Clin Chem Lab Med 2007;45(4):487–492 © 2007 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2007.103

# The c.–292C>T promoter polymorphism increases reticulocyte-type 15-lipoxygenase-1 activity and could be atheroprotective

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## Abstract

**Background**: Reticulocyte-type 15-lipoxygenase-1 (ALOX15) has anti-inflammatory and inflammatory effects and is implicated in the development of asthma, arthritis and atherosclerosis. Previously, we screened the human *ALOX15* gene for variations because genetic variability in *ALOX15* might influence these diseases. We found a C>T substitution at position c.–292 in the *ALOX15* promoter that created a novel binding site for the transcription factor SPI1 and increased *ALOX15* mRNA levels in monocytes from c.–292CT heterozygous volunteers.
**Methods**: To test whether the higher mRNA levels led to higher ALOX15 activity, we performed an activity assay and measured the arachidonic acid metabolite 15(S)-hydroxy-eicosatetraenoic acid [15(S)-HETE] by HPLC analysis. To test whether this polymorphism was associated with coronary artery disease (CAD), we investigated its association in a case-control study involving 498 Caucasians.
**Results**: The c.–292C>T polymorphism was associated with higher enzyme activity in heterozygous carriers. Intriguingly, this polymorphism also showed a tendency to be protective against atherosclerosis.
**Conclusions**: These results suggest that increased ALOX15 activity may attenuate inflammation, which could be caused by an increase in 15(S)-HETE and eventually by its metabolites, the lipoxins.
Clin Chem Lab Med 2007;45:487–92.

**Keywords**: inflammation; lipoxin; 15-lipoxygenase (15-LOX); polymorphism; PU.1; reticulocyte-type 15-lipoxygenase-1 (ALOX15); SFPI1; SPI1; transcription factor.

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## Introduction

Reticulocyte-type 15-lipoxygenase-1 (ALOX15) (MIM# 152392) is a lipid-peroxidizing enzyme that inserts molecular oxygen into free and esterified polyunsaturated fatty acids to produce an array of bioactive lipids (1). The oxidation of arachidonic acid by ALOX15 leads to 15-hydroxy-eicosatetraenoic acid (15-HETE). This metabolite can either directly serve as a peroxisome proliferator-activated receptor-γ (PPARγ) activator (2) or be further metabolized to lipoxins, which are involved in the resolution of inflammation (3). Because these lipoxins were shown to inhibit chemotaxis, adhesion and transmigration of neutrophils, and to antagonize the pro-inflammatory effects of leukotrienes (4), ALOX15 is thought to play an anti-inflammatory role in the pathology of inflammatory diseases (5).

Besides the oxidation of arachidonic acid, ALOX15 oxidizes linoleic acid to 13-hydroxy-octadecadienoic acid (13-HODE), which is an activator of the PPARα, PPARγ and PPARδ receptors (6). Intriguingly, ALOX15 can also oxidize esterified linoleic acid on low-density lipoprotein (LDL) particles to produce the pro-atherogenic oxidized LDL (oxLDL) (7), which has pro-inflammatory and immunogenic properties (8). Hence, it is not clear whether the production of pro-atherogenic oxLDL outweighs the anti-inflammatory effects of ALOX15 in atherosclerosis and other inflammatory diseases.

Because functionally relevant polymorphisms in the *ALOX15* gene could indicate whether altered ALOX15 activity does indeed influence disease progressions in humans, we recently screened the *ALOX15* gene for polymorphisms and detected 11 variations (9, 10). One of the polymorphisms, a C>T substitution at position c.–292 in the *ALOX15* promoter, creates a novel binding site for the myeloid and B-cell transcription factor SPI1 (9, 10). Binding of SPI1 to the c.–292T promoter increased *ALOX15* transcription in human primary macrophages, but not in lung epithelial cells. Stimulated macrophages from heterozygous c.–292CT carriers had three-fold higher *ALOX15* mRNA levels than macrophages from homozygous c.–292CC non-carriers (9, 10).

