

A single nucleotide polymorphism of interleukin-6 gene is related to plasma adrenomedullin levels

Short title: IL6 SNP and plasma ADM level

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Abstract

Objective: Elevated plasma adrenomedullin (ADM) levels are associated with cardiovascular diseases. Single nucleotide polymorphisms (SNPs) in the gene encoding ADM (*ADM*) are associated with plasma ADM levels. The presence of a nuclear factor for interleukin-6 (IL-6) expression binding site in the promoter region of the *ADM* gene suggests a possible relationship between the expression of the ADM and IL-6. Therefore, we investigated if plasma ADM levels are related to SNPs in the gene encoding IL-6 (*IL6*).

Methods: Plasma ADM levels were measured in 476 subjects in the Hong Kong Cardiovascular Risk Factor Prevalence Study-2 (CRISPS2). The subjects were genotyped for three tagging SNPs in the *IL6* gene.

Results: The minor allele frequencies of the *IL6* SNPs rs17147230, rs1800796 and rs2069837 were 41.8%, 20.0% and 15.4% respectively. The tagging SNP, rs17147230, was associated with plasma ADM levels after adjusting for age and sex ($\beta=-0.096$, $P=0.034$). The association was significant in women ($\beta=-0.115$, $P=0.021$) but not in men. Amongst all subjects, plasma ADM levels decreased with an increasing number of minor alleles of rs17147230 in multivariate analysis ($P=0.034$). Compared to subjects with the AA genotype, subjects with the TT genotype had plasma ADM levels 12.8% lower (95% CI: 0.6%-23.5%, $P=0.041$). Haplotype analysis demonstrated a significant association of the haplotype ACA with plasma ADM levels in women ($P<0.05$).

Conclusion: Plasma ADM levels are related to the SNP rs17147230 in *IL6* gene. The effect of the polymorphism on inflammation and cardiovascular disease remains to be determined.
(242 words)

Introduction

Adrenomedullin (ADM) is a vasodilatory peptide initially discovered in human pheochromocytoma tissues (1). The gene encoding ADM (*ADM*) is located on chromosome 11p15.4, which encodes prepro-ADM, the precursor form of circulating ADM. ADM is both a paracrine hormone (2) and an adipokine (3), expressed and secreted in different tissues such as the adrenal medulla, heart, kidney and pancreatic islets (4). Plasma ADM levels are elevated in hypertension, renal failure, septic shock and type 2 diabetes (T2DM) (5). ADM also regulates acute and chronic inflammatory responses (6, 7).

Recently, plasma ADM levels have been suggested as a biomarker to predict cardiovascular events or diseases (8). It is important to understand the factors that may account for the change in plasma ADM levels, which may be influenced by genetic variants (9). As such, factors that could modulate ADM gene expression may alter plasma ADM levels. In the discovery of the *ADM* gene, a nuclear factor for the interleukin-6 expression (NF-IL6) binding site was found in the *ADM* gene promoter region (10). This arouses interest in the possibility of an interaction between ADM and IL-6. Indeed, in our previous study, plasma IL-6 levels decreased significantly with increasing plasma ADM tertiles (9).

Plasma ADM levels have been shown to correlate with single nucleotide polymorphisms

(SNPs) in its gene. Given the presence of a NF-IL6 binding site in *ADM* gene, which implies the regulation of IL-6 and ADM expression by a common nuclear factor, we hypothesized that SNPs in the gene encoding IL-6 (*IL6*) may have an effect on plasma ADM levels. The human *IL6* gene is located on chromosome 7p21, where several common SNPs have been identified in the promoter region that could affect *IL6* transcription and circulating IL-6 levels (11, 12). Therefore, we investigated the association between SNPs in the *IL6* gene with plasma ADM levels.

Methods

Subjects

The Hong Kong Cardiovascular Risk Factor Prevalence Study (CRISPS) is a prospective cohort study of cardiovascular risk factors in a general population. In 1995-1996, a random sample of 2,895 subjects were first recruited for CRISPS1. In the period from 2000-2004, 1,944 subjects were followed up in CRISPS2 (13, 14). Plasma ADM was measured in 476 randomly selected subjects from the sub-cohort of 1,944 subjects. Written informed consent was given by all participants. The study protocol was approved by the Ethics Committee of the University of Hong Kong and the institutional review board of the Hong Kong West Cluster of Hospitals.

