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Aspirin and statin medication decreases the risk of myocardial infarction associated with LTA and NFKBIL1 polymorphisms

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Abstract: Lymphotoxin-α (LTA) is a cytokine involved in inflammatory reactions. NFKBIL1 is a regulator of the NF-κB complex. The study investigated the associations of LTA 804 C>A and NFKBIL1–63 T>A polymorphisms with the use of statin and acetylsalicylic acid (ASA) treatment in relation to myocardial infarction (MI). The study population comprised of 600 Finnish individuals who underwent coronary angiography volunteering for the Angiography and Genes Study. Genotypes were detected by the TaqMan 5' nuclease assay. We found a interaction between the LTA genotype (p=0.002) and the NFKBIL1 genotype (p=0.012) and statin treatment in relation to MI. Subjects with the LTA AA or the NFKBIL1 AA genotype were at a 2.77 (95% CI:1.22-6.24) and 2.85 (95% CI:1.22-6.66) times higher risk, respectively, of suffering an MI when compared to other genotypes among statin non-users. ASA treatment also modulated associations between LTA and NFKBIL1 genotypes and MI (p=0.015 and p=0.028 respectively). The NFKBIL1-A-LTA-A haplotype showed a 61% increase in the risk of MI compared to the NFKBIL1-T-LTA-C haplotype among statin non-users. Anti-inflammatory medication modifies the genotype-related risk of MI, suggesting that subjects with LTA and NFKBIL1 AA haplotype might especially benefit from the treatment.

Keywords: Lymphotoxin-α, inhibitor of κB-like gene, myocardial infarction

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1 Introduction

Increasing evidence suggests that atherosclerosis is an inflammatory disease [1]. Cytokines contribute both to the atheroma formation and rupture which can result in myocardial infarction (MI) [1]. Aspirin and statin treatment, shown to decrease the risk of MI by affecting thrombogenesis and cholesterol levels [2]. Inflammation in the coronary wall begins with an endothelial injury which increases the adhesiveness of endothelium to leukocytes and platelets. As the inflammation continues, macrophages and lymphocytes migrate from the blood, multiply within the lesion [1] and release cytokines such as lymphotoxin-α (LTA). LTA (also called tumour necrosis factor-β) is secreted by activated T-lymphocytes and acts as a modulator in the immune response. LTA initiates a local vascular inflammatory response and contributes to the progression of atheromatous plaques by stimulating adhesion molecule production, thrombogenesis, smooth muscle cell proliferation, platelet activation and the release of vasoactive agents [3]. The LTA gene is located in the major histocompatibility complex (MHC) class III region in chromosome 6 which harbours many immunologically relevant genes [4–6]. It has a common 804 C-to-A polymorphism in exon 3 which causes a threonine-to-asparagine substitution at codon 26 (26 T/N). This functional polymorphism has previously been connected to an increased risk of MI [7].

The inhibitor of the κB-like (IKBL or NFKBIL1) gene lies next to the LTA gene in the HLA class III region of the same chromosome [8]. The biological function of the NFKBIL1 protein is unclear, but its similarity to IκBα in the sequence and cellular localization presumes an analogous function. IκBα regulates the nuclear localization of NF-κB which stimulates the transcription of many immunologically and inflammatorily relevant genes, including LTA and other cytokines [9–11]. Polymorphism in the promoter area of NFKBIL1 (-63 T>A) could affect the transcriptional activity of the gene, and it has also been connected to the risk of MI [7].

This cross-sectional study was designed to study whether the LTA 804 C>A or the NFKBIL1 -63 T>A polymorphisms, or their haplotypes, are associated either directly or through an interaction with commonly used anti-inflammatory medication – i.e., statins or acetylsalicylic acid (ASA)—with the severity of coronary artery disease or the risk of MI in 600 Finnish subjects who underwent a coronary angiography examination because of clinically suspected coronary artery disease.

