* 13 (2004) 2361-2368.

27. M.J. Tierney, R.L. Medcalf, Plasminogen Activator Inhibitor Type 2 Contains mRNA Instability Elements within Exon 4 of the Coding Region. Sequence homology to coding region instability determinants in other mRNAs, *J.Biol.Chem.* 276 (2001) 13675-13684.
28. J. Duan, M.S. Wainwright, J.M. Comeron, N. Saitou, A.R. Sanders, J. Gelernter, P.V. Gejman, Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor, *Hum.Mol.Gen.* 12 (2003) 205-216.
29. R. Di Paola, L. Frittitta, G. Miscio, M. Bozzali, R. Baratta, M. Centra, D. Spampinato, M.G. Santagati, T. Ercolino, C. Cisternino, T. Soccio, S. Mastroianno, V. Tassi, P. Almgren, A. Pizzuti, R. Vigneri, V. Trischitta, A variation in 3' UTR of hPTP1B increases specific gene expression and associates with insulin resistance, *Am.J.Hum.Genet.* 70 (2002) 806-812.
30. E. Frisdal, A.H. Klerkx, W. Le Goff, M.W. Tanck, J.P. Lagarde, J.W. Jukema, J.J. Kastelein, M.J. Chapman, M. Guerin, Functional interaction between -629C/A, -971G/A and -1337C/T polymorphisms in the CETP gene is a major determinant of promoter activity and plasma CETP concentration in the REGRESS Study, *Hum.Mol.Genet.* 14 (2005) 2607-2618.
31. A.H. Klerkx, M.W. Tanck, J.J. Kastelein, H.O. Molhuizen, J.W. Jukema, A.H. Zwinderman, J.A. Kuivenhoven, Haplotype analysis of the CETP gene: not TaqIB, but the closely linked -629C-->A polymorphism and a novel promoter variant are independently associated with CETP concentration, *Hum.Mol.Genet.* 12 (2003) 111-123.
32. M. Corbex, O. Poirier, F. Fumeron, D. Betoulle, A. Evans, J.B. Ruidavets, D. Arveiler, G. Luc, L. Tiret, F. Cambien, Extensive association analysis between the CETP

- gene and coronary heart disease phenotypes reveals several putative functional polymorphisms and gene-environment interaction, *Genet Epidemiol* 19 (2000) 64-80.
33. J.M. Ordovas, L.A. Cupples, D. Corella, J.D. Otvos, D. Osgood, A. Martinez, C. Lahoz, O. Coltell, P.W. Wilson, E.J. Schaefer, Association of cholesteryl ester transfer protein-TaqIB polymorphism with variations in lipoprotein subclasses and coronary heart disease risk: the Framingham study, *Arterioscler Thromb Vasc Biol.* 20 (2000) 1323-1329.
34. F.G. Schaap, P.C. Rensen, P.J. Voshol, C. Vriens, H.N. van der Vliet, R.A. Chamuleau, L.M. Havekes, A.K. Groen, V.D. Willems, ApoAV reduces plasma triglycerides by inhibiting very low density lipoprotein-triglyceride (VLDL-TG) production and stimulating lipoprotein lipase-mediated VLDL-TG hydrolysis, *J.Biol.Chem.* 279 (2004) 27941-27947.
35. M. Merkel, J. Heeren, Give me A5 for lipoprotein hydrolysis!, *J.Clin.Invest* 115 (2005) 2694-2696.
36. M. Merkel, B. Loeffler, M. Kluger, N. Fabig, G. Geppert, L.A. Pennacchio, A. Laatsch, J. Heeren, Apolipoprotein AV accelerates plasma hydrolysis of triglyceride-rich lipoproteins by interaction with proteoglycan-bound lipoprotein lipase, *J.Biol.Chem.* 280 (2005) 21553-21560.
37. S.K. Nilsson, A. Lookene, J.A. Beckstead, J. Gliemann, R.O. Ryan, G. Olivecrona, Apolipoprotein A-V interaction with members of the low density lipoprotein receptor gene family, *Biochemistry* 46 (2007) 3896-3904.
38. A. Dichlberger, L.A. Cogburn, J. Nimpf, W.J. Schneider, Avian apolipoprotein A-V binds to LDL receptor gene family members, *J.Lipid Res.* 48 (2007) 1451-1456.



