Genes, inflammation, and age-related diseases

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Chapter 2

Lymphotoxin-alpha C804A polymorphism is a risk factor for stroke. The PROSPER study

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Abstract

Inflammation plays a prominent role in the development of atherosclerosis, which is the most important risk factor for vascular events. Lymphotoxin alpha (LTA) is a pro-inflammatory cytokine and is found to be expressed in atherosclerotic lesions. We investigated the association between the C804A polymorphism within the LTA gene and coronary and cerebrovascular events in 5804 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). The primary endpoint was the combined endpoint of death from coronary heart disease, non-fatal myocardial infarction, and clinical stroke. Secondary endpoints were the coronary and cerebrovascular components separately. All associations were assessed with a Cox-proportional hazards model adjusted for sex, age, pravastatin use, and country. Our overall analysis showed a significant association between the C804A polymorphism and the primary endpoint (p=0.03). After stratification for gender, this association was found only in males. Furthermore, we found that the association between the C804A polymorphism and the primary endpoint was mainly attributable to clinical strokes (p=0.02). The C804A polymorphism in the LTA gene associates with clinical stroke, especially in men. But further research is warranted to confirm our results.

Introduction

Inflammation plays a prominent role in the development of atherosclerosis, which is the most important risk factor for vascular events (1-3). Lymphotoxin-alpha (LTA), also known as tumor necrosis factor beta (TNFβ), is a pro-inflammatory cytokine which activates a cytokine cascade by inducing interleukin-1 (4;5). LTA is expressed in atherosclerotic lesions and induces the expression of a number of molecules involved in atherogenesis (6;7). Moreover, atherosclerotic lesions in LTA knock-out mice are significantly smaller compared to LTA wild-type mice (7).

Genetic variation in the LTA gene has been associated with vascular disease, like myocardial infarction (MI) and stroke (6;8-12). For example, Laxton *et al* have reported an association between the LTA C804A polymorphism and the severity of atherosclerosis in patients with coronary artery

disease (6). They found that carriers of the 804A variant had a higher risk for severe atherosclerosis. Furthermore, they found that only the male carriers had this higher risk.

Based on this evidence we hypothesized that genetic variation in the LTA gene is associated with vascular disease, especially in men. We assessed the association between the LTA C804A polymorphism and coronary and cerebrovascular events in participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER).

Methods

A detailed description of the protocol of the PROSPER study has been published elsewhere (13;14). Here a short outline is provided.

Participants

The PROSPER study was a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo for an average 3.5-year intervention period. The primary endpoint in the study was the combined endpoint of death from coronary heart disease (CHD), non-fatal myocardial infarct (MI), and occurrence of clinical stroke, either fatal or non-fatal. When death occurred following a non-fatal stroke within a period of 28 days, it was regarded as a fatal stroke. Secondary endpoints were the separate coronary and cerebrovascular components of the primary endpoint. All endpoints were adjudicated by the study endpoint committee. More details about the diagnosis of the cerebrovascular and coronary events within the PROSPER study has been published elsewhere (13).

Genotyping

The single nucleotide polymorphism (SNP) C804A (rs1041981) in the LTA gene was selected based on its allele frequency and available literature. A genome wide scan showed two SNPs within the LTA gene that were associated with vascular disease (15). An additional study showed that the LTA C804A polymorphism is indeed functional and results in an amino-acid change T26N (8). The SNP was genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA). Amplification reactions and parameters were based on the manufacturer's instructions. Genotyping of the LTA C804A polymorphism was successful in 5389 participants. The results of the remaining patients are missing due to lack of DNA or inconclusive genotyping.