2 Subjects and methods

2.1 Subjects

The Angiography and Genes Study (ANGES) comprises a prospective series of patients referred to the Tampere University Hospital from September 2002 to March 2004 for coronary angiography because of chest pain and clinically suspected CAD. The study group consisted of 600 Finnish individuals (393 men and 207 women, mean age 62.4 ±

10.5). All patients were interviewed by a study nurse, and general information—including age, sex, body mass index (BMI), alcohol use, smoking, medication as well as traditional risk factors of atherosclerosis and MI—was collected systemically with a questionnaire (Table 1). Information about previous surgical operations and MIs was collected from patient records at Tampere University Hospital. The Ethics Committee of the Tampere University Hospital approved the study, and written informed consent was obtained from each patient.

2.2 Laboratory measurements

Blood samples for biochemical analyses were collected into tubes containing EDTA, after the subjects had fasted overnight. Plasma was separated by centrifugation (2000 rpmin, 20 min) and stored frozen at -80°C until analysis. Plasma triglycerides as well as total and high-density lipoprotein cholesterol (HDL-C) concentrations were analysed with a Cobas Integra 700 automatic analyser with reagents and calibrators, according to the manufacturer's recommendations (Hoffmann-LaRoche Ltd, Base, Switzerland). Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using Friedewald's formula [12].

2.3 LTA and NFKBIL1 genotyping

DNA was isolated from lymphocytes with a commercially available kit (Qiagen Inc. Valencia, CA). DNA samples were genotyped by employing the 5' nuclease assay in combination with specific fluorogenic TaqMan MGB probes using the ABI Prism 7900HT Sequence Detection System (Applied Biosystesm, Foster City, CA, USA). The nucleotide sequences of primers and allele-specific wild-type and variant probes, labelled with the reporter dyes FAM or VIC, were deduced from sequences deposited in the GenBank database and synthesized in accordance with Applied Biosystems. PCR reaction containing genomic DNA, 1 X Universal Master Mix, 900 nM of each primer and 200 nM of each probe was performed in 96-well plates using the standard method. Endpoint readings of the fluorescence generated from each probe during the PCR amplification were measured with the allelic discrimination analysis module, resulting in clear identification of three genotypes for both genes [13]. Negative and positive controls and random duplicates were used as a quality control.

Table 1 Characteristics of the study subjects.

	NFKBIL1 genotype				LTA genotype			
	TT	TA	AA	P*	CC	CA	AA	P*
Number of subjects, n (%)	252 (42.5)	271 (45.3)	73 (12.2)		239 (39.8)	281 (46.8)	80 (13.3)	
Sex (men/women), n	172/83	182/89	38/35	0.037	165/74	184/97	44/36	0.073
Body mass index (kg/m^2)	28.2 ± 4.4	27.7 ± 3.9	28.0 ± 4.2	0.408	28.1 ± 4.4	27.9 ± 3.9	27.8 ± 4.1	0.716
Age (years)	62.9 ± 9.8	61.8 ± 10.3	62.4 ± 9.9	0.433	63.08 ± 9.9	61.70 ± 10.2	62.61 ± 10.1	0.288
Diabetes mellitus (yes/no)	65 / 190	71 / 200	17 / 56	0.879	61 / 178	74 / 207	18 / 62	0.786
Hypertension (yes/no)	152 / 102	153 / 116	42 / 31	0.783	141 / 97	160 / 119	46 / 34	0.903
Smoking (yes/no)	32 / 222	43 / 228	12 / 61	0.505	31 / 207	44 / 237	13 / 67	0.640
Total cholesterol (mmol/l)	4.59 ± 0.93	4.66 ± 1.11	4.70 ± 0.11	0.689	4.56 ± 0.90	4.69 ± 1.11	4.56 ± 1.01	0.326
Triglycerides (mmol/l)	1.59 ± 0.72	1.65 ± 1.51	1.61 ± 1.17	0.804	1.56 ± 0.71	1.66 ± 1.46	1.66 ± 1.19	0.562
LDL cholesterol (mmol/l)	2.74 ± 0.80	2.75 ± 0.05	2.78 ± 0.84	0.903	2.72 ± 0.78	2.76 ± 0.82	2.76 ± 0.81	0.823
HDL cholesterol (mmol/l)	1.14 ± 0.31	1.17 ± 0.34	1.17 ± 0.32	0.674	1.14 ± 0.29	1.17 ± 0.35	1.15 ± 0.32	0.433
Myocardial infarction (yes/no)	93/155	79/184	25/47	0.201	86/145	88/186	24/55	0.374
Statins (yes/no)	169 / 86	186 / 85	43 / 30	0.294	160 / 79	190 / 91	49 / 31	0.558
Acetylicsalicylic acid (yes/no)	217 / 38	216 / 55	58 / 15	0.230	204 / 35	226 / 55	61 / 19	0.132

*p-values by ANOVA or chi-square ♯. Values are mean ± SD.