In this study, we investigated the influence of elevated mRNA levels in c.–292CT carriers on ALOX15 enzyme activity and asked whether there is an association of the polymorphism with coronary artery disease (CAD). We show that the c.–292C>T promoter polymorphism leads to an increase in ALOX15 enzyme activity upon stimulation and demonstrate that the c.–292T allele shows a trend towards an athe-

roprotective effect in a case-control study for CAD involving 498 participants.

## Materials and methods

### Mutation analysis

DNA was extracted from EDTA blood samples using a QIAmp® DNA Mini Blood Kit (Qiagen AG, Basel, Switzerland) and genotyping was performed according to Wittwer et al. (9, 10).

### Nomenclature

All *ALOX15* numbering is based on the cDNA sequence NM_001140.3 with the A of the ATG translation initiation codon being taken as +1. The intronic numbers are further based on the genomic sequence NT_010718.15. For example, c.136-35C>T means a C>T substitution in the 35th base on the intron upstream of the next exon starting with base number 136 according to the cDNA sequence. Sequences identical to NM_001140.3 and NT_010718.15 are called *ALOX15* throughout the article.

### Preparation of human peripheral blood monocytes

Lymphocytes from either c.–292CC homozygous or c.–292CT heterozygous volunteers were extracted from 60 mL of heparinized blood using a HISTOPAQUE-1077 gradient (Sigma-Aldrich, Buchs, Switzerland). Peripheral blood monocytes were purified by capture on anti-CD14 antibodies coupled to MACS MicroBeads according to the manufacturer's instructions (CD14 Microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were differentiated into macrophages by stimulation for 3 days with 670 pM IL-4 in RPMI-1640 medium (Sigma) supplemented with 5% FCS (Fisher Scientific, Wohlen, Switzerland), 5% human AB serum (Sigma) and 5% $CO_2$.

### Enzymatic assay of ALOX15 activity

To boost IL-4 stimulation at 65 h after seeding the monocytes, 670 pM IL-4 was added; 72 h after seeding, cells were collected and counted. For each sample, $5\times10^5$ cells were transferred to 1.5 mL of PBS, spiked with 100 μM arachidonic acid and incubated for 15 min at 37°C. The suspension was centrifuged and the supernatant from each sample was stored at −20°C until analysis.

### HPLC analysis

15(R,S)-Hydroxy-eicosatetraenoic acid [15(R,S)-HETE] and 15(R)-HETE were purchased from Cayman Europe (Tallinn, Estonia). n-Hexane, methanol, ethanol, 2-propanol (all HPLC grade) were from Carl Roth (Karlsruhe, Germany). Acetic acid and ethyl acetate were from Grüssing (Filsum, Germany). The equipment consisted of a Waters 510 HPLC pump (Vienna, Austria), a 7725i Rheodyne injector (Riemerling, Germany) with a 20-μL sample loop, a column protection SecurityGuard™ cartridge (silica; 4 mm×3 mm ID) from Phenomenex (Aschaffenburg, Germany) and a Merck Hitachi L-4500A diode-array detector (Darmstadt, Germany). Data were analyzed using D-6500 DAD system manager software from Hitachi (Hamburg, Germany). All chromatographic runs were monitored at a wavelength of 235 nm (conjugated diene system) and measurements of the absorption maximum ($\lambda_{max}$) were performed after the run was completed.

### HPLC sample preparation

Samples were extracted twice with 1.5 mL of ethyl acetate. The combined organic layers were vaporized under a nitrogen stream. The residue was redissolved in 60 μL of n-hexane and subjected to normal-phase HPLC.

### Normal-phase HPLC

A LiChrospher® Si 60 column with pure silica gel as the stationary phase from Merck (250 mm×4 mm ID, particle size 5 μm) was used. The column was eluted with n-hexane/2-propanol/acetic acid (100:2:0.1 by vol.) at a flow rate of 1 mL/min. The 15-HETE fractions were collected and the solvent was evaporated under a nitrogen stream. The residue was redissolved in 25 μL of n-hexane and subjected to enantioselective HPLC.