Statistical analysis

The program Haploview (16) was used to estimate the allele frequency and to test the consistency of the genotype frequency at the SNP locus with Hardy-Weinberg equilibrium. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated with Cox-proportional hazards model. Subjects who withdrew consent or deceased during the study were censored on the date they left the study. All analyses were adjusted for sex, age, pravastatin use, and country. All analyses were additionally sex-stratified performed. To assess whether the PROSPER study is large enough to gain statistical power in a sex-stratified analysis, we performed power calculations (Quanto software, http://hydra.usc.edu/gxe). Based on a total number of 124 cases with a fatal or non-fatal stroke in males (n=2617), we calculated that with a minor allele frequency (MAF) of 20% in a log-additive model, a baseline risk of fatal or non-fatal stroke of 4%, and a gene effect of 1.5, the statistical power to detect the association between the polymorphism and fatal or non-fatal stroke is 98% for a p-value of 5×10^{-2}.

The SPSS software (version 12.0.1, SPSS Inc, Chicago, Ill) was used for all statistical analyses. P-values lower than 0.05 were considered statistically significant.

Results

Genotyping of the LTA C804A polymorphism was successful for 5389 subjects, the results of the remaining subjects were missing because of insufficient DNA or incomplete genotyping (success rate 93.2%). Table 1 represents the baseline characteristics of all 5389 participants divided over categories of the C804A polymorphism. About 50% of the participants were male (N=2617) and the mean age of all subjects at study entry was 75.3 years. The mean follow-up time was 3.2 years (range 2.8-4.0) for participants who did not die or withdrew consent. There were no differences in baseline characteristics between genotype groups.

Table 1: Baseline characteristics of the participants of the Prosper study (N=5389).

	Lymphotoxin-alpha C804A		
	Wt/Wt (N=2102)	Wt/Var (N=2547)	Var/Var (N=740)
Continous variates (mean, SD)			
Age, (years)	75.4 (3.3)	75.3 (3.4)	75.2 (3.3)
Body Mass Index, (kg/m2)	26.8 (4.2)	26.9 (4.2)	26.7 (4.3)
Total cholesterol , (mmol/L)	5.7 (0.9)	5.7 (0.9)	5.6 (0.9)
LDL cholesterol, (mmol/L)	3.8 (0.8)	3.8 (0.8)	3.8 (0.8)
HDL cholesterol, (mmol/L)	1.3 (0.4)	1.3 (0.4)	1.3 (0.4)
Categorical variates (N, %)			
Female	1067 (51)	1317 (52)	388 (52)
Current smoker	547 (26)	716 (28)	193 (26)
History of diabetes	219 (10)	270 (11)	93 (13)
History of hypertension	1327 (63)	1550 (61)	440 (60)
History of angina	580 (28)	666 (26)	194 (26)
History of claudication	129 (6)	187 (7)	47 (6)
History of myocardial infarction	265 (13)	366 (14)	88 (12)
History of vascular disease	938 (45)	1127 (44)	313 (42)
History of stroke or TIA	253 (12)	279 (11)	75 (10)

The major allele frequency of the C804A polymorphism was 63% in all participants. The C804A polymorphism showed no significant deviation from Hardy-Weinberg equilibrium ($p=0.77$). The genotype frequencies between the three countries differed significantly ($p<0.01$, data not shown), for Scotland the major allele frequency was 62%, for Ireland 61% and for the Netherlands 66%. Therefore all analyses were adjusted for country to control for population stratification.

Figure 1 shows the association between the C804A polymorphism and the primary endpoint. In the overall analysis a significant relation with the primary endpoint was found (p=0.03). The significant association of the overall analysis was mainly due to homozygous carriers of the variant (HR 1.27, 95%CI 1.03-1.55). Furthermore, after stratification for gender, the relation with the primary endpoint was especially present in males (HR 1.36, 95%CI 1.04-1.77) and not in females (HR 1.15, 95%CI 0.84-1.58), although the interaction term for gender with genotype was not significant (p=0.60).