2.4 Angiography measurements and the diagnostic criteria of MI

Selective coronary angiography by femoral or radial route was performed in all patients by digital x-ray equipment. The left coronary artery was evaluated from at least four projections (left and right anterior oblique, anteroposterior cranial and caudal), and the right coronary artery (RCA) from at least two projections. A significant stenosis in the coronaries, including the left main coronary artery (LMCA), was defined as $> 50\%$ diameter obstruction of the coronary artery lumen diameter according to the American Heart Association classification system.

The site of the culprit lesion in patients with acute coronary syndrome was determined by the presence of total or subtotal occlusion; if this was not present, then by the presence of residual thrombus or an ulcerated lesion; if these also were not present, then by the severity of stenosis alone. Any disagreement between the investigators was resolved by consensus.

The clinical diagnosis of MI was based on Finnish guidelines. The criterion of MI includes clinical symptoms like chest pain, electrocardiographic findings such as ST-segment elevation and biochemical marker tests measuring troponin I and creatinekinase [14].

2.5 ASA and statin treatment

Subjects were administered ASA in daily dose of 75-150 mg according to the recommendation of European guidelines in CVD events prevention [16]. Two most commonly used statins were simvastatin and atrovastatin. Daily dose of simvastatin varies from 20 mg to 80 mg and daily dose of atrovastatin from 10 mg to 40 mg. When survey interview was done most subjects had used ASA and statins for several months.

2.6 Statistical analysis

Categorical variables were compared using the chi-square test, and the odd ratios were calculated using logistic regression analysis. Confounding factors—including age, sex, total cholesterol, HDL-C, BMI, smoking (yes/no), diabetes mellitus (yes/no) and drug treatments—were taken into account by adding them into the regression analysis as covariates. The continuous variables were compared between genotype groups using analysis of variance (ANOVA) or after adjusting the analysis with covariates (ANCOVA).

The use of statins and ASA were added to the models because they both have anti-inflammatory effects, although the main indication for the use of statins is the need to lower LDL-C [15]. Interactions between genotypes and medicines were investigated by multinominal regression analyses after dividing the subjects into two groups: subjects who had used ASA or statins and subjects who had not.

Haplotype analyse phase was resolved by applying E-M algorithm, after which the frequencies of different haplotype combinations were estimated from the saturated model (i.e., in which the polymorphisms of the two loci were found to be in linkage disequi-

librium). Haplotype-specific odds ratios (OR) and their confidence intervals (CI) were calculated comparing the haplotypes which entail of the common alleles with haplotypes comprising the polymorphic alleles for the different outcome variables.

Analyses were conducted using the statistical software STATA (version 8.0, STATA Corporation, Texas, USA) and SPSS (version 11.5, SPSS Inc., Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant.

3 Results

3.1 Descriptive data

The NFKBIL1 genotypes were distributed as follows: TT 252 (42.5%), TA 271 (45.3%), and AA 73 (12.2%). For LTA, the distribution was: CC 239 (39.8%), CA 281 (46.8%), and AA 80 (13.3%). With regard to both genotypes, we sought to focus on AA homozygotes, i.e., the risk genotype, and therefore we pooled both the NFKBIL1 TT and TA genotypes (T-allele carriers) as well as the LTA CC and CA genotypes (C-allele carriers) into one group and compared these groups to NFKBIL1 AA or LTA AA homozygote groups. All genotypes were, however, also analysed separately. Both genotype distributions followed the Hardy-Weinberg equilibrium. The characteristics of the subjects by the LTA and NFKBIL1 genotype groups are presented in Table 1. The LTA genotype information was available from 600 subjects, NFKBIL1 genotype information from 596 subjects. In LTA genotype group information about MI's was available from 584 subjects and in NFKBIL1 group from 583 subjects. Statin and ASA information was available from 599 subjects in NFKBIL1 group and from 600 subjects in LTA group. No differences in these baseline characteristics between genotype groups were found when subjects with MI or without MI were analyzed separately (data not shown). There was a significant association between MI and angiographic findings. If there was a stenosis ($> 50\%$) in three or more of coronary arteries subjects were likely to have had myocardial infarction when comparing to group in which there was no stenonis found ($p<0.001$). However we failed to determine an association between genotype and angiographic findings. For the LTA or NFKBIL1 loci there were no differences between genotype groups regarding these variables. 356 (59.3%) subjects used both statin and ASA medication. No other anti-inflammatory drug usage was reported.