SNP selection

Tagging SNPs in *IL6* were selected from the HapMap Han Chinese (phase II data, release 24) as described previously (15). Four tagging SNPs could capture all six SNPs from the 5kb region upstream to 2kb downstream region of the gene. The SNP rs2069852 was excluded for analysis due to the deviation from Hardy Weinberg equilibrium ($P < 0.05$) after genotyping. Three tagging SNPs, which could capture the remaining five out of six genotyped SNPs, were selected (position 22,728,345-22,740,141, namely rs17147230, rs1800796, and rs2069837) with $r^2 \geq 0.9$ and minor allele frequency (MAF) ≥ 0.05 (Figure 1). Genotyping was performed using the MassARRAY system (Sequenom, San Diego, CA) with the iPLEX assay.

Plasma ADM levels and other variables

Plasma samples underwent extraction and ADM levels were measured by radioimmunoassay (RIA) using a method adapted from previous studies (16, 17). Plasma ADM immunoreactivity was measured using a commercially available RIA kit from Peninsula Laboratories (Belmont, CA, USA) in 235 subjects and Phoenix Pharmaceuticals (Burlingame, CA, USA) in 241 subjects, both with the same protocol and internal controls were included. Plasma IL-6 was measured in 438 subjects in duplicate using high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits (Bender MedSystems

GmbH, Vienna, Austria) (18).

Other clinical parameters such as body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), post oral glucose tolerance test (OGTT) glucose level, homeostatic model assessment of insulin resistance index (HOMA-IR), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, adiponectin, high-sensitivity C-reactive protein (hsCRP), fibrinogen, alkaline phosphatase (ALP) and γ -glutamyl transferase (GGT) were measured as previously described (9, 13, 14, 18). Full measurement of glucose level and plasma IL-6 level, complete information on regular drinking and exercise status were obtained in 377 out of 476 subjects. Regular drinking was defined as drinking alcohol at least once a week. Regular smoking was defined as smoking cigarettes currently, based on the criteria of having a habit of consuming cigarettes weekly. Regular exercise was defined as doing exercise for at least 30 minutes at least once a week in the previous month. Presence of cardiovascular diseases was based on self-reported stroke or ischemic heart diseases status from the questionnaire.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL., USA). Normally distributed data were presented as mean \pm SD. Variables with skewed

distribution were log-transformed before analysis and were presented as geometric mean (95% confidence interval). The PLINK program (version 1.0.7) was used to assess the overall association of various *IL6* SNPs with ADM levels using additive allelic model (19). The independent association of plasma ADM level with *IL-6* SNPs was assessed by multiple linear regression analysis, with adjustment for age and sex in the initial model, followed by other biochemical and demographic parameters in the final adjustment model. Relationship of ADM level with other confounding factors was also assessed. Correction of multiple SNP testing was performed using permutation testing with the simulation repeated 1,000 times. Assessment of linkage disequilibrium (LD) and haplotype analysis was performed using PLINK software. Only haplotypes with frequency > 5% were tested. Omnibus testing was first applied to assess the overall P value of the global variation at the locus. Then the specific effect of each haplotype was tested by comparing with all other haplotypes combined. Sex-specific analysis was carried out using the regression modeling and haplotype analysis. The interaction effect of sex was also investigated. For highly correlated variables such as BMI and waist circumference, only one was entered into the model for analysis to avoid multicollinearity. Percentage changes were expressed for plasma IL-6 and ADM for ease of interpretation.

Results

Genotyping of SNPs in IL6 gene

The MAFs of the *IL6* SNPs rs17147230, rs1800796 and rs2069837 were 41.8%, 20.0% and 15.4% respectively. None of the three SNPs showed significant deviation from the Hardy-Weinberg equilibrium ($P>0.05$). The pairwise linkage disequilibrium (r^2) between rs17147230 and rs1800796, rs1800796 and rs2069837, and rs17147230 and rs2069837 was 0.150, 0.728 and 0.081 respectively.