### Enantioselective HPLC

A Chiralpak® AD column of amylose Tris-(3,5-dimethylphenylcarbamate) coated on silica gel from Chiral Technologies Europe (Illkrich, France; 250 mm×4.6 mm ID, particle size 10 μm) was used. The column was eluted with n-hexane/ethanol/methanol/acetic acid (93:4:3:0.1, by vol.) at a flow rate of 1 mL/min.

### Case-control study

A total of 342 men and 156 women from Zurich volunteered to participate in the study (11, 12). Written informed consent was obtained from all participants and the local Ethics Committees approved the study. The case group consisted of 260 consecutive Caucasian patients with angiographically documented CAD with more than 50% stenosis in at least one coronary artery. The control group consisted of 238 Caucasians with no history of CAD, stroke, or peripheral vascular disease and was recruited from the general population. Angiographically negative individuals were also included in the control group. A cholesterol-lowering regimen was being followed by 81% of the cases and 8% of the controls. Risk factors and the use of medication were assessed by questionnaire. Clinical chemistry analysis was carried out on a Roche-Hitachi Modular Clinical Chemistry analyzer using commercial tests from Roche Diagnostics (Rotkreuz, Switzerland).

### Statistical analyses

Statistical analysis was performed using StatView 5.0.1 and SAS 9.1.3. (SAS Institute, Heidelberg, Germany). Continuous variables between cases and controls were compared using a two-sided t-test. Association of *ALOX15* polymorphisms with CAD was assessed by exact logistic regression, using an additive genetic model and adjusting for age and sex. Differences in C-reactive protein (CRP) levels between carriers and non-carriers of the c.–292T allele were assessed by Mann-Whitney test.

The peak areas for 15-HETE and the S/R-enantiomeric ratios were compared by ANOVA using Scheffe's F-test between c.–292CC homozygous and c.–292CT heterozygous volunteers. All p-values are two-sided, with $p<0.05$ considered statistically significant.

## Results

### The c.–292C>T polymorphism is functional and increases ALOX15 activity

To investigate whether an increase in *ALOX15* mRNA would lead to higher enzyme activity, we measured the product 15-HETE from isolated primary macrophages from individuals with different c.–292 genotypes. Owing to a lack of c.–292TT homozygous volunteers (0.2% of the population), we used monocytes from c.–292CT heterozygous and c.–292CC homozygous volunteers. Primary blood monocytes were extracted from whole blood and IL-4 was used to stimulate differentiation into macrophages and expression of *ALOX15* before stimulation with arachidonic acid. This stimulation led to a significant release of the ALOX15-mediated arachidonic acid metabolite 15-HETE into the extracellular solution by macrophages of both genotypes (Figure 1). However, four-fold higher 15-HETE levels were observed in supernatants from c.–292CT heterozygous macrophages ($p<0.0001$) compared to c.–292CC homozygous macrophages (Figure 2).

Since arachidonic acid can undergo spontaneous oxidation by oxygen radicals, it is essential to distinguish *ALOX15* enzyme activity from spontaneous oxidation. The product of spontaneous oxidation is a racemic mixture of 15(R)-HETE and 15(S)-HETE, while ALOX15 oxidizes arachidonic acid specifically to 15(S)-HETE. To assess ALOX15 activity, we separated the 15-HETE fractions collected into their enantiomers using enantioselective HPLC (Figure 3). Macrophages from c.–292CT heterozygous volunteers produced more 15(S)-HETE compared to those from c.–292CC homozygous volunteers (Figure 3A). The average 15(S)-HETE/15(R)-HETE enantiomer ratio was 1.6 in c.–292CC homozygous volunteers and 9.6 in c.–292CT heterozygous volunteers ($p<0.0001$) (Figure 3B).