3.2 LTA and NFKBIL1 genotype interactions with statins in relation to MI

When we analyzed all subjects regardless of their medication use, no statically significant relation between MI and LTA or NFKBIL1 genotypes was detected. However, both the LTA and NFKBIL1 AA genotype subjects tended to have a higher risk of MI, although the association was not statistically significant.

Table 2 Odds ratios for myocardial infarction among statin and acetylsalicylic acid users and non-users by LTA and NFKBIL1 AA genotypes, according to logistic regression analysis.

	Acetylsalicylic acid				Statins			
	Non-users (n =108)	p	Users (n =491)	p	Non-users (n =201)	p	Users (n =399)	p
OR (95% CI) for MI with LTA AA	3.01 (1.02-9.17)	0.046	0.63 (0.35-1.14)	0.126	2.77 (1.22-6.24)	0.025	0.49 (0.25-0.97)	0.039
LTA AA carriers with MI, n (%)	7 (38.9)		17 (27.9)		11 (35.5)		13 (27.1)	
OR (95% CI) for MI with NFKBIL1 AA	3.75 (1.14-12.39)	0.030	0.82 (0.46-1.47)	0.502	2.85 (1.22-6.66)	0.015	0.69 (0.36-1.37)	0.290
NFKBIL1 AA carriers with MI, n (%)	6 (42.9)		19 (32.8)		11 (36.7)		14 (33.3)	

Abbreviations: LTA AA lymphotoxin-α AA genotype; NKKBIL1, inhibitor of κB-like AA genotype; MI, myocardial infarction; OR, odds ratio; p, p-values.
Please note: In the regression analysis, subjects who carry the LTA-C or NFKBIL-T allele are used as a reference category.

In the further analysis (Table 2), we found a significant LTA genotype-by-statin treatment interaction (p=0.002) in relation to MI. In statin non-users with the LTA AA genotype, the odds ratio for MI was 2.77 (95% CI 1.22-6.24, p=0.025) when compared to C-allele carriers. In a similar analysis for statin users, subjects with the AA genotype were not at an increased risk to develop an MI. In fact, statin therapy reduced the risk of MI among these AA homozygotes to a lower level (OR 0.49, 95% CI 0.25-0.97, p=0.039) than it did among the C-allele carriers. In addition, we found a significant NFKBIL1 genotype-by-statin treatment interaction (p=0.012) in relation to MI. In statin non-users with the NFKBIL1 AA genotype, the risk of MI was almost three times higher (OR 2.85, 95% CI 1.22-6.66, p=0.015) when compared to the T-allele carriers. Again, in statin users, the same genotype was not associated with a risk of MI (p=0.290).

3.3 LTA and NFKBIL1 genotype interactions with ASA in relation to MI

Similarly to statin treatment, we found a statistically significant NFKBIL1 genotype-by-ASA interaction with MI (p=0.028). Among the patients who had not used ASA, NFKBIL1 AA genotype carriers had an odds ratio of 3.75 (95% CI 1.14-12.39, p=0.030) for MI when compared to other genotypes. Among the ASA users, the AA genotype was not associated with an increased risk of MI (p=0.502). Furthermore, we found an LTA genotype-by-ASA interaction with MI (p=0.015). Among ASA non-users, subjects with the LTA AA genotype had an odds ratio of 3.01 (95% CI 1.02-9.17, p=0.046) for MI when compared to the subjects who carried the C-allele. However, in ASA users, the AA genotype was not associated with increased risk of MI (p=0.126). (Table 2)

3.4 Haplotype analyses

Haplotypes analysis showed that the two polymorphisms were tightly in linkage disequilibrium (-2 log likelihood value between saturated and unsaturated models = 850.90 with 1 d/f, $p < 0.00001$).