Association analysis and clinical characteristics

Among the three *IL6* SNPs, only rs17147230 was significantly associated with plasma ADM levels ($\beta=-0.096$, $P=0.034$) (Figure 2). As shown in Table 1, plasma ADM levels significantly decreased from 12.1 pmol/L to 10.6 pmol/L with an increasing number of minor T alleles present. The association remained significant after adjusting for other clinical parameters shown in Table 1, but further adjustment for plasma IL-6 resulted in loss of significance in the association (Table 2). Adjusting for plasma creatinine alone did not result in great change in effect size (adjusted $r^2=0.022$), while the association became marginally insignificant ($P=0.053$, data not shown). The association of rs17147230 with plasma ADM was significant in women and not in men, but there was no significant interaction with sex ($P=0.207$).

The other two SNPs were not significantly associated with plasma ADM. For rs1800796, plasma ADM in subjects with CC, CG and GG genotypes were 11.6, 11.2 and 13.1 pmol/L respectively whereas the corresponding levels in subjects with AA, AG and GG genotypes at rs2069837 were 11.7, 10.9 and 12.6 pmol/L respectively (both $P>0.05$ after adjusting for age and sex). Other clinical characteristics according to the genotypes of rs17147230 are shown in Table 1. HOMA-IR decreased with increasing number of the minor allele T ($P=0.045$), and carriers of the minor T allele of rs17147230 tended to have less regular exercise than non-carriers ($P=0.042$). There was no significant difference in other clinical characteristics across different genotypes.

Multivariate analysis of rs17147230

In multivariate analysis using *IL6* SNP as a categorical variable (Supplementary Table S1), the presence of two minor T alleles of rs17147230 resulted in -12.8% (95% CI: -23.5%, -0.6%) change in plasma ADM levels ($P=0.041$), after adjusting for age and sex. In sex-specific analysis, TT genotype in females resulted in -21.4% (95% CI = -35.7%, -4.0%) change in ADM levels ($P=0.019$), with an effect size much greater than males who only showed a 2.2% decrease ($P=0.799$). The association remained significant after adjusting for other clinical parameters, but further adjustment for plasma IL-6 resulted in loss of

significance in the association ($P=0.076$, 0.969 and 0.064 for all subjects, men and women respectively).

Haplotype analysis

After constructing haplotypes for rs17147230, rs1800796 and rs2069837, three haplotypes with frequency $> 5\%$ were found, namely, ACA, TCA and TGG with a frequency of 54.5% , 25.5% and 12.0% respectively. Haplotype analysis revealed similar results in which the association tended to be more prominent in women than in men (overall $P=0.056$ and 0.92 respectively), although the association did not reach statistical significance. In women, the haplotype ACA was associated with ADM levels that were higher by 10.6% (95% CI: 0.2% , 22.1% , $P=0.047$) compared to all other haplotypes. This compared to a 1.4% increase associated with the same haplotype in men (95% CI: -6.4% , 9.8% , $P=0.736$).

Discussion

Plasma ADM levels are elevated in cardiovascular disease and diabetes mellitus, and are subject to multi-factorial control. The presence of an NF-IL6 binding site in the *ADM* promoter region suggests that the expression of IL-6 and ADM may be related. IL-6 has been found to be a regulator of the production of acute phase proteins like CRP and fibrinogen (19), which are also inflammatory markers. Genetic variants in *IL6* gene are also

associated with CRP and fibrinogen levels (16, 21, 22). This study identifies for the first time an association between an *IL6* SNP and plasma ADM levels. This deepens our understanding of the interactions between ADM and other markers of inflammation and cardiovascular disease.

Previous studies on *IL6* and *ADM* SNPs have identified SNPs located in the promoter region. Due to the presence of a NF-IL6 binding site on both *ADM* and *IL6* genes in the promoter region, we may suspect that the association could be due to induction of the nuclear factor by plasma IL-6. For instance, a mutation in the NF-IL6 binding site could reduce promoter activity and gene transcription (10). The SNP rs17147230 is located at 411 bp near the 5' end of *IL6* gene while the NF-IL6 binding site is located between -145 and -158 region (23). Hence, the SNP is not located at the binding site for NF-IL6. Nevertheless, this study demonstrates that genetic influence from the *IL6* gene outside the promoter region could modulate plasma ADM levels. This effect may not be mediated through the activation of the NF-IL6 site. We speculate that the effect of rs17147230 may be mediated through interacting with an *ADM* SNP rs4910118 reported in our previous study, since interaction analysis showed that the dominant allele of rs4910118 interacted with rs17147230 ($P<0.025$). Further studies are needed to reveal the detailed mechanism of the effect on ADM levels.