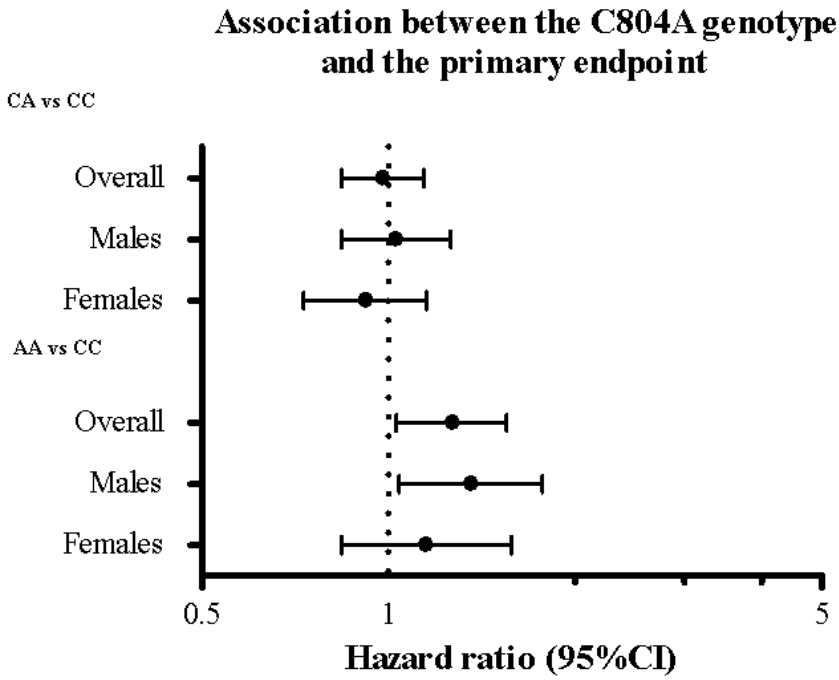

Figure 1: Association between the lymphotoxin-alpha C804A genotype and the primary endpoint in the participants of the Prosper study (n=5389).
The primary endpoint included coronary heart disease death, non-fatal myocardial infarct, and fatal or non-fatal stroke. In the overall group a significant association between the C804A genotype and primary endpoint was found (p=0.03), namely because an increased risk for the primary endpoint in males (HR 1.36, 95%CI 1.04-1.77).

We assessed the association of the C804A variant with the coronary and cerebrovascular endpoints separately. The association with the primary endpoint in men was mainly attributable to occurrence of clinical strokes and not to coronary events (figure 2). The increased risk for clinical stroke for the heterozygous carriers was 1.43 (95%CI 0.95-2.15) and for the homozygous male carries 2.07

(95%CI 1.24-3.44) (p-trend=0.02). In women, there was no significant association for clinical stroke for both the heterozygous carriers (HR 0.94, 95%CI 0.63-1.41) and the homozygous carries (HR 1.47, 95%CI 0.89-2.44).