The NFKBIL1-A-LTA-A risk haplotype showed a 61% increase in the risk of MI (OR 1.61, 95% CI 1.21–2.82, $p<0.05$) when compared to the NFKBIL1-T-LTA-C control haplotype among statin non-users, but no such association was found among the statin users. The risk of MI was particularly high among the non-users of both ASA and statin in the risk haplotype group, compared to the control haplotype group (OR 7.57, 95% CI 2.36–34.52, p=0.001). The risk of MI was not elevated among subjects using both of these medications.

4 Discussion

The use of statin and/or ASA medication is very common among subjects with CAD and MI, but the interaction analysis between genotypes and inflammatory drugs have usually

been neglected in genetic association studies in the field of cardiovascular diseases. The aim of the present study was to examine the interactions between the LTA 804 C>A and NFKBIL -63 T>A polymorphisms, haplotypes and anti-inflammatory medication in relation to the risk of MI in Finnish middle-aged or elderly subjects. In our study, we could not determine a direct association between LTA and NFKBIL1 genotypes and MI, but instead we found a strong interaction between these genotypes and the use of anti-inflammatory drugs, ASA and statins, in relation to MI.

Inflammation in atherosclerosis can lead to atheroma formation and rupture of plaque, which can result in thrombosis and MI [1]. Activated inflammatory cells secrete inflammatory cytokines, which are active in processes that weaken the connective tissue framework of the plaque's fibrous cap [17, 18]. When the rupture occurs, the core of the plaque filled with lipid droplets and smooth muscle cells leaks to circulating blood. This initiates platelet aggregation, which is almost immediately followed by coagulation. These events finally lead to the formation of thrombus [17, 19].

LTA is involved in many inflammation processes, including stimulation of adhesion molecule production, thrombogenesis and smooth muscle proliferation [6]. The LTA gene 804 C>A polymorphism, leading to the asparagine-to-threonine substitution at codon 26, has been reported to be functional and involved in the development of postoperative sepsis and subsequent complications [20, 21]. In addition, this polymorphism has been previously associated with coronary artery disease risk factors, i.e., hyperinsulinaemia and fastened glycemia [22]. The relation between LTA polymorphisms and MI has not been extensively studied. Padovani et al. found that the +252 A→G polymorphism in the LTA gene increases the risk of MI in smokers and that the -308 G→A polymorphism may, in turn, increase the risk of MI conferred by obesity [3]. Ozaki et al. showed evidence that 804 C>A polymorphisms of the LTA gene affect susceptibility to MI in the Japanese population and that the subjects carrying the AA genotype are at an increased risk of MI [7]. In line with this study, the European multicentre PROCARDIS study found an association of the LTA 804 A allele with coronary artery disease [23]. In our study, we found a similar connection between the LTA 804 AA genotype and MI in subjects who did not use medications which may affect the inflammatory process. In subjects who used ASA or statins or both, the genotype association was not significant, even though the tendency existed. Our results suggest that LTA is involved in cascades of atherosclerosis but that its effects can be counteracted by the use of anti-inflammatory or lipid lowering drugs.

NF-κB complex is known to be a regulator of cell responses, and it seems certain that this complex is involved in the development of atherosclerosis and other cardiovascular diseases by inducing transcription factors and adhesion molecules [10, 24]. The NF-κB complex is inhibited by I-kappa-B proteins which inactivate NF-kappa-B by trapping it in the cytoplasm. Phosphorylation of serine residues on the I-kappa-B proteins by kinases marks them for destruction via the ubiquitination pathway, thereby allowing activation of the NF-kappa-B complex. The activated NF-κB complex translocates into the nucleus, binds DNA and activates gene transcription. NFKBIL1, also known as

IKBL, may interact with members of NF-κB [8], which makes the role of NFKBIL1 important in NF-κB inhibition/activation responses. NFKBIL1 and its polymorphisms in the promoter region (-63 T>A and -263 A>G) are involved in resistance to type 1 diabetes in the Japanese population [25]. Ozaki et al. showed that the -63 T>A polymorphism slightly decreased the transcriptional activity of the NFKBIL1 gene, indicating a possible functional role of this polymorphism [7]. Therefore, it may be hypothesized that the NFKBIL1 AA genotype decreases NFKBIL1 transcription, which in turn makes the NF-κB pathway more active, thus enhancing the inflammatory processes of atherosclerosis. This would explain the association that was found in the present study between the NFKBIL1 -63 AA genotype and MI among medication non-users.

