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Modulation of TNF-*α* plasma levels in coronary artery disease [CAD] and non-CAD male patients by lncRNA UCA1 and aspirin

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Abstract: The aim of this study was to investigate the expression of long non-coding RNA urothelial carcinoma-associated 1 (UCA1) and its role in TNF-α production as one of the main inflammatory cytokines, in peripheral blood mononuclear cells (PBMCs) of patients with coronary artery disease (CAD) and healthy non-CAD (NCAD) individuals as the control group. Fifteen CAD and 15 NCAD individuals were enrolled in the study. UCA1 expression in PBMCs and the plasma concentrations of interleukin (IL)-6, IL-22 and tumor necrosis factor (TNF)-α were assessed by real-time PCR and flowcytometry, respectively. UCA1 expression was not significantly different between the CAD and NCAD groups, however, its level was higher in PBMCs of regular aspirin users of both study groups. Furthermore, aspirin users showed a significantly lower plasma level of TNF-α in comparison to non-aspirin users. In addition, UCA1 expression was negatively correlated with the level of TNF-α in the total sample of the examined population. It seems that the increased levels of UCA1 may be an underlying mechanism for downregulation of TNF-α in aspirin users.

Keywords: aspirin; noncoding RNA; UCA1; TNF-α; coronary artery disease

INTRODUCTION

Coronary artery disease (CAD) is the most common type of heart disease and remains the principal cause of death worldwide [1]. However, CAD mortality rates sharply declined by 50% in most industrialized countries in the second half of the 20th century. About half of this decrease is attributed to improved management of major risk factors, while the other half is related to evidence-based medical therapies with aspirin, other platelet function inhibitors, angiotensin converting enzyme (ACE) inhibitors and statins [2-5].

It is commonly accepted that systemic uncontrolled inflammation is the underlying process in different metabolic diseases such as diabetes and CAD. The inflammatory process is characterized by the activation of immune cells along with an increase in levels of inflammatory protein mediators such as interleukin IL-6 and tumor necrosis factor TNF-α [6].

Aspirin, as an antiinflammatory and antiplatelet aggregation drug, remains one of the most widely used medications. Currently, about 40% of US adults older than 50 use aspirin for primary or secondary prevention

of cardiovascular diseases [7]. Randomized clinical trials indicate that aspirin reduces by about 20% acute myocardial infarction (MI) events [4]. It was shown that aspirin decreases the need for coronary angioplasty intervention by about 53%; simultaneously, it reduces the risk of unstable angina by about 46% [8].

Although the antiatherosclerotic molecular mechanisms underlying the effects of aspirin have been extensively investigated, the involvement of a large number of cell components, such as long non-coding RNAs (lncRNAs), is still not completely understood. LncRNAs are a class of non-protein-coding transcripts longer than 200 nucleotides that have recently been shown to be involved in the regulation of various pathophysiological conditions at all levels of gene regulation [9]. Several lncRNAs, such as SNHG16 [10], LINC00305 [11], ANRIL [12], MALAT1 [13], lincRNA-H19 [14] and lncRNA-p21 [15], are associated with atherosclerosis and related cellular processes, including the inflammatory responses of macrophages, apoptosis, modulation of atherogenic cell functions through trans-regulation of gene networks, endothelial cell function and upregulation of acid phosphatase 5 expression resulting in increased risk of ischemic stroke and neointima formation.

LncRNA urothelial carcinoma-associated 1 (UCA1), which is located at 19p13.12, was first detected in human bladder carcinoma and was later found to be overexpressed in many types of cancer. UCA1 plays a crucial role in the pathophysiology of cardiovascular diseases. Thus, UCA1 contributes to cardiomyocyte apoptosis and hypertrophy [16,17]. The plasma level of UCA1 has been reported to be elevated in patients with chronic heart failure (CHF) and it could be an excellent diagnostic parameter for CHF [18]. Furthermore, a recent *in vitro* study in human macrophages suggested that knockdown of UCA1 could inhibit atherosclerosis progression [19].

