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Associations of the apolipoprotein A1/C3/A4/A5 gene cluster with triglyceride and HDL cholesterol levels in women with type 2 diabetes

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Abstract

The apolipoprotein gene cluster (APOA1/C3/A4/A5) was recently associated with triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) in non-diabetic population. Little is known whether the variations in these genes affect lipid homeostasis in patients with type 2 diabetes. We examined the associations of 10 polymorphisms at APOA1/C3/A4/A5 gene cluster with blood lipids among 902 diabetic women. A linkage disequilibrium (LD) breakdown was observed between APOA5 and other genes. APOA5 S19W was associated with significantly higher fasting TG levels ($P=0.001$). Two common haplotypes encompassing four APOA5 polymorphisms (SNP1, SNP2, S19W, and SNP3) were associated with 35.6 mg/dL (haplotype 2212, APOA5*2, $P=0.016$) and 57.8 mg/dL (haplotype 1121, APOA5*3, $P=0.0002$) higher fasting TG levels compared with the most common (haplotype 1111, APOA5*1), respectively. Adjustment for age, BMI, and other covariates did not appreciably change such associations. In addition, APOC3 promoter polymorphism $-455T/C$ showed significant associations with fasting TG levels ($P=0.006$), whereas APOA4 $+347T/A$ showed significant associations with lower levels of HDL-C ($P=0.017$). Our results indicate that the variability in APOA1/C3/A4/A5 gene cluster may affect TG and HDL levels in women with type 2 diabetes.

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Keywords: Apolipoprotein; Gene cluster; Triglycerides; HDL cholesterol; Diabetic patients

1. Introduction

Patients with type 2 diabetes are featured by substantially increased risk of atherogenesis [1]. In diabetic patients, elevated triglycerides (TG) and lowered high-density lipoprotein cholesterol (HDL-C), which often occur together and are known as abnormalities of the TG-HDL axis [2], are the major proatherogenic factors. Both TG and HDL-C levels are to some extent controlled by genetic factors [3]. However, the specific loci involved have not been well defined. In recent years, a cluster of apolipoprotein (APO) genes that contains four components (APOA1/C3/A4/A5) in a genomic interval of ~60 kb on chromosome 11q23 has been repeatedly impli-

cated as potential genetic determinants for inter-individual differences of both TG and HDL-C levels in general populations and patients with familial combined hyperlipidemia (FCH) [4,5].

The apolipoproteins encoded by this gene cluster are involved in the metabolism of TG-rich lipoprotein and HDL particles. ApoAI (coded by APOA1) and apoAIV (coded by APOA4) are major components of intestinally derived lipoproteins and HDL. ApoCIII (coded by APOC3) is a major component of very low-density lipoprotein (VLDL), chylomicron remnants, and HDL-C [6,7]. ApoAV (coded by APOA5) is a newly identified member of apolipoprotein family that may inhibit VLDL-TG production and stimulate lipoprotein lipase (LPL)-mediated VLDL-TG hydrolysis [8]. In the general population, sequence variants at APOA1 locus primarily affect HDL-C concentration [9,10], while variants

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in APOC3 and APOA5 are primarily associated with TG levels [11–13]. Thus far, no study has examined the relationship of the variability of APOA1/C3/A4/A5 gene cluster and the levels of TG and HDL-C in patients with type 2 diabetes.

In this study, we aimed to determine whether 10 common polymorphisms identified at APOA1/C3/A4/A5 gene cluster were associated with the levels of TG and HDL-C in a cohort of diabetic women from a large prospective study—the Nurses' Health Study (NHS).

2. Subjects, materials and methods

2.1. Study population

NHS began in 1976 with the recruitment of 121,700 female registered nurses (>95% Caucasian) between the ages of 30 and 55 years. About 32,826 women provided blood between 1989 and 1990. Incident cases were defined as self-reported diabetes confirmed by supplementary questionnaire. We used National Diabetes Data Group criteria to define diabetes because most of our subjects were diagnosed before the release of the American Diabetes Association criteria in 1997 [14]. A case of diabetes was considered if at least one of the following was reported on the supplementary questionnaire: (1) classic symptoms plus elevated fasting plasma glucose ≥ 7.8 mmol/l, random plasma glucose ≥ 11.1 mmol/l, and/or plasma glucose ≥ 11.1 mmol/l after ≥ 2 h during an oral glucose tolerance test; (2) no symptoms but at least two elevated plasma glucose concentrations (by the above criteria) on different occasions; or (3) treatment with oral hypoglycemic agents or insulin. Our study included the 902 diabetic women who were free of fatal coronary heart disease, nonfatal MI, coronary artery bypass grafting (CABG) or percutaneous transluminal coronary angioplasty (PTCA), fatal stroke, and nonfatal stroke at blood collection.