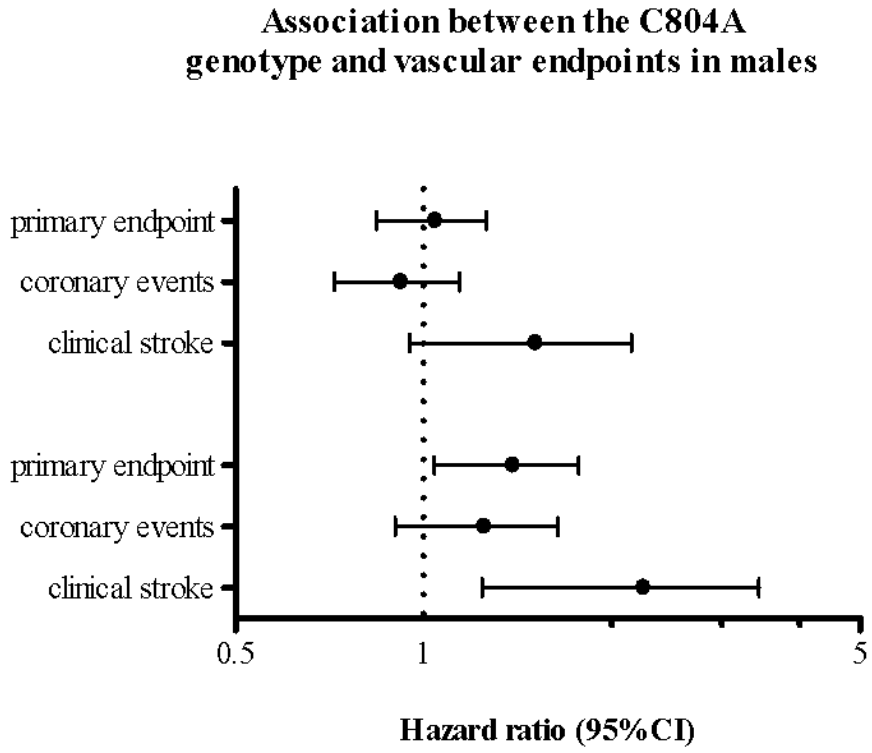

Figure 2: Association between the lymphotoxin-alpha C804A genotype and vascular endpoints in male participants (N=2617).
The primary endpoint included coronary heart disease death, non-fatal myocardial infarct, and fatal or non-fatal stroke. Coronary events are coronary heart disease death and non-fatal myocardial infarct. Clinical stroke consists of fatal or non-fatal stroke. The association in males with the primary endpoint is namely due to an increased risk of clinical stroke (p=0.02). The increased risk for clinical stroke for the heterozygous carriers was 1.43 (95%CI 0.95-2.15) and for the homozygous male carries 2.07 (95%CI 1.24-3.44).

Discussion

We assessed the association between the C804A polymorphism in the LTA gene and vascular events in an elderly population at risk for vascular disease. Our results indicate that carriers of the 804A allele have an increased risk for the primary study endpoint consisting of coronary events and clinical strokes. After stratification for gender, this association was only significant in men.

Furthermore, we found that the association between the C804A polymorphism and the primary endpoint in males was mainly attributable to incident strokes.

Although we found no statistically significant interaction with gender, the association between the C804A polymorphism and clinical strokes was only significant in men. Such a sex-specific effect has been reported previously (6). Men who were homozygous for the 804A allele were more likely to develop atherosclerosis than homozygous females. This finding is in line with our results and fits well within the widely recognized difference in susceptibility and severity of atherosclerosis between men and women. Men have a higher predisposition to atherosclerosis compared to females (17). Likewise, several other genes, like apolipoprotein E, have been shown to have gender specific effects on cardiovascular outcomes (18;19). However, further research is necessary to confirm our results.

Three studies have previously investigated the association between the LTA gene and the susceptibility for stroke (9;10;12). The study of Hagiwara *et al* found no higher frequency of the LTA C804A polymorphism in stroke patients (12). Um *et al* found an increase of the homozygous 252G allele in subjects with cerebral infarction compared to controls (9). Szolnoki *et al* also found that the homozygous LTA allele with the 252G and 804A SNPs is more frequent in stroke patients than in controls (10). These studies combined with our findings, indicate that carriers of the variant allele are indeed at a higher risk for the development of clinical strokes.

We do not have information about the separate ischemic and hemorrhagic strokes. In our study both types of strokes were combined into one clinical endpoint. Because we know from previous studies in elderly populations that approximately 80 percent of all strokes is attributable to ischemic events (20;21), the association between the C804A polymorphism and clinical stroke is probably driven by an association between the polymorphism and ischemic stroke. If there is no association with the polymorphism and hemorrhagic stroke, then the association we found is an underestimation of the true relative risk for ischemic stroke.

The whole LTA gene is in strong linkage disequilibrium, therefore the 252G allele naturally coexists with the 804A allele (22). Ozaki *et al* investigated the functionality of the A252G and C804A SNPs in the LTA gene (15).The C804A polymorphism causes an amino-acid change from threonine (T) to asparagine (N) at codon 26. They found that the variant protein 26N is associated with a two-fold increase in the induction of cell-adhesion molecules in vascular smooth muscle cells (15). Adhesion molecules are implicated in cardiovascular disease because elevated levels have been observed in atherosclerotic lesions (23;24). This might explain the association of the polymorphisms in the LTA gene and the increased risk for incident stroke.