Recent studies have revealed that different types of lncRNAs were expressed in certain cancers as a response to aspirin treatment. For example, 28 lncRNAs were statistically upregulated more than two-fold by aspirin in human colorectal cancer cells [20]. After aspirin treatment, lncRNA-H19 expression was dramatically decreased in both papillary thyroid carcinoma [21] and breast cancer stem cells [22]. However, little is known about the regulation and functions of lncRNAs in the prevention and treatment of cardiovascular diseases with aspirin. Therefore, the aim of this study was to evaluate UCA1 expression as well as its association with the levels of IL-6, IL-22 and TNF-α in the group of aspirin users among the CAD and non-CAD [NCAD] individuals.

MATERIALS AND METHODS

Participants

Between October 2018 and January 2019, peripheral blood samples were collected from thirty male patients who had undergone CT angiography or coronary angiography for suspected coronary artery disease at the Al Zahra Heart Hospital affiliated to Shiraz University of Medical Sciences, Shiraz, Iran. The patients were categorized into 2 equal groups: CAD and NCAD. The CAD patients were visited by a cardiologist and were characterized by more than 50% of stenosis in at least one coronary artery, which was confirmed by angiography. The demographic and clinical characteristics of the study groups are summarized in Supplementary Table S1. All the samples were collected from the participating volunteers after they signed informed consent forms. The study protocol was approved by the local Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1397.687). Individuals who had received immunosuppressive drugs, as well as individuals with any chronic renal or liver failure, diabetes mellitus, blood diseases, malignancy, infections and history of inflammatory diseases, were excluded from the study. The demographic and anthropometric data, including age and body mass index (BMI), were collected via a questionnaire. Using the hospital patient records, the participants were categorized into "aspirin users" or "aspirin non-users".

Sample collection and PBMCs isolation

Venous blood samples were collected (10 mL) from the participants and poured into a sterile tube containing EDTA as anticoagulant. PBMCs were separated by a Ficoll-Hypaque (Lympholyte-H; Cedarlane Laboratories, Canada) gradient and centrifuged at 400 ×g for 20 min. Plasma and PBMCs were stored at -80°C.

Cytokine assay

The plasma concentrations of IL-6, IL-22 and TNF-α were measured by LEGENDplex™ Human Th22 Panel (BioLegend, USA) using a flow cytometer (BD FACSCalibur, USA), according to the manufacturer's instruction.

Quantitative real-time polymerase chain reaction [qRT-PCR]

Following the manufacturer's instructions, total RNA was extracted from PBMCs using the One Step-RNA Reagent (Bio Basic, Germany). For detecting the relative gene expression of lncRNA-UCA1, 2 μg of the total RNA was reverse transcribed into a cDNA template using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA), and qRT-PCR was performed in triplicate using the ABI 7500 Sequence Detection System (Applied Biosystems, USA) with specific primers. The primers for human lncRNA-UCA1 amplification used in the study were: forward: 5'-TTAGGCTGGCAACCATCAGATC-3', reverse: 5'-TGTTGTCCTGGATGCTGGTCT-3'; β-actin: sense: 5'-GCCTTTGCCGATCCGC-3', anti-sense: 5'-GCCGTAGCCGTTGTCG-3'. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed using SPSS ver. 22.0 (IBM Inc., USA) and GraphPad Prism ver. 8.2 (San Diego, CA, USA). Categorical data were tested by Fisher's exact test and presented as the frequency and percentile. Continuous variables are presented as the mean±standard error of the mean (SEM) and tested by Mann-Whitney's U test and one-way ANOVA. The Spearman correlation test was used to determine the relationship between the variables. A P value < 0.05 was considered as statistically significant.