2.2. Assessment of lipids and biomarkers

Blood was collected between 1989 and 1990. The concentrations of total cholesterol, HDL-C, and TG were simultaneously measured on the Hitachi 911 analyser using reagents and calibrates from Roche Diagnostics (Indianapolis, IN, USA); coefficients of variation for these measurements were below 1.8%. LDL-C cholesterol concentrations were measured by a homogenous direct method from Genzyme Corporation (Cambridge, MA, USA). The day-to-day variability was below 3.1%. Apolipoprotein B (apoB)-100 concentrations were measured via an immunonephelometric assay using reagents and calibrators from Wako Chemicals USA (Richmond, VA, USA) with a day-to-day variability of less than 5%. HbA_{1c} concentrations were determined based on turbidimetric immunoinhibition using haemolysed whole blood or packed red cells. The day-to-day variability at HbA_{1c} concentrations of 5.5 and 9.1% was 1.9 and 3.0%, respectively.

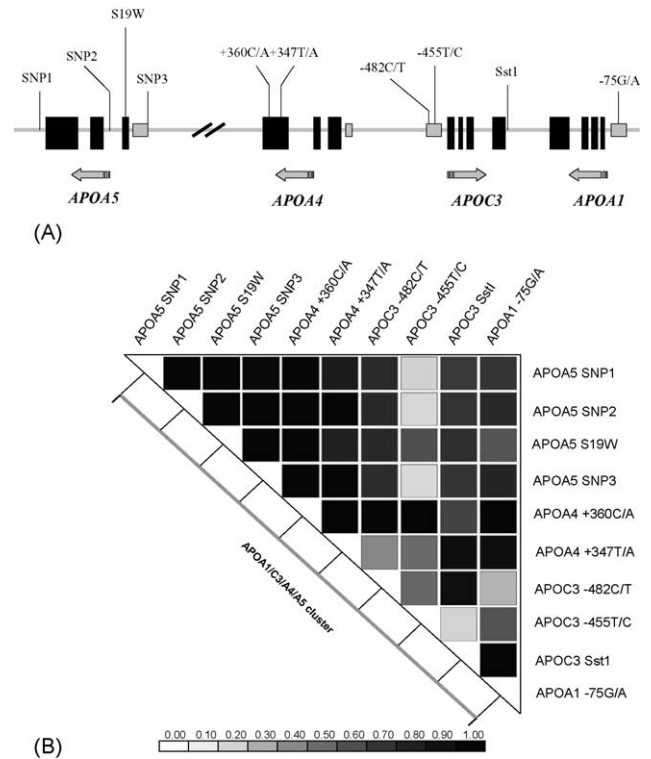


Fig. 1. (A) Location of the genetic markers in APOA1/C3/A4/A5 gene cluster (not drawn to scale). Exons are depicted by black boxes and the promoters are denoted by smaller gray boxes. The direction of transcription is labeled with lines; (B) pairwise linkage disequilibrium matrix. D' is used for matrix creation. D' of 1 is indicated by black while D' of 0 is indicated by white.

2.3. Genotype determination and assessment of covariates

DNA was extracted from the buffy coat fraction of centrifuged blood using the QIAmp Blood Kit (Qiagen, Chatsworth, CA). Ten common SNPs at APOA1/C3/A4/A5 gene cluster (APOA5 SNP1 [+1891A/G, rs2266788], SNP2 [+751C/T, rs2072560], S19W [rs3135506], and SNP3 [−1131T/C, rs662799]; APOA4 +360C/A [Q360H, rs5110] and +347T/A [T347S, rs675]; APOC3 −482C/T [rs2854117], −455T/C [rs2854116], and Sst1 [rs5128]; and APOA1 −75G/A [rs670]) (Fig. 1A) were genotyped using Taqman SNP allelic discrimination by means of an ABI 7900H T (Applied Biosystems, Foster City, CA). Replicate quality control samples were included and genotyped with 100% concordance.

Baseline body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Physical activity was expressed as metabolic equivalent task (MET)-hours based on self-reported types and durations of activities over the previous year.