ADM has been shown to predict dysglycemia development (24), and mid-regional pro-adrenomedullin (MR-proADM) had good prognostic value in patients with heart failure after an acute myocardial infarction (25). In the light of our new findings, the association of ADM levels with IL-6 may help refine the predictive value of ADM in cardiovascular diseases. Both plasma ADM and IL-6 levels are elevated in various diseases like heart failure and could reflect progression and deterioration of diseases (26, 27). Therefore, a high IL-6 level may indicate possible elevation of ADM levels or vice versa. However, we previously demonstrated that plasma IL-6 levels correlated with plasma ADM (9), in which IL-6 levels decreased with increasing ADM tertiles. This raises the possibility that IL-6 or ADM may counter-regulate each other. Determining the sequence of elevation in IL-6 and ADM levels might shed light on the causal relationship between IL-6 and ADM and help to understand the regulation of inflammation. Interestingly, rs17147230 was associated with ADM but not IL-6 levels, the latter was associated with another *IL6* SNP rs1800796 in our previous study (15). Therefore studying the interaction between the two SNPs may help understand the mechanism of the regulation of ADM level by *IL-6*.

This report is in agreement with previous studies showing a role for the *IL6* gene in regulating inflammatory biomarkers (20). Various SNPs such as -572G/C and -174G/C in

the *IL6* promoter have been reported to be associated with inflammation and cardiovascular disease (28, 29). But for rs17147230 in this study, it is not a significant predictor of cardiovascular diseases according to our results. Nevertheless, genetic polymorphisms could only explain a small part of the variation in ADM levels. Clearly, the regulation of ADM is subject to other genetic and environmental influences. Recently a report suggested the effect of several confounders including age, sex, BMI and estimated glomerular filtration rate (eGFR) on plasma MR-proADM level (30). However our results did not show a significant confounding effect by plasma creatinine on the association of *IL6* SNP and ADM level (Table 2), while the association appears stronger in women without a significant sex interaction. There have been experimental studies reporting the effect of sex hormones on ADM expression. In rats, female sex hormone has been found to increase the expression of adrenomedullin-2 receptor (ADM2) and increase ADM-induced vasodilation (31). Plasma ADM levels are known to be raised in pregnancy (32), but none of our subjects were pregnant. ADM is expressed in the female gonads and reproductive tract (33). However, ADM is also expressed in the male reproductive organs including the testis, prostate and epididymis, and moreover, testosterone at physiological concentrations is reported to increase adrenomedullin production in human endothelial cells (34). Hence, the effect of sex on such an association cannot be confirmed in this study. The insufficient sample size may limit the statistical power to detect a significant interaction effect and significant

association in men. Therefore a larger sample is necessary to clarify the effect of sex on the association. Nevertheless, as ADM plays a key role in inflammation, cardiovascular disease and cancer, and its plasma level is an indicator of prognosis, elucidating the regulation of ADM warrants further studies.

The minor allele of rs1717230 was associated with decreased HOMA-IR and less regular exercise. Although we expect a relationship between insulin resistance and frequent exercise (35), our study showed no correlation between HOMA-IR and frequent exercise ($P=0.320$).

A possible reason for the discrepancy is that the regular exercise status in our analysis only takes the duration into account, but not the nature and intensity of exercise.

In conclusion, plasma ADM levels are associated with the *IL6* SNP rs17147230 in a Hong Kong Chinese population. Further work is needed in order to elucidate the mechanism and interactions of the SNP with the *ADM* gene to give rise to the change in plasma ADM levels.

Conflict of Interest

The authors declare that there is no conflict of interest.

Financial Disclosure

Nothing to declare.

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Figure Legends

Figure 1. A diagram showing the interleukin-6 gene (*IL6*). Exons and introns are shown as boxes and horizontal lines with the respective sizes indicated. The minor allele frequencies of the tagging single nucleotide polymorphisms (SNPs) in HapMap Han Chinese are indicated in brackets as shown above.

Figure 2 Association of single nucleotide polymorphism (SNP) with plasma adrenomedullin levels. The error bars show 95% CI., of the mean. P values were calculated using natural log-transformed levels after adjusting for age and sex, and remained significant after multiple testing correction by permutation method.