RESULTS

In this cross-sectional study, a total of 30 participants were included (15 CAD and 15 NCAD). Supplementary Table S1 presents the demographic and clinical characteristics of the study groups. As can be seen, HC, Echo EF and DBP were significantly lower in patients with CAD compared to NCAD.

Comparison of UCA1 level between the study groups

Analysis of UCA1 expression in the PBMCs of CAD and NCAD groups revealed that there were no significant differences between the groups (P = 0.907) (Fig. 1). When the participants were classified into aspirin and aspirin non-users, the level of UCA1 was significantly higher in the aspirin users (P = 0.003), as shown in Fig. 2C. Furthermore, comparison of the UCA1 levels between CAD and NCAD groups based on the aspirin consumption showed that UCA1 expression was significantly higher in the group of NCAD aspirin users compared to the NCAD aspirin non-users (P = 0.013). However, the difference between CAD aspirin and CAD aspirin non-users in the level of UCA1 was not statistically significant (P = 0.090) (Fig. 2D).

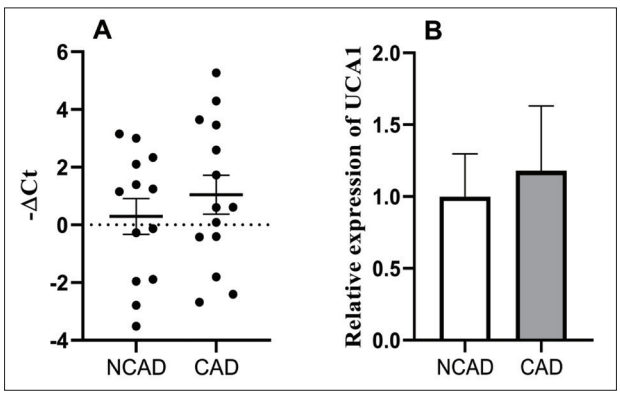

Fig. 1. Comparison of UCA1 expression in PBMCs from patients with coronary artery disease (CAD) and non-CAD (CAD) groups. **A** – Scatter plot of the -ΔCT of UCA1 (the difference between CT value for UCA1 and CT value for beta-actin). **B** – Relative expression of UCA1.

Comparison of the cytokine levels between the study groups

Measuring the cytokine levels revealed that the production of IL-6, IL-22 and TNF-α was similar for the CAD and NCAD groups. Comparison of the concentrations of cytokines between groups showed that although the concentrations of IL-6 and IL-22 cytokines were lower in the aspirin users (Fig. 3A and C)], the concentration of TNF-*α* was significantly lower (P = 0.034) (Fig. 3E). When the CAD and NCAD groups were divided based on aspirin intake, the cytokine concentrations were lower in the group of aspirin users, however, this difference was not statistically significant (Fig. 3B, D and F).