2.4. Statistical analyses

A chi-square test was used to assess whether the genotypes were in Hardy–Weinberg equilibrium (HWE). General

Table 1
Associations between single SNPs and plasma levels of fasting TG and HDL-C among diabetic women^a

Polymorphisms	Fasting TG (mg/dL)			HDL-C (mg/dL)		
	<i>n</i>	Means	<i>P</i>	<i>n</i>	Means	<i>P</i>
APOA5 SNP1, +1891A/G, rs2266788						
AA	504	201	0.17	747	51.3	0.65
AG+GG	72	215		117	50.6	
APOA5 SNP2, +751C/T, rs2072560						
CC	523	201	0.07	776	51.3	0.63
CT+TT	62	225		108	50.5	
APOA5 S19W, +170C/G, rs3135506						
CC	526	195	0.001	786	51.5	0.08
CG+GG	68	252		104	49.0	
APOA5, SNP3, −1131T/C, rs662799						
TT	525	201	0.05	779	51.3	0.72
TC+CC	67	222		116	50.8	
APOA4 +360C/A (Q/H), rs5110						
CC	507	206	0.68	754	51.2	0.68
CA+AA	82	189		128	51.7	
APOA4 +347T/A (T/S), rs675						
TT	394	200	0.41	595	52.0	0.017
TA+AA	198	202		296	49.6	
APOC3 −482C/T, rs2854117						
TT	312	208	0.07	467	51.0	0.87
CT	237	192		352	51.6	
CC	34	224		56	51.1	
APOAC3 −455T/C, rs2854116						
TT	216	210	0.006	325	51.5	0.43
TC	272	182		407	51.7	
CC	77	259		114	49.8	
APOAC3 SstI, C/G, rs5128						
CC	482	204	0.30	725	50.8	0.12
CG	94	195		143	53.2	
GG	9	235		13	48.8	
APOA1 −75G/A, rs670						
GG	407	205	0.49	612	51.0	0.56
GA	153	193		232	51.9	
AA	12	223		19	53.5	

^a The associations were examined in women who were successfully genotyped and measured on TG and HDL cholesterol. The associations with TG levels were analyzed only in the fasting samples. Analysis was adjusted for age, BMI, smoking, alcohol consumption, physical activity, HbA_{1c}, history of hypertension, diabetes duration, and postmenopausal hormone use.

linear models were used to compare geometric mean values of quantitative traits across allele/genotype groups, adjusting for age, BMI, smoking (never/past/current), alcohol consumption, physical activity (in quartiles), HbA_{1c}, diabetes duration (at 1990), history of hypertension, and postmenopausal hormone use. To reduce the skewness of the data, TG level was logarithmically transformed. Stepwise-selection regression analyses with a nominal *P*-value cut-points of 0.05 were used to evaluate the independent effects of the investigated genetic variants. The SAS statistical package was used for the analyses (SAS, Version 8.2 for UNIX). Haplotype frequencies were inferred using the expectation-maximization (EM) algorithm and the haplotypic associations were examined using the omnibus chi-square test implemented in SAS/genetics and THESIAS program that is based on the Stochastic-EM

algorithm (SEM) [15]. In these analyses, the reference haplotype was chosen as the haplotype combining the most frequent allele at each site [15]. All *P*-values are two-sided.

3. Results

3.1. Allele frequency and linkage disequilibrium

The allele frequencies of the examined polymorphisms of APOA1/C3/A4/A5 cluster ranged from 0.066 (APOA5 S19W) to 0.37 (APOC3 −455T/C). The genotype distribution of these polymorphisms did not significantly deviate from HWE ($P > 0.05$). To determine the extent of LD in our study sample, standardized LD coefficients D' was calcu-

lated for all pairs of polymorphisms. Fig. 1B shows the LD matrix generated using D' . By setting a criterion of $D' = 0.8$, it appears that the four APOA5 polymorphisms were within a LD block. None of polymorphisms of APOA1/C3/A4 had D' above 0.8 with all the four APOA5 polymorphisms, and, the APOA1/C3/A4 polymorphisms were not within a single block (Fig. 1B).