In our study we found no association between the LTA polymorphism and myocardial infarction (MI). A genome-wide association study identified two functional polymorphisms in the LTA gene associated with MI (A252G and C804A) (8). A case-control association study by Ozaki *et al* found that subjects homozygous for the mutant allele (804AA) had an almost two-fold higher risk for MI (15). However, three observational studies did not find any association between the LTA polymorphisms and myocardial infarction (22;25;25;26). Moreover, a meta-analysis of six studies investigating this association found no significant result (22). The association between the LTA gene and incident stroke has not been replicated recently. Further research into this association is warranted before we can draw definite conclusions from our results.

That we found an association between the LTA C804A genotype and incident stroke and not with coronary events is understandable based on available literature (27). Recently, Vanderlaan *et al* suggested that the variation of lesion development at different vascular beds is sensitive to various parameters (28). For example, hypertension is one of the main risk factors for atherosclerosis in the carotid arteries and for incident stroke whereas smoking is a stronger risk factor for coronary atherosclerosis (27). This indicates that cerebrovascular disease has other risk factors than coronary disease, which also suggests a different genetic background. LTA 804AA carriers could therefore have an increased risk for incident stroke and not for coronary events.

A possible weakness of our study is that we have measured only one SNP in the LTA gene. But because the SNPs of the LTA gene are in strong linkage disequilibrium, information of one SNP is sufficient for analyses. Moreover, we have an enrichment of the variant allele in our study population compared to European populations reported in the NCBI database (www.ncbi.nlm.nih.gov). However, this does not affect the internal validity.

The strength of our study is that it is a prospective study which is not affected by population stratification (29). Because the genotype frequencies differed in the three countries we performed a stratified analysis for each country. This analysis showed consistent but not significant results, because of lack of statistical power. Another strength is our population size. We had sufficient cases of incident stroke to reach a high power for statistical analyses. Furthermore, all participants were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes, which makes this study population suitable for investigating coronary and cerebrovascular diseases.

In conclusion, we found an association of the C804A polymorphism in the LTA gene with the primary endpoint, which seems primarily due to an association in men. After separating the coronary and cerebrovascular events, we found that the association with the primary endpoint and the C804A variant was mainly attributable to clinical stroke. This study is a further argument that the LTA gene is associated with cerebrovascular disease, especially in males, but further research is warranted.

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Reference List

(1) Kaperonis EA, Liapis CD, Kakisis JD, Dimitroulis D, Papavassiliou VG. Inflammation and atherosclerosis. Eur J Vasc Endovasc Surg 2006 Apr;31(4):386-93.

(2) Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation 2002 Mar 5;105(9):1135-43.

(3) Libby P. Inflammation in atherosclerosis. Nature 2002 Dec 19;420(6917):868-74.

(4) Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, et al. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 1984 Dec 20;312(5996):721-4.

(5) McDevitt H, Munson S, Ettinger R, Wu A. Multiple roles for tumor necrosis factor-alpha and lymphotoxin alpha/beta in immunity and autoimmunity. Arthritis Res 2002;4 Suppl 3:S141-S152.

(6) Laxton R, Pearce E, Kyriakou T, Ye S. Association of the lymphotoxin-alpha gene Thr26Asn polymorphism with severity of coronary atherosclerosis. Genes Immun 2005 Sep;6(6):539-41.

(7) Schreyer SA, Vick CM, LeBoeuf RC. Loss of lymphotoxin-alpha but not tumor necrosis factor-alpha reduces atherosclerosis in mice. J Biol Chem 2002 Apr 5;277(14):12364-8.

(8) Ozaki K, Tanaka T. Genome-wide association study to identify SNPs conferring risk of myocardial infarction and their functional analyses. Cell Mol Life Sci 2005 Aug;62(16):1804-13.

(9) Um JY, An NH, Kim HM. TNF-alpha and TNF-beta gene polymorphisms in cerebral infarction. J Mol Neurosci 2003;21(2):167-71.

(10) Szolnoki Z, Havasi V, Talian G, Bene J, Komlosi K, Somogyvari F, et al. Lymphotoxin-alpha gene 252G allelic variant is a risk factor for large-vessel-associated ischemic stroke. J Mol Neurosci 2005;27(2):205-11.