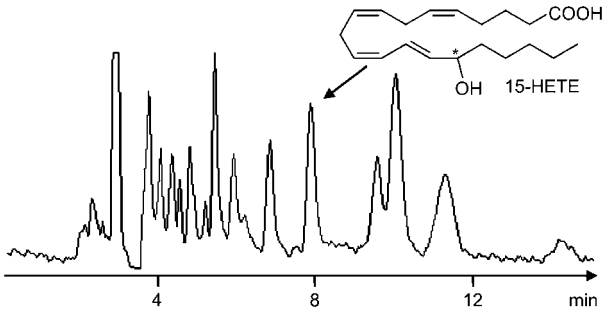


**Figure 1** 15-HETE detection in supernatant from macrophages.
Extracts of the supernatant of c.–292CC homozygous and c.–292CT heterozygous macrophages following the activity assays were loaded on a normal-phase HPLC non-chiral LiChrospher Si 60 column and the elution profile was followed at a wavelength of 235 nm. The retention time for 15-HETE (7.95 min) was identified using a 15-HETE reference standard. The arrow depicts the 15-HETE peak. The chemical structure of 15-HETE is indicated. Other peaks were not identified.

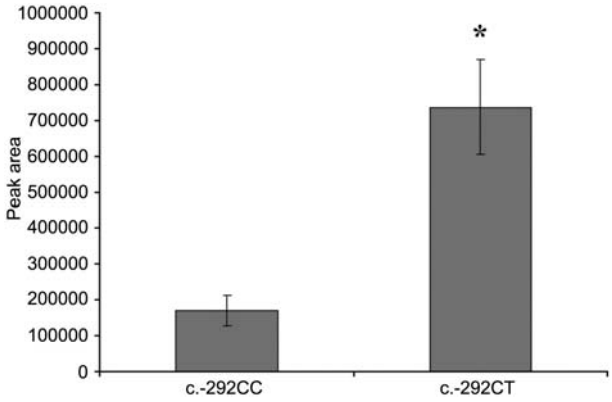


**Figure 2** Increased 15-HETE levels in supernatants from c.–292CT heterozygous macrophages.
The areas of 15-HETE peaks in supernatants from c.–292CC and c.–292CT macrophages were determined. Five 15-HETE determinations were performed for c.–292CC and four for c.–292CT genotypes. Error bars indicate the standard deviation. The asterisk indicates a significant difference ($p<0.0001$).

### Association study of *ALOX15* genetic variants with CAD

To investigate the influence of this *ALOX15* polymorphism on atherosclerosis, we investigated the association of all *ALOX15* mutations and polymorphisms with CAD in a case-control study including 260 Caucasian CAD cases and 238 healthy controls (12). The characteristics of the controls and cases are shown in Table 1. No significant correlation between genetic variants detected in the *ALOX15* gene and CAD was observed (Table 2). However, the c.–292C>T polymorphism showed a trend toward association with CAD (odds ratio 0.45, confidence interval 0.16–1.17). Intriguingly, the c.–292T allele was more frequent in controls (4.6%) than in cases (2.7%), suggesting that ALOX15 could be atheroprotective in humans.

To investigate whether this atheroprotective effect of the c.–292T allele could be explained by the anti-inflammatory action of ALOX15, we compared CRP levels between carriers and non-carriers of the c.–292T allele in controls. There was no difference in CRP levels between carriers and non-carriers [2.4 (0.8–3.8) vs. 1.3 (0.6–2.6) mg/L, median (interquartile range); $p=0.3$].

## Discussion

Previously, we screened the *ALOX15* gene for variations and found a c.–292C>T polymorphism in the *ALOX15* promoter that leads to higher *ALOX15* mRNA levels in primary macrophages from heterozygous carriers through the formation of a novel binding site for the transcription factor SPI1 (9, 10). In this study, we demonstrate that this higher *ALOX15* mRNA level leads to higher enzyme activity and that the same polymorphism shows a trend towards an atheroprotective effect in a CAD case-control study.