39. M. Dammerman, L.A. Sandkuijl, J.L. Halaas, W. Chung, J.L. Breslow, An apolipoprotein CIII haplotype protective against hypertriglyceridemia is specified by promoter and 3' untranslated region polymorphisms, *Proc.Natl.Acad.Sci U.S.A.* 90 (1993) 4562-4566.
40. W.W. Li, M.M. Dammerman, J.D. Smith, S. Metzger, J.L. Breslow, T. Leff, Common genetic variation in the promoter of the human apo CIII gene abolishes regulation by insulin and may contribute to hypertriglyceridemia, *J Clin Invest* 96 (1995) 2601-2605.
41. E. Olano-Martin, E.C. Abraham, R. Gill-Garrison, A.M. Valdes, K. Grimaldi, F. Tang, K.G. Jackson, C.M. Williams, A.M. Minihane, Influence of ApoA5 gene variants on postprandial triglyceride metabolism: Impact of gender, *J.Lipid Res.* (2008) epub.
42. N. Ahituv, J. Akiyama, A. Chapman-Helleboid, J. Fruchart, L.A. Pennacchio, In vivo characterization of human APOA5 haplotypes, *Genomics* (2007) epub .

**FIGURES LEGENDS**

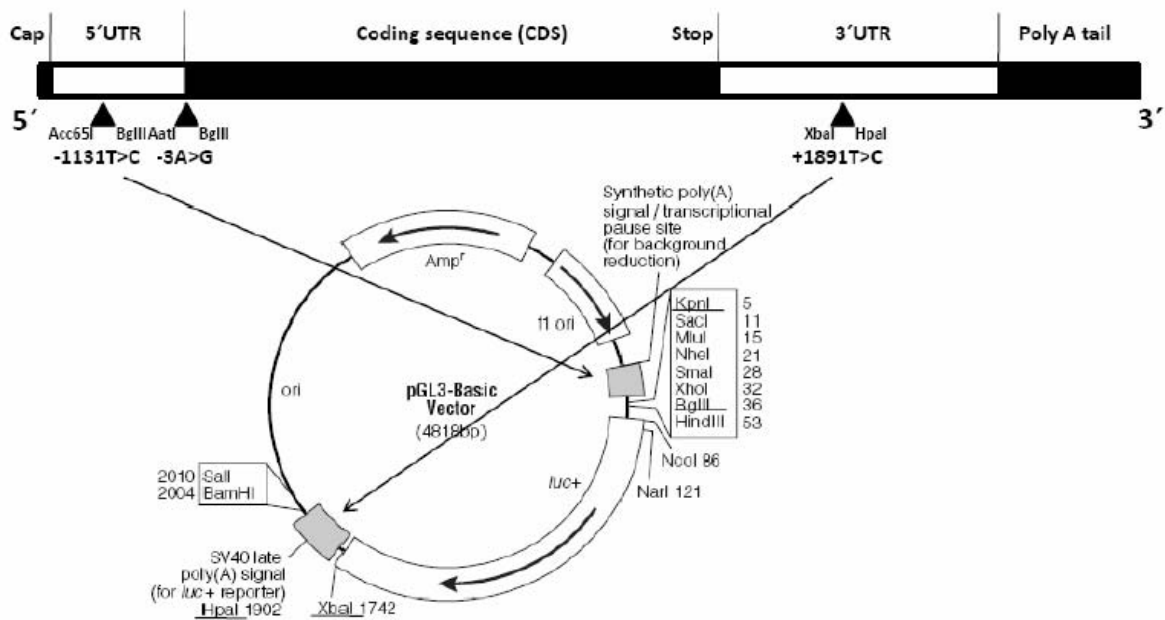
**Figure 1.** Schematic representation of pGL3 basic constructs including the *APOA5* promoter and 3' UTR fragments.

**Figures 2a, b.** Effects of the *APOA5* promoter and 3'UTR fragments including sites -1131T>C, -3A>G (1176bp promoter sequence) and +1891T>C (816 bp 3'UTR sequence), in different combinations, on relative luciferase (Luc) activity, compared to wildtype construct TAT (set at 100). Data were corrected for transfection efficiency. Results represent data from 30 repeat experiments each with 8 replicates for TAT vs. CGC, 5 repeats each with 8 replicates for the rest, from at least two different DNA preparations (Bars = SEM). Data for 2a and 2b are identical but the comparisons changed.

**Figure 3.** Pair-wise comparison of deletion constructs with one site missing in each construct on relative luciferase (Luc) activity. Mutant construct compared to the wildtype construct in each case. Data were corrected for transfection efficiency. Results represent data from 3 repeat experiments each with 8 replicates from two different DNA preparations (Bars = SEM).

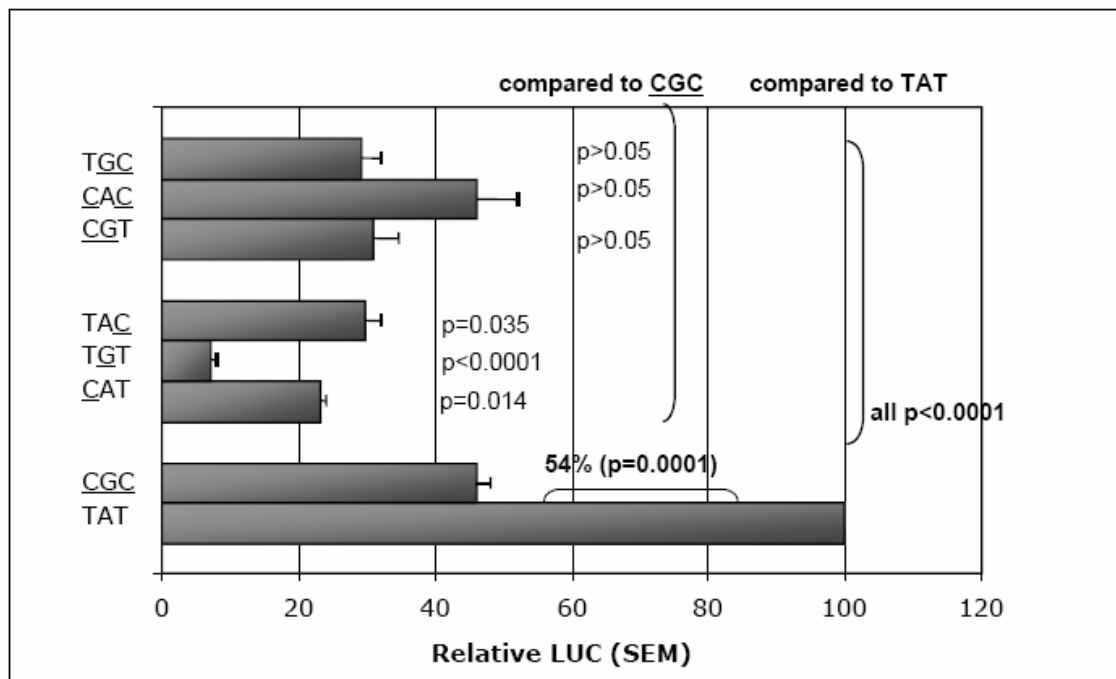
**Figure 4.** EMSA to investigate differential binding abilities of *APOA5* variants using IL-6 stimulated nuclear extract. See text for details of probes and binding conditions. Lane 1: Sp1 consensus probe; Lane 2: Sp1 consensus probe with excess unlabelled oligo; Lanes 3-6: -1131C/T probes with excess unlabelled competitor in 4/6; Lanes 7-10: -3 A/G probes with excess unlabelled competitor in 8/10; Lanes 11-14: 1891 C/T probes with excess unlabelled competitor in 12/14; Lane 15: Sp1 consensus sequence with no extract.

Figure 1



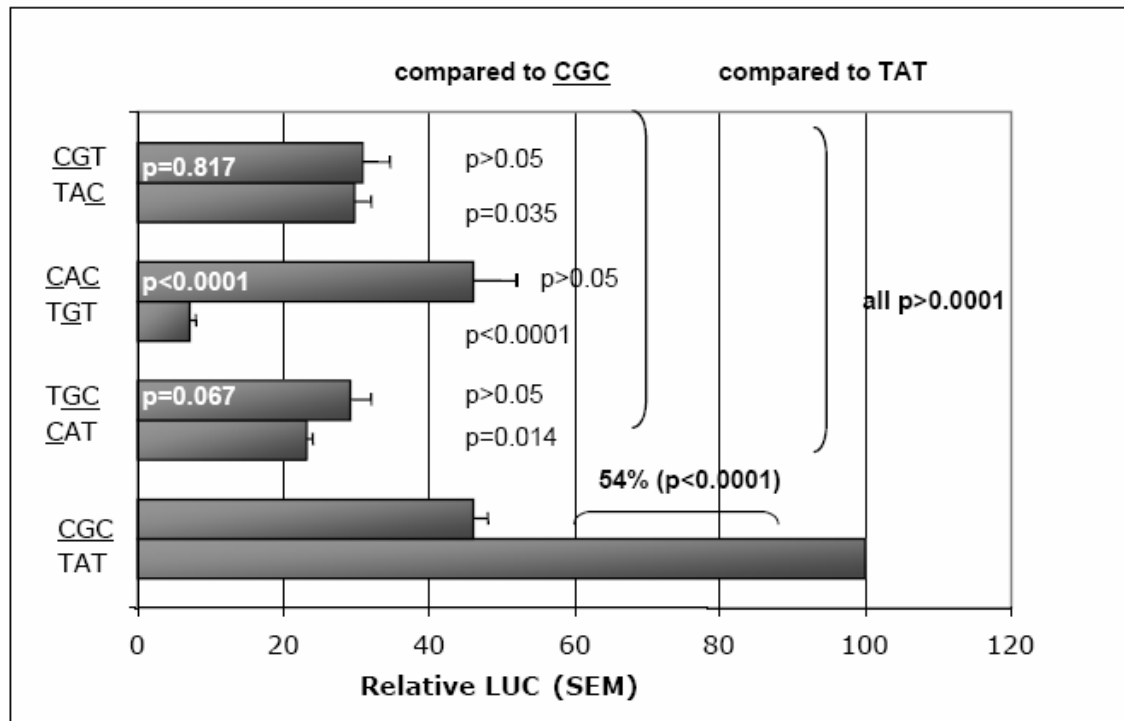
ACCEPTED

Figure 2a

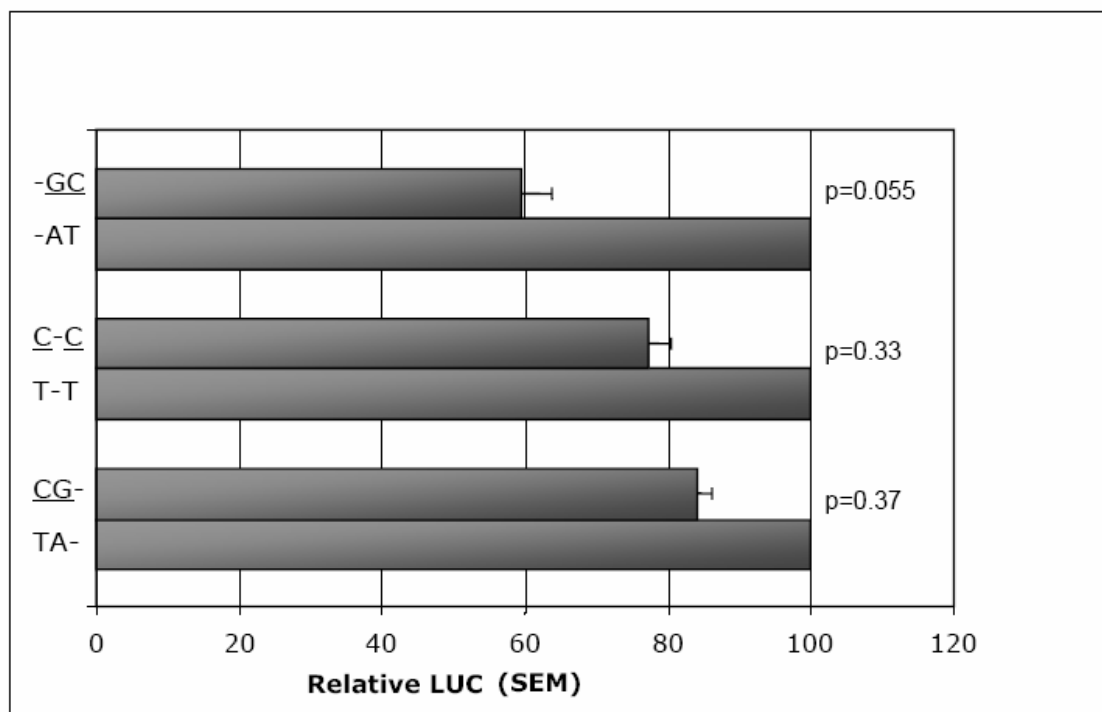


ACCEPT

Figure 2b



ACCEP

**Figure 3**

ACCEPTED

