

Dynamic Changes in Plasma Urotensin II and its Correlation with Plaque Stability

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Abstract

Background: Urotensin II (Ull) is involved in the formation of atherosclerosis, but its role in the stability of atherosclerotic plaque is undetermined. The purpose of this study was to observe the dynamic change of plasma Ull and analyze its relationship with the stability of atherosclerotic plaques.

Methods: The plasma Ull concentration in patients with acute coronary syndrome (ACS) was detected. A vulnerable plaque model was established by local transfection of a recombinant *P53* adenovirus into plaques of rabbits fed with a high-cholesterol diet and subjected to balloon injury, to evaluate the stability of the atherosclerotic plaques.

Results: Our results showed that the level of plasma Ull was increased in ACS patients compared with healthy subjects. However, it was significantly decreased in ST-segment elevation myocardial infarction patients (STEMI) and increased again in acute myocardial infarction (AMI) patients that were discharged after three months. Ull dynamic change and its correlation with plaques stabilities were further verified in rabbit with atherosclerotic vulnerable plaque. The Ull level in rabbits was significantly decreased after *P53* gene transfection which can lead to of plaque instability.

Conclusions: In conclusion, the level of plasma Ull was significantly decreased in ACS with STEMI, which may serve as a reliable biological marker to reflect the progression and