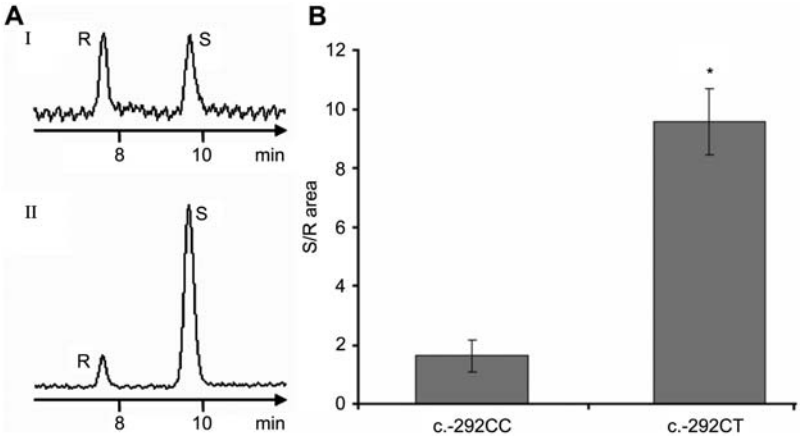


**Figure 3** Increased 15(S)-HETE levels result from increased ALOX15 activity in c.–292T allele carriers.
15-HETE eluted from the non-chiral HPLC column was separated into S- and R-enantiomers on a chiral HPLC column. (A) Chromatograms of the separation of 15-HETE S- and R-enantiomers on the chiral column: (I) supernatant from c.–292CC homozygous macrophages with an S/R-enantiomeric ratio of 1.1; (II) supernatant from c.–292CT heterozygous macrophages with an S/R-enantiomeric ratio of 10. Retention times for the R-enantiomer (7.5 min) and S-enantiomer (9.5 min) were identified by comparison with 15-HETE reference standards. (B) Average S/R-enantiomeric ratios for 15-HETE in supernatants from macrophages of five c.–292CC homozygous and four c.–292CT heterozygous carriers. Error bars indicate the standard deviation. The asterisk indicates a significant difference ($p < 0.0001$).

ALOX15 directly catalyzes formation of the arachidonic acid metabolite 15(S)-HETE, which is an endogenous inhibitor of inflammation. For example, 15(S)-HETE directly inhibits 5-LOX and superoxide production (13), and inhibits polymorphonuclear neutrophil (PMN) degranulation following stimulation with leukotriene B4 (14). 15(S)-HETE production also inhibits PMN migration across cytokine-activated endothelium in vitro (15). This inhibition of migration is further assisted by the metabolism of 15(S)-HETE

**Table 1** Characteristics of controls and cases.

| | Controls (n = 238) | Cases (n = 260) | p-Value |
|---|---|---|---|
| Age, years | 59 (53–66) | 64 (57–71) | <0.0001 |
| Male, % | 58.2 | 78.9 | <0.0001 |
| History of hypertension, % | 28.9 | 46.7 | <0.0001 |
| History of diabetes, % | 3.6 | 20.7 | <0.0001 |
| History of high cholesterol, % | 19.7 | 51.3 | <0.0001 |
| Smoking, % | 47.4 | 73.6 | <0.0001 |
| Body mass index, kg/m$^2$ | 25.7 (23.0–28.4) | 26.8 (24.5–29.8) | <0.0001 |
| Creatinine, μmol/L | 90 (83–99) | 89 (80–99) | 0.14 |
| CRP, mg/L | 2 (1–3) | 2 (1–4) | <0.0001 |
| Cholesterol, mmol/L | 5.9 (5.1–6.5) | 5.1 (4.4–5.9) | <0.0001 |
| HDL, mmol/L | 1.6 (1.3–2.0) | 1.3 (1.1–1.4) | <0.0001 |
| LDL, mmol/L | 3.5 (2.8–4.0) | 3.2 (2.5–3.9) | 0.0096 |
| Triglycerides, mmol/L | 1.4 (0.9–2.0) | 1.3 (1.0–1.8) | 0.79 |

Results are presented as median (interquartile range) or percentage.