The LTA and NFKBIL1 genes are both located in chromosome 6p, which harbours many immunologically relevant genes in the HLA class III region. The other genes in this particular region seem to be highly polymorphic both in their promoters and exons, and they may be in linkage disequilibrium with the LTA or NFKBIL1 polymorphisms studied [26]. Since LTA and NFKBIL1 genes lie next to each other in this region, it is not surprising that, in the present study, NFKBIL and LTA polymorphisms exhibited strong linkage disequilibrium and thus formed a risk haplotype (i.e., NFKBIL1-A-LTA-A) for MI among subjects who did not use aspirin or statins

In our study, we found a strong interaction between LTA and NFKBIL1 gene polymorphisms and the use of statins or ASA in relation to MI. The risk of MI was particularly high among the non-users of both ASA and statin who carry the risk haplotype (NFKBIL1-A-LTA-A) when compared to the control haplotype carriers. Statin and ASA both reduced this genotype-related risk, leading to similar odds ratios for MI among AA homozygotes and carriers of the other genotypes. These results imply that the risk allele carriers would benefit greatly from the use of anti-inflammatory or lipid lowering drugs. The main reasons for using statins in hyperlipidemia are related to their ability to reduce cholesterol levels. Statins inhibit HMG-CoA reductase and the body's own cholesterol synthesis. This, in turn, increases LDL uptake to cells, leading to decreased LDL concentration in plasma. However, statins have biological functions beyond LDL cholesterol level reduction, including anti-inflammatory effects. These so-called pleiotropic effects may offer protection against atherosclerotic plaque growth and the precipitation of acute events [27, 28]. On the other hand, ASA inhibits platelet aggregation, which is mainly based on blocking thromboxane A2 synthesis in platelets. Understandably, this beneficial mechanism of ASA helps in the prevention of coronary artery thrombosis [29, 30]. It seems likely that the use of these anti-inflammatory drugs can counteract the weaker inflammatory effects of the genotypes studied.

The major limitation of the present study is the lack of data on the duration of the subjects' medication therapy. We do not know for how long the subjects have been using ASA or statins, or how regular their use has been. Therefore, this study is a cross-sectional study containing both long-term users and subjects who have failed to use their medication regularly. Moreover, we cannot eliminate the possibility that the polymorphisms studied and MI may be related to each other through a linkage disequilibrium

with other nearby polymorphisms. Another limitation is the scale of the study. The study population (n=600) is rather small when dealing with a heterogeneous disease like myocardial infarction. Furthermore, as the cohort consisted of real patients that underwent to angiographic examination due to different clinical indications, majority of the study subjects have cardiac risk profile (i.e. preexisting CAD). Though using actual cardiac patients as study population can be clinically considered as a strength, these facts naturally limit the generalization of results.

In conclusion, our results suggest that both the 804 C>A LTA and –63 T>A NFKBIL1 polymorphisms are involved in the inflammatory processes occurring in advanced atherosclerotic plaques. According to our results, carriers of the AA genotype of the LTA and NFKBIL1 genes are at increased risk to have an MI if they do not use statin or ASA therapy. Among statin and ASA users, however, the risk is similar for the AA homozygotes and the carriers of the other genotypes. This implies that particularly the carriers of the AA haplotype are likely to benefit from regular use of ASA and statins in preventing MIs. According to our results, it would, therefore, be important to find those younger but still asymptomatic subjects with increased, genetically determined risk for MI in the early stage of disease progression and treat them effectively with statins or ASA, or with their combination. Large-scale, genotype-stratified statin and ASA intervention trials in relation to MI and coronary end-points are, however, required before such treatment recommendations can be issued.

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