3.2. Single polymorphism associations

Firstly, we examined the individual associations between each polymorphism and lipid levels. The associations were examined in women who were successfully genotyped and measured on TG (fasting sample) and HDL cholesterol. The heterozygotes and homozygotes for the minor allele were pooled together for some polymorphisms (all APOA5 polymorphisms and APOA4 +360 C/A) in our analysis due to the low allele frequency. After adjustment for age, BMI, and other covariates, APOA5 S19W was associated with significantly higher fasting TG levels ($P = 0.001$) (Table 1). Two other APOA5 polymorphisms, SNP2 (+751C/T) and SNP3 (–1131A/G), were also associated with higher fasting TG levels with marginal significance ($P = 0.07$ and $P = 0.05$, respectively). The homozygosity of APOC3 –455T/C was also associated with significantly higher fasting TG levels ($P = 0.006$). Other polymorphisms were not associated with fasting TG in diabetic women. In addition, APOA4 +347T/A showed significant association with lower HDL-C levels in a dominant inheritance model ($P = 0.017$). Other polymorphisms were not associated HDL-C levels.

We performed stepwise-selection regression analyses using a nominal P -value cut-point of 0.05 to evaluate whether APOC3 and APOA5 polymorphisms were independently associated with TG levels. Both APOC3 –455T/C ($P = 0.01$) and APOA5 S19W ($P = 0.0009$) remained significant in the model, suggesting the independent effects of APOA5 and APOC3 on TG levels. We further examined the associations of the variants of APOA5 and APOC3 separately among women without the effective genotypes of either gene (Table 2). Although the number of individuals decreased, the associations of all APOA5 polymorphisms were significant ($P = 0.005$ for SNP1, $P = 0.0007$ for SNP2, $P = 0.004$ for S19W, and $P = 0.0006$ for SNP3). Moreover, the associations of APOC3 –455T/C became stronger ($P = 0.0018$).

3.3. Haplotype associations

Because of the nature of the LD pattern obtained in this and earlier studies [16], we examined the haplotype associations separately for APOA5 gene and APOA1/C3/A4 genes. The cluster genes are not transcribed in the same direction. For simplicity, our haplotype inference followed the same direction from APOA5 SNP1 to APOA1 –75G/A (Fig. 1A). To label the haplotypes, we generally used ‘1’ to represent the common allele and used ‘2’ to represent the minor allele. On the basis of the four APOA5 polymor-

Table 2

Independent associations of the polymorphisms of APOC3 and APOA5 genes with fasting TG (mg/dL) among diabetic women^a

	Genotypes			<i>P</i>
	11	12	22	
In sample without APOA5 variants				
APOC3 –482C/T (<i>n</i>)	198 (264)	191 (223)	228 (32)	0.09
APOC3 –455T/C (<i>n</i>)	202 (177)	175 (249)	260 (74)	0.0018
APOC3 SstI, C/G (<i>n</i>)	197 (432)	199 (88)	240 (8)	0.24
	Genotypes			<i>P</i>
	11	12 + 22		
In sample without APOC3 –455T/C homozygotes				
APOA5 SNP1 (<i>n</i>)	201 (447)	229 (55)	/	0.005
APOA5 SNP2 (<i>n</i>)	201 (463)	239 (46)	/	0.0007
APOA5 S19W (<i>n</i>)	200 (465)	238 (52)	/	0.004
APOA5, SNP3 (<i>n</i>)	200 (451)	235 (64)	/	0.0006

^a The associations were examined in women who were successfully genotyped and measured on fasting TG levels. Analysis was adjusted for age, BMI, smoking, alcohol consumption, physical activity, HbA1c, history of hypertension, diabetes duration, and postmenopausal hormone use.

phisms, we inferred three common haplotypes (frequency >5%) which represented >99% allelic variance. In the univariate analysis, both haplotypes 2212 (APOA5*2, $P = 0.016$) and 1121 (APOA5*3, $P = 0.0002$) showed significantly associations with higher fasting TG levels compared with the most common haplotype 1111 (APOA5*1) (Table 3). The haplotype associations remained significant after adjustment for covariates and correction for the multiple comparisons (two comparisons). Haplotypes comprising the non-APOA5 polymorphisms were not associated with TG levels.

We further examined the associations of the haplotypes inferred from all the cluster genes (Fig. 2). Hap-

Table 3

Haplotype associations with fasting TG (mg/dL) among diabetic women

Haplotypes ^a	Frequency	Crude		Multivariate ^b	
		Hap diff	<i>P</i>	Hap diff	<i>P</i>
APOA5 (<i>n</i> = 550) ^a					
1111	0.86	Ref.		Ref.	
2212	0.069	35.6	0.016	40.1	0.015
1121	0.067	57.8	0.0002	70.4	0.0002
APOA1/C3/A4 (<i>n</i> = 505) ^a					
111111	0.55	Ref.		Ref.	
122211	0.11	–12.7	0.43	–11.2	0.48
112222	0.09	4.6	0.78	3.6	0.67
121211	0.08	30.2	0.87	28.7	0.44
211111	0.07	–13.6	0.72	–12.3	0.48
112211	0.06	–25.6	0.35	–26.0	0.23

Abbreviation: ref, reference; hap diff, difference with the most common haplotype.