**Table 2** Exact logistic regression models predicting CAD status for cases.

| Variation | Genotype, % | | Odds ratio | 95% CI | p-Value |
|---|---|---|---|---|---|
| | Controls (n = 238) | Cases (n = 260) | | | |
| c.–635A>G | 48.5/43.9/7.5 | 53.3/40.2/6.5 | 0.82 | 0.61–1.11 | 0.16 |
| c.–511A>G | 97.1/2.9/0.0 | 98.1/1.9/0.0 | 0.65 | 0.18–2.24 | 0.46 |
| c.–292C>T | 95.4/4.2/0.4 | 97.3/2.7/0.0 | 0.45 | 0.16–1.17 | 0.084 |
| c.–272G>T | 64.4/30.1/5.4 | 60.9/34.5/4.6 | 1.11 | 0.81–1.52 | 0.55 |
| c.–186G>C | 66.1/29.7/4.2 | 61.3/34.1/4.6 | 1.24 | 0.90–1.72 | 0.18 |
| c.136-35C>T | 100/0.0/0.0 | 98.8/1.2/0.0 | 3.07[a] | 0.45–∞ | 0.16 |
| c.337+8C>T | 51.9/41.4/6.7 | 57.1/36.0/6.9 | 0.96 | 0.71–1.30 | 0.79 |
| c.543-16C>T | 97.5/2.5/0.0 | 96.9/3.1/0.0 | 1.14 | 0.35–3.86 | 0.89 |
| c.1564C>T | 100/0.0/0.0 | 99.6/0.4/0.0 | 0.35[a] | 0.02–∞ | 0.63 |
| c.1849C>T | 98.7/1.3/0.0 | 99.6/0.4/0.0 | 0.52 | 0.02–5.35 | 0.82 |
| c.2076+200C>T | 65.7/30.5/3.8 | 68.2/28.7/3.1 | 0.87 | 0.62–1.22 | 0.41 |

[a]Unbiased median value.

into its derivatives lipoxin A4 and B4. The lipoxins have also been shown to antagonize the pro-inflammatory effects of leukotrienes, reduce neutrophil chemotaxis and block neutrophil entry across the intestinal epithelium and to be involved in the resolution of inflammation (16).

This anti-inflammatory effect may play a role in atherosclerosis, since the c.–292C>T polymorphism showed a trend towards an atheroprotective effect in a human CAD case-control study. In this study, the c.–292T allele was less frequent in patients with CAD (2.7%) compared to controls (4.6%), and carriers of the c.-292T allele showed a non-significant reduction in risk for atherosclerosis (odds ratio 0.45, confidence interval 0.16–1.17). These results are in line with the atheroprotective role of the monocyte-specific *ALOX15* expression in transgenic rabbits (17, 18), which produced higher levels of the anti-inflammatory factor lipoxin A4 (19). This led to an overall enhancement of endogenous anti-inflammation, which resulted in a lower degree of atherosclerosis upon feeding of a cholesterol-rich diet (19). However, not all animal models showed an atheroprotective role of ALOX15. In *apoE*$^{-/-}$ mice, conditional macrophage-specific and general disruption of the *ALOX15* homologue gene *12(S)/15(S)-LOX* reduced atherosclerosis (20, 21). In line with the pro-atherogenic effect of ALOX15 in mice, endothelial overexpression of human ALOX15 enhanced atherosclerosis (22). This discrepancy in the function of ALOX15 between different species can be explained by the finding that each animal has a different set of 12-LOX and 15-LOX isoenzymes with different enzymatic properties. When these enzymes are knocked out or overexpressed in different animals and cells, this can cause divergent effects. In this context, higher expression of ALOX15 in human macrophages seems to be atheroprotective, as in transgenic rabbits that overexpress reticulocyte-type 15(S)-LOX (17, 18).

Although our results indicate a trend towards an atheroprotective effect for the c.–292T allele, there was no significant association, which may be due to the low allele frequency (2.5%) of the mutant c.–292T allele in the study population (9, 10) and the small sample size. Our sample with 498 participants is too small to detect a significant association with enough power. The sample size necessary to detect an odds ratio of 0.5 with statistical power of 80% and a c.–292T allele carrier frequency of 4.6% is 1170 subjects. Hence, larger studies to analyze the association of this polymorphism with CAD are required to investigate whether increased ALOX15 expression and activity in the physiological range is anti-atherogenic in humans.

## Acknowledgements

We thank Erika Schläpfer-Nadal from the Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich for kind help with isolation of human monocytes. This work was supported by the EMDO Stiftung, the Forschungskommission of the University of Zurich and the OPO Stiftung to M.H.

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Received October 13, 2006, accepted January 4, 2007