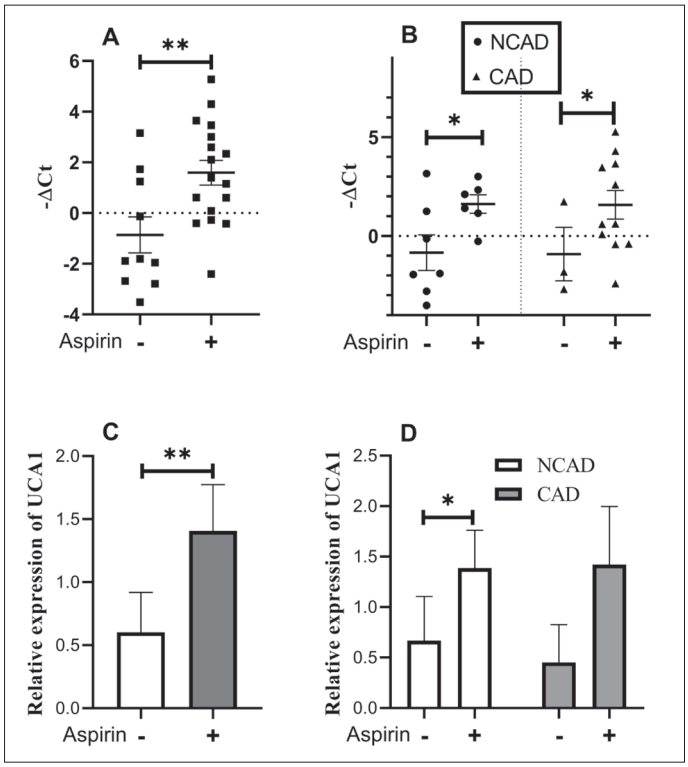

Fig. 2. Comparison of UCA1 expression in PBMCs (**A** and **C**) from the total population of aspirin and non-aspirin users, respectively. **B** and **D** – aspirin and non-aspirin users among CAD and NCAD groups, respectively. *P value < 0.05; **P value < 0.01. CAD – coronary artery disease; NCAD – non-CAD.

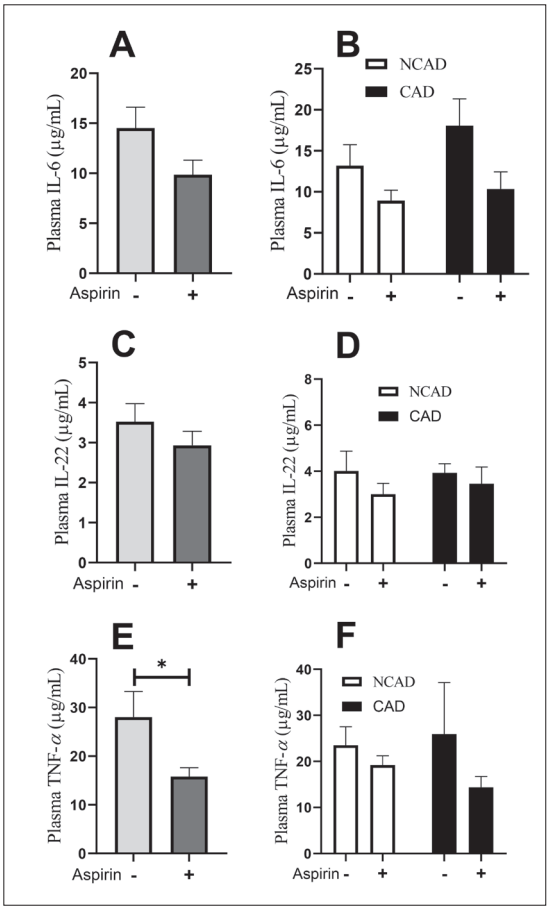

Fig. 3. Comparison of inflammatory cytokine concentrations (**A**, **C** and **E**) in the plasma of the total population of aspirin users and non-aspirin users; inflammatory cytokine concentrations (**B**, **D** and **F**) in the plasma of aspirin and non-aspirin users in coronary artery disease (CAD) and non-CAD (CAD) groups. *P value < 0.05. IL – interleukin; TNF – tumor necrosis factor.

Investigation of the relationship between UCA1 and cytokine levels

Investigation of the correlation between UCA1 and IL-6, IL-22 and TNF-α revealed a significant negative correlation between UCA1 expression and the plasma concentration of TNF-α ($r = -0.419$, $P = 0.047$) in the total population (Fig. 4C). However, no significant correlations between IL-6, IL-22 and UCA1 were observed (Fig. 4A and B).

DISCUSSION

There is growing evidence supporting the role of lncRNAs in the pathogenesis of almost all types of diseases. LncRNA UCA1 has generated great interest among cardiologists because it is abundantly expressed in the heart and plays critical roles in promoting the development of human cancers [23]. However, the association between UCA1 and inflammatory cytokines in CAD patients as related to aspirin intake remains unknown. Accordingly, the present study investigated the effects of aspirin on the expression of UCA1 as well as on the production of IL-6, IL-22 and TNF-α cytokines by PBMCs, and their concentrations in the plasma among patients with CAD as compared to NCAD individuals.