^a The order of polymorphisms on the haplotype were APOA5 SNP1, SNP2, S19W, and SNP3; the order of polymorphisms on the haplotype were APOA4 +360C/A, APOA4 +347T/A, APOC3 –482C/T, APOC3 –455T/C, APOC3 SstI, and APOA1 –75G/A.

^b Adjusted for age, BMI, smoking, alcohol consumption, physical activity, HbA1c, history of hypertension, diabetes duration, and postmenopausal hormone use.

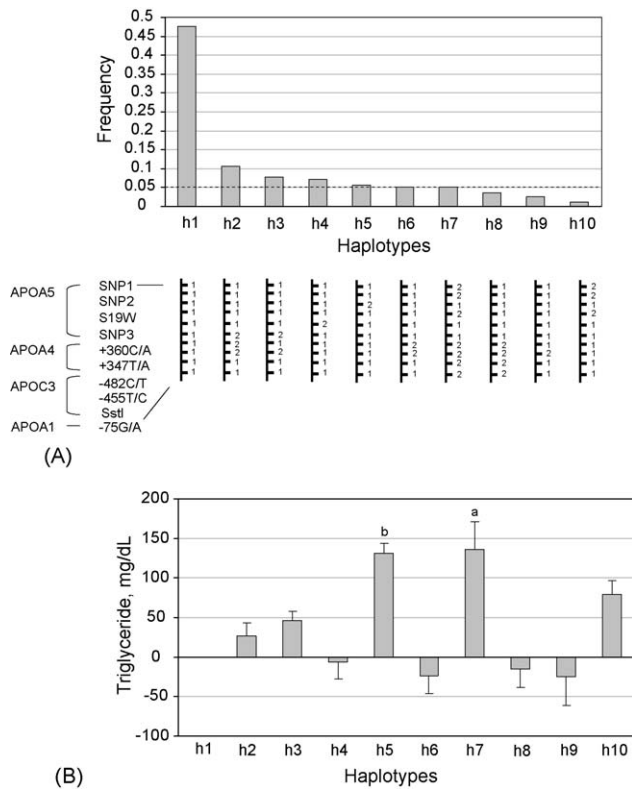


Fig. 2. (A) Haplotypes (frequency >0.01) inferred from 10 polymorphisms at APOA1/C3/A4/A5 gene cluster (APOA5 SNP1, SNP2, S19W, and SNP3; APOA4 +360C/A and +347T/A; APOC3 –482C/T, –455T/C, and SstI; and APOA1 –75G/A). Symbols indicating the location and allele identity of polymorphisms are presented under the frequency figure. ‘h’ represents haplotype; ‘0’ represents the common allele and ‘1’ represents the minor allele; (B) the mean difference of fasting triglyceride levels and standard error between other haplotypes and the reference haplotype h1; ^a $P < 0.05$; ^b $P < 0.01$.

lotype 112111111 (h7, $P = 0.0008$) and 2212112222 (h5, $P = 0.045$) were associated with significantly higher fasting TG levels compared with the most common haplotype 111111111 (h1), after adjusting for covariates. The associations of haplotype 112111111 remained significant after correcting for the multiple comparisons (nine comparisons). There were not significant associations between the haplotypes and HDL-C levels in diabetic women.

4. Discussion

Our findings of the associations of the APOA5 variants with higher TG levels in diabetic women are consistent with the associations observed in the general population [17]. APOA5 is a newly identified gene that has shown substantial effects on TG levels in animal and humans [8]. APOA5 transgenic mice displayed significantly reduced TG concentration whereas APOA5 knockouts had four-fold increased TG [18]. ApoAV reduces plasma TG by inhibiting VLDL-TG production and stimulating LPL-mediated VLDL-TG hydrolysis [19,20]. In humans, serum Apo A-V is associ-

ated with VLDL, HDL, and chylomicrons [21]. Among the non-diabetic populations, APOA5 polymorphisms have been repeatedly associated with TG levels [13,22]. Our findings demonstrate that the genetic effect of APOA5 on TG levels holds in diabetic patients.