Table 1. Subject characteristics according to genotypes of rs17147230

Characteristics	All subjects	AA	AT	TT	P for trend
n	476	151	252	73	-
Age (years)	50.9±10.8	51.7±11.2	50.5±10.9	50.9±9.4	0.461
Women (%)	50.4	49.7	50.4	52.1	0.802
BMI (kg/m ²)	23.9±3.8	23.7±3.3	24.1±3.7	23.6±3.2	0.845
Waist circumference (cm)	79.7±10.1	79.4±9.5	80.2±10.7	78.9±9.5	0.815
SBP (mmHg)	122.8±18.1	123.4±17.7	122.6±18.0	122.2±19.4	0.869
DBP (mmHg)	76.8±10.1	76.7±9.4	77.2±10.9	75.6±10.2	0.746
Triglycerides (mmol/L)*	1.19 (1.10-1.27)	1.18 (1.08-1.28)	1.24 (1.16-1.33)	1.03 (0.92-1.16)	0.318
HDL cholesterol (mmol/L)	1.37±0.39	1.41±0.41	1.35±0.38	1.39±0.35	0.360
LDL cholesterol (mmol/L)	3.26±0.84	3.34±0.89	3.21±0.80	3.26±0.87	0.400
Glucose 2 hours after OGTT (mmol/L)*	6.83 (6.55-7.11)	6.79 (6.36-7.24)	7.00 (6.71-7.31)	6.38 (5.79-7.02)	0.478
HOMA-IR*	1.72 (1.56-1.88)	1.82(1.65-2.01)	1.74(1.60-1.89)	1.48 (1.28-1.70)	0.045
ADM (pmol/L)*	11.52 (11.03-12.00)	12.14 (11.26-13.02)	11.45 (10.78-12.12)	10.56 (9.44-11.67)	0.034

Adiponectin (mg/L)*	6.89 (6.44-7.34)	7.12(6.39-7.94)	6.50(6.00-7.04)	7.88(6.89-9.01)	0.588
Fibrinogen (g/L)	2.92±0.53	2.90±0.56	2.93±0.54	2.95±0.47	0.331
hsCRP (mg/L)*	0.56 (0.47-0.65)	0.53 (0.46-0.62)	0.57(0.51-0.65)	0.58(0.47-0.72)	0.423
GGT (U/L)*	23.89 (21.47-26.31)	23.54 (21.29-26.02)	24.48(22.66-26.44)	22.64(19.59-26.16)	0.992
ALP (U/L)*	68.18 (66.39-69.96)	67.68 (65.15-70.52)	69.13 (64.24-71.67)	65.94 (64.16-70.84)	0.997
IL-6 (pg/L)*	0.47 (0.41-0.53)	0.45 (0.40-0.52)	0.51(0.46-0.57)	0.38(0.29-0.51)	0.504
Creatinine (umol/L)	90.61±15.40	90.45±14.99	90.73±15.72	90.53±15.30	0.543
Cardiovascular diseases (%)	4.6	4.6	5.2	2.7	0.844
Current smoking (%)	18.9	20.5	18.3	17.8	0.583
Regular drinking (%)	10.3	10.7	10.2	10.0	0.901
Regular Exercise (%)	29.3	37.0	26.0	25.0	0.042

Data are expressed as mean±SD or percentage

P values were adjusted for age and sex

*Variables with skewed distribution are expressed as geometric mean (95% CI).

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OGTT, oral glucose tolerance test; HOMA-IR, homeostatic model assessment of insulin resistance index; ADM,

adrenomedullin; hsCRP, high-sensitivity C-reactive protein; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; IL-6, interleukin-6

Table 2. Association of the number of minor alleles in rs17147230 with plasma ADM

	Model 1	Model 2	Model 3	Model 4
n	476	436	411	377
β^*	-0.096	-0.104	-0.109	-0.092
P	0.034	0.031	0.030	0.077
Adjusted r^2	0.025	0.037	0.028	0.038

Analysis was performed with plasma ADM (ln-transformed) as the dependent variable.

Only the standardized β coefficient for the number of minor alleles present is shown.

Model 1: Adjusted for age and sex

Model 2: Further adjusted for biochemical parameters including BMI, HDL

cholesterol ,SBP, fibrinogen and natural log of triglycerides, , HOMA-IR, post OGTT

glucose level, hsCRP, adiponectin, ALP, GGT and plasma creatinine level

Model 3: Further adjusted for lifestyle factors including regular drinking, smoking and exercise, and presence of cardiovascular diseases

Model 4: Further adjusted for plasma IL-6 level (In-transformed)

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; HDL, high-density

lipoprotein; OGTT, oral glucose tolerance test; HOMA-IR, homeostatic model assessment

of insulin resistance index; hsCRP, high-sensitivity C-reactive protein; GGT,

gamma-glutamyltransferase; ALP, alkaline phosphatase; IL-6, interleukin-6

Figure 1

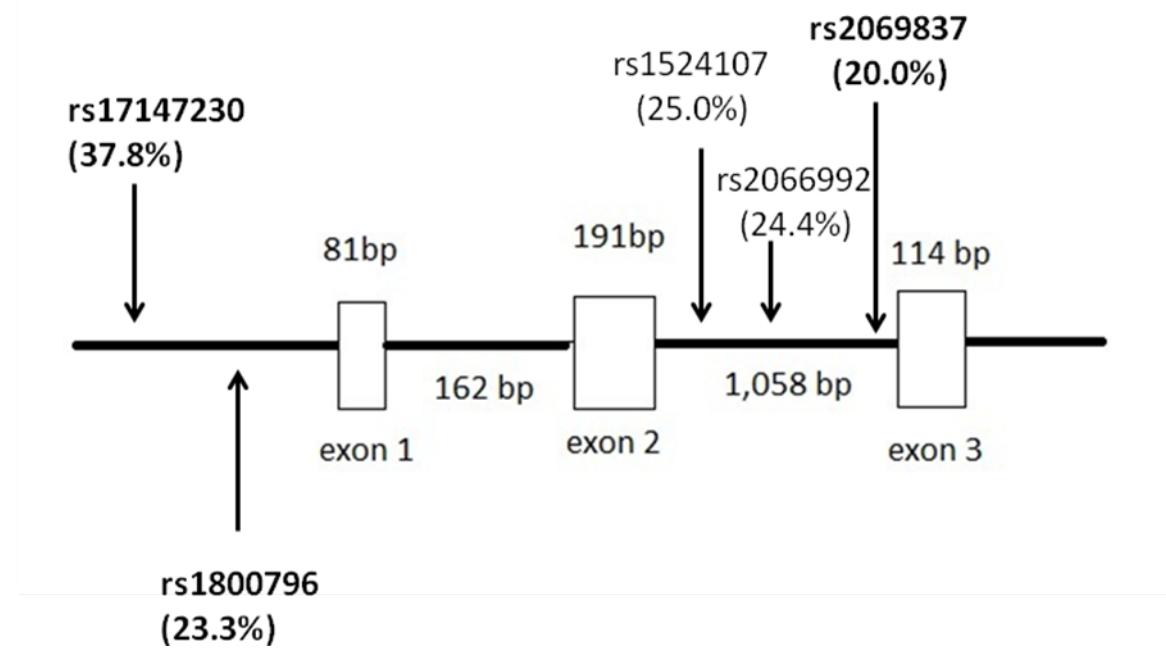
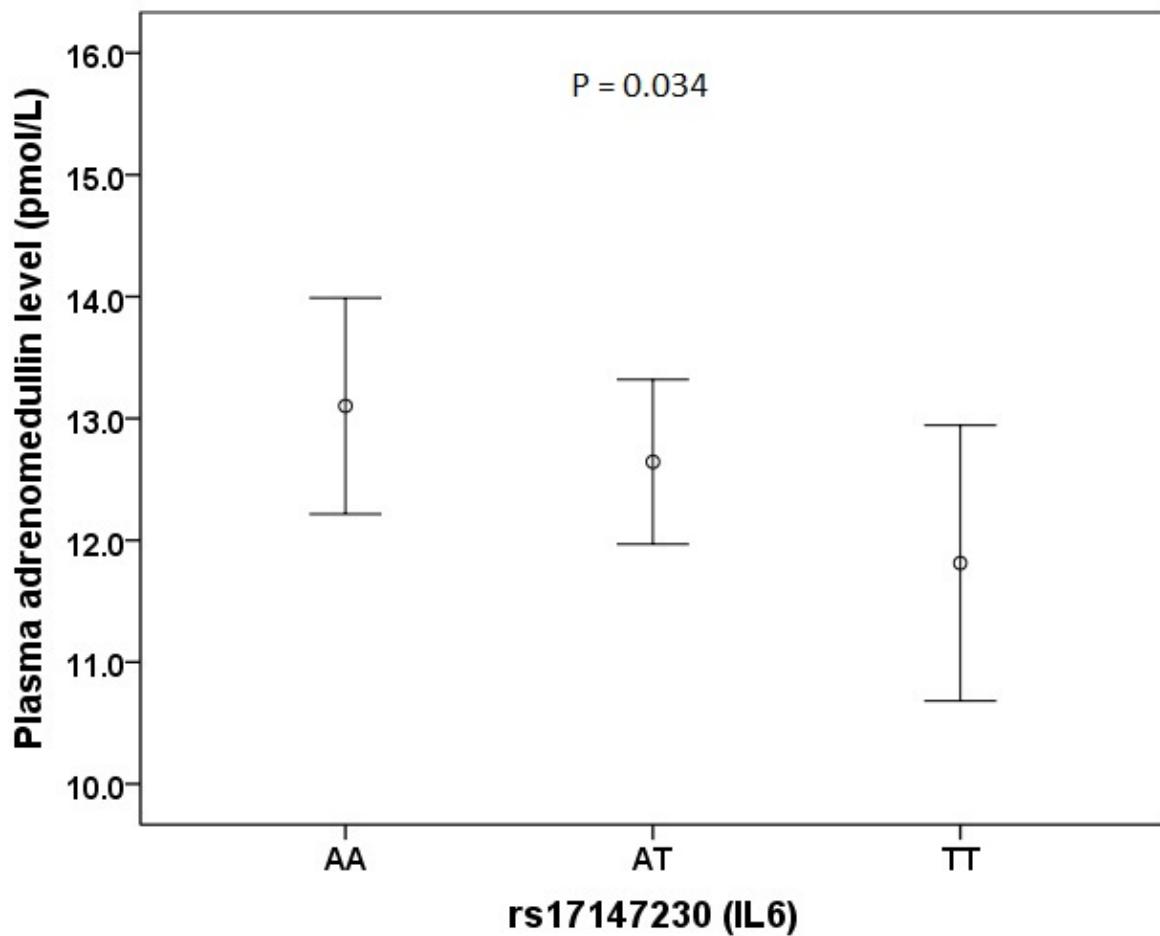


Figure2



Online Supplementary Tables

Supplementary Table S1. Multivariate analysis of rs17147230 for plasma ADM levels (ln-transformed)

Model	Genotype	All subjects (n = 476)		Men (n = 236)		Women (n = 240)	
		B (SE)	P value	B (SE)	P value	B (SE)	P value
Model 1	TT	-0.137 (0.067)	0.041	-0.022 (0.086)	0.799	-0.241 (0.102)	0.019
	AT	-0.069 (0.048)	NS	-0.039 (0.061)	NS	-0.089 (0.075)	NS
	AA	referent	-	referent	-	referent	-
Model 2	TT	-0.149 (0.070)	0.033	-0.028 (0.090)	NS	-0.266 (0.108)	0.014
	AT	-0.068 (0.051)	NS	-0.020 (0.067)	NS	-0.082 (0.078)	NS
	AA	referent	-	referent	-	referent	-

Model 1: Adjusted for age and sex (adjusted for age only in sex-specific analysis)

Model 2: Further adjusted for biochemical parameters including BMI, HDL cholesterol ,SBP, fibrinogen and natural log of triglycerides, ,

HOMA-IR, post OGTT glucose level, hsCRP, adiponectin, ALP, GGT and plasma creatinine level

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; HDL, high-density lipoprotein; OGTT, oral glucose tolerance test;

HOMA-IR, homeostatic model assessment of insulin resistance index; hsCRP, high-sensitivity C-reactive protein; ALP, alkaline phosphatase;

GGT, gamma-glutamyltransferase

Supplementary Table S2. Association of haplotype with plasma ADM level in women

Haplotype	rs17147230	rs1800796	rs2069837	Frequency	Beta	P value ^a	Overall P*
1	A	C	A	0.539	0.101	0.0471	0.056
2	T	C	A	0.259	-0.114	0.0555	
3	T	G	G	0.117	-0.143	0.0693	
4	T	G	A	0.047	0.151	0.195	
5	A	G	G	0.033	0.0579	0.692	

*Omnibus test was initially performed to assess the P value of the overall variation at the locus.

^aAdjusted for age only