Based on the presented results, although the levels of UCA1 were higher in the CAD group, its upregulation was not significantly different from the NCAD group, suggesting that this is due to the low sample size. However, the role of this lncRNA has been shown in the pathogenesis of several diseases such as chronic heart failure [18], atherosclerosis [19] and many types of cancer [16]. Recently, it was revealed that UCA1 might

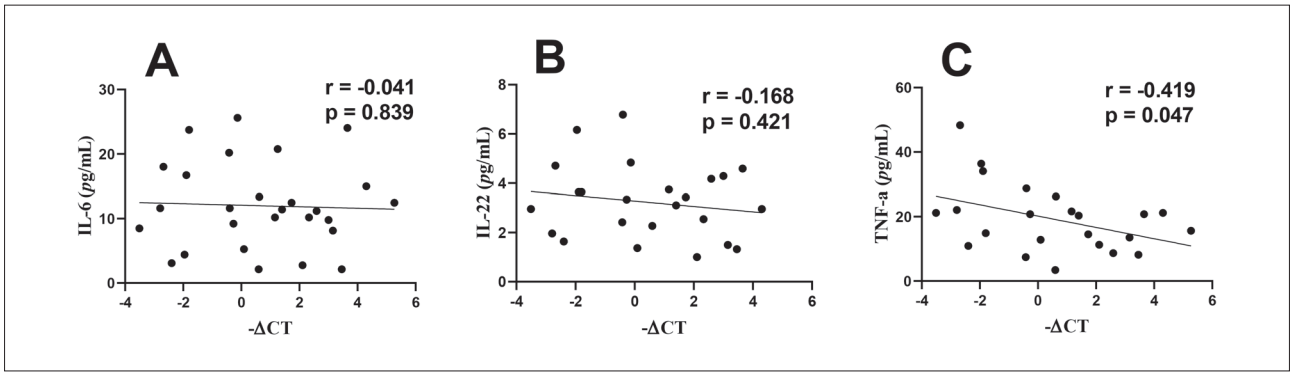

Fig. 4. Correlation analyses between plasma cytokine levels and UCA1 expression in PBMCs of the total population. UCA1 expressions are presented as minus CT levels (the difference between the CT value for UCA1 and the CT value for control β-actin). The results show significant negative correlations between the concentration of TNF-α and UCA1 expression. IL – interleukin; TNF – tumor necrosis factor.

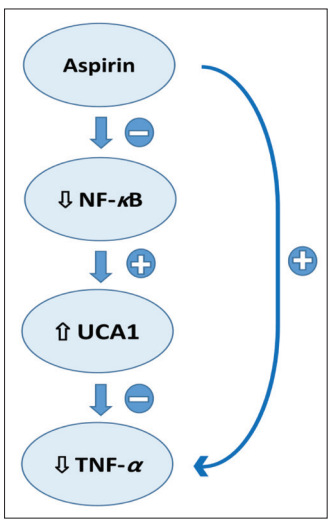

Fig. 5. The potential mechanism of the effect of aspirin on UCA1 expression among aspirin users. Modulating the NF-κB/UCA1 pathway might be an underling mechanism for down-regulation of TNF-α in regular aspirin users. IL – interleukin; TNF – tumor necrosis factor.

protect cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by suppressing miR-143, which modulates the MDM2/p53 signaling pathway [24]. The presented findings also demonstrated the protective effect of UCA1 against ischemia/reperfusion-induced oxidative stress and mitochondrial dysfunction in H9C2 cardiomyocytes [25]. In addition, a novel mechanism for UCA1 that can provide therapeutic targets or promising biomarkers related to heart failure and myocardial infarction was reported [24].