(11) Porto I, Leone AM, Crea F, Andreotti F. Inflammation, genetics, and ischemic heart disease: focus on the major histocompatibility complex (MHC) genes. Cytokine 2005 Mar 7;29(5):187-96.

(12) Hagiwara N, Kitazono T, Kamouchi M, Kuroda J, Ago T, Hata J, et al. Polymorphisms in the Lymphotoxin Alpha Gene and the Risk of Ischemic Stroke in the Japanese Population. The Fukuoka Stroke Registry and the Hisayama Study. Cerebrovasc Dis 2008 Mar 17;25(5):417-22.

(13) Shepherd J, Blauw GJ, Murphy MB, Cobbe SM, Bollen EL, Buckley BM, et al. The design of a prospective study of Pravastatin in the Elderly at Risk (PROSPER). PROSPER Study Group. PROspective Study of Pravastatin in the Elderly at Risk. Am J Cardiol 1999 Nov 15;84(10):1192-7.

(14) Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002 Nov 23;360(9346):1623-30.

(15) Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. Nat Genet 2002 Dec;32(4):650-4.

(16) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005 Jan 15;21(2):263-5.

(17) Joakimsen O, Bonaa KH, Stensland-Bugge E, Jacobsen BK. Age and sex differences in the distribution and ultrasound morphology of carotid atherosclerosis: the Tromso Study. Arterioscler Thromb Vasc Biol 1999 Dec;19(12):3007-13.

(18) Desvarieux M, Schwahn C, Volzke H, Demmer RT, Ludemann J, Kessler C, et al. Gender differences in the relationship between periodontal disease, tooth loss, and atherosclerosis. Stroke 2004 Sep;35(9):2029-35.

(19) Reilly SL, Ferrell RE, Sing CF. The gender-specific apolipoprotein E genotype influence on the distribution of plasma lipids and apolipoproteins in the population of Rochester, MN. III. Correlations and covariances. Am J Hum Genet 1994 Nov;55(5):1001-18.

(20) Melcon CM, Melcon MO. Prevalence of stroke in an Argentine community. Neuroepidemiology 2006;27(2):81-8.

(21) Sagui E, M'Baye PS, Dubecq C, Ba FK, Niang A, Gning S, et al. Ischemic and hemorrhagic strokes in Dakar, Senegal: a hospital-based study. Stroke 2005 Sep;36(9):1844-7.

(22) Clarke R, Xu P, Bennett D, Lewington S, Zondervan K, Parish S, et al. Lymphotoxin-alpha gene and risk of myocardial infarction in 6,928 cases and 2,712 controls in the ISIS case-control study. PLoS Genet 2006 Jul;2(7):e107.

(23) Belch JJ, Shaw JW, Kirk G, McLaren M, Robb R, Maple C, et al. The white blood cell adhesion molecule E-selectin predicts restenosis in patients with intermittent claudication undergoing percutaneous transluminal angioplasty. Circulation 1997 Apr 15;95(8):2027-31.

(24) Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr., et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 1997 Dec 16;96(12):4219-25.

(25) Kimura A, Takahashi M, Choi BY, Bae SW, Hohta S, Sasaoka T, et al. Lack of association between LTA and LGALS2 polymorphisms and myocardial infarction in Japanese and Korean populations. Tissue Antigens 2007 Mar;69(3):265-9.

(26) Sedlacek K, Neureuther K, Mueller JC, Stark K, Fischer M, Baessler A, et al. Lymphotoxin-alpha and galectin-2 SNPs are not associated with myocardial infarction in two different German populations. J Mol Med 2007 Sep;85(9):997-1004.

(27) Caprie Study group. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 1996 Nov 16;348(9038):1329-39.

(28) VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. Arterioscler Thromb Vasc Biol 2004 Jan;24(1):12-22.

(29) Beaty TH, Fallin MD, Hetmanski JB, McIntosh I, Chong SS, Ingersoll R, et al. Haplotype diversity in 11 candidate genes across four populations. Genetics 2005 Sep;171(1):259-67.