The haplotype associations of APOA5 gene in this study broadly confirmed those observed in previous studies [13,22]. Although the polymorphisms included are not exactly identical, the LD pattern in our study sample is similar to that identified in other Caucasian populations [13], in which APOA5 S19W tags a unique common haplotype (reported as APOA5*3), whereas APOA5 SNP1, SNP2, and SNP3 tag another common haplotype (APOA5*2). Although S19W is physically amid other polymorphisms, it has been repeatedly observed that the genetic effect driven by S19W is independent of the others [22]. A potential explanation is that the minor allele of APOA5 S19W occurred at a different chromosome from those of the other polymorphisms. For haplotype APOA*2, because the examined variants showed strong LD, it is difficult to tease out whether the observed associations are caused by either polymorphism.

Fasting TG levels also tended to be higher in diabetic women carrying the homozygotes of allele APOC3 –455C. ApoC-III is a 79-amino-acid protein synthesized by liver and intestine, which is an essential constituent of circulating particles rich in TG [7]. Earlier studies indicate that variants in APOC3 gene are primarily associated with TG levels [11,12]. Variant –455T/C falls within a previously identified insulin response element and FOXO1 binding site of APOC3 gene promoter [22]. Our results are consistent with an earlier association between this variant and elevated TG levels in patients with metabolic syndrome [23].

Because of the physical proximity of APOA5 gene and A1/C3/A4 cluster, one question arose as to whether the observed genetic associations with APOA5 and APOC3 genes are actually due to the common causal mutation. Our data did not support such a hypothesis. First, in this study, a LD breakdown was observed between APOA5 and APOC3 genes. Another study examined the LD structure in the APOA1/C3/A4/A5 region by typing 49 SNPs in Caucasian of northern European origin also indicates that the APOA5 haplotype block is separated from the other genes by a region of significantly increased recombination [16]. These data together suggest that the associations observed for APOA5 and APOC3 polymorphism are unlikely accounted by the allelic correlation. Second, using a subset analysis, we also demonstrated that the genetic effects of APOA5 and APOC3 polymorphisms are independent of each other. Such an inference is also in agreement with the findings from animal models [24].

In this study, we also found that APOA4 +347T/A (T347S) was associated with decreased levels of HDL-C in a dominant inheritance model. ApoA-IV is a 46 kDa glycoprotein that circulates freely or in association with chylomicrons and HDLs [25]. T347S substitution generate apoA-IV-1A isoform of lower lipid affinity [26] and has been associated

with changes in the concentration of both apo B and apo A-I-containing lipoproteins and cholesterol levels [27]. Our finding is in line with the association of this variant with an increased risk of coronary heart disease [28].

Because multiple genetic markers and phenotypes were tested, it may be argued that Bonferroni correction should be applied. Bonferroni correction assumes that some numbers of independent test are performed. However, considering that the comparisons were not independent of each other due to tight LD among the polymorphisms and high correlation between the phenotypes, Bonferroni correction may be overly conservative. Moreover, the high consistency between our findings with previous evidence supports a robust relation between the variability in apolipoprotein gene cluster and the levels of TG and HDL-C.

As a potential limitation, restriction of the analysis to fasting samples (TG) and missing data in both genotype and lipids may somewhat affect the power of this study. Population stratification may influence the observed associations. However, our populations are racially homogeneous, with the majority of the participants being white (~96%). Further adjustment for ethnicity or removing the minorities from the analyses did not change our results. In addition, our findings are limited to diabetic women and may be not generalized to the general population.

Patients with type 2 diabetes have increased risk of coronary heart disease [1]. In diabetic patients, elevated TG and lowered HDL-C are the major proatherogenic factors. To the best of our knowledge, this study is among the first investigating the associations of the variability of APOA1/C3/A4/A5 gene cluster and the lipids in patients with type 2 diabetes. Our results indicate that two gene components of the cluster (APOA5 and APOC3) may independently contribute to the variance of TG levels in diabetic patients. Also, the variability at APOA4 gene may affect HDL-C levels. Genotyping of polymorphisms in the APOA1/C3/A4/A5 gene cluster can be of clinical interest to identify diabetic women at highest risk to develop diabetic dyslipidemia and thus be directed toward a more frequent monitoring of the lipid homeostasis and/or more aggressive lipid management.

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