In the present study, we observed that UCA1 expression was not associated with CAD, however, its expression was significantly higher in the PBMCs of aspirin users compared with that in aspirin non-users. This is the first study indicating that UCA1 expression is increased in the PBMCs of aspirin users and that this association is independent of CAD status. While the molecular mechanisms underlying increased expression of UCA1 are not well understood, the antiinflammatory effect of aspirin could be a determining factor for this. As already mentioned, the relationship between UCA1 and inflammatory pathways has been reported in several studies [26,27], and the protective effects of aspirin on colon and lung cancers through modulation of some lncRNAs have previously been suggested [28,29]. Aspirin is known as a nonsteroidal antiinflammatory drug, and it has also been reported that at the antiplatelet activity dose, it can change cyclooxygenase-2 or COX-2 to the antiinflammatory mediator 15-epi-lipoxin.

In addition, aspirin, by acetylating the lysine in endothelial nitric oxide synthase (eNOS), leads to the production of heme oxygenase (HO-1), a molecule with an antioxidative property in vascular cells [30]. Other potentially antiinflammatory effects of aspirin have been described, such as inhibition of inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, suppression of fractalkine, also known as chemokine (C-X3-C motif) ligand 1 (CX3CL1), in atherosclerotic lesions, reduction of aortic NF-κB activity with a decrease in intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein 1 (MCP1), TNF-α and interleukin-12 subunit p40 (IL-12p40), suppression of COX-2 gene transcription, inhibition of the expression of lectin-type oxidized LDL receptor 1 (LOX-1) and matrix metalloproteinase-1 (MMP-1), and improvement of insulin sensitivity [30].

Accordingly, upregulation of UCA1 might contribute to the antiinflammatory effects of aspirin. To

test this hypothesis, we assessed the levels of cytokines involved in inflammation, such as IL-6, IL-22 and TNF-α, and their associations with the levels of UCA1 and aspirin consumption. Although the levels of these cytokines were not significantly different between the study groups, aspirin users displayed lower plasma concentrations of all inflammatory cytokines [IL-6 (32%), IL-22 (17%), TNF-α (44%)] in comparison to aspirin non-users, which was statistically significant only for TNF-α production. Interestingly, the concentration of TNF-α exhibited a negative correlation with UCA1 expression. Given the inflammatory nature of CAD, the effects of aspirin on the level of inflammatory cytokines were investigated in different studies. It was reported that aspirin could prevent the production of proinflammatory cytokines (IL-6 and TNF-α) via direct inhibition of the IκB/NF-κB pathway [31,32]. LncRNA UCA1 can also suppress inflammation via inhibition of NF-κB and the myocyte-specific enhancer factor 2C (MEF2C)/NF-κB signaling pathway by influencing the production of IL-6, TNF-α and miR-203 [26]. Therefore, we concluded that in response to aspirin, increased levels of UCA1 can act as a compensatory mechanism to decrease the level of TNF-α, as summarized in Fig. 5. Thus, one of the possible routes of the beneficial effects of aspirin on injury [33] can be through increased UCA1 expression. Regarding the controversies around the effects of aspirin on the downregulation [34] or upregulation [35] of TNF-α expression, further *in vitro* and *in vivo* studies are needed to elucidate the exact molecular mechanism that would explain the effect of aspirin on UCA1.

CONCLUSION

The upregulated level of UCA1 in aspirin users among cardiovascular patients may be an underlying mechanism for downregulation of one of the main inflammatory cytokines such as TNF-α. However, further studies are needed to explore the precise molecular mechanism of UCA1 upregulation and its association with aspirin in the context of suppression of inflammatory responses.

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Author contributions: P.N-S performed all of the experiments, analyzed the data and contributed to writing the manuscript. P.I. and M.K. analyzed the CA and CTA reports. H.A. and R.F. contributed to the concept and design. A.S. and M.K contributed to the concept and design, the financial support, supervision of practical performance and the final approval of the manuscript. M.E contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Conflict of interests disclosure: The authors have no interests to declare.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Nowrouzi-Sohrabi%20et%20al_5497_Supplementary%20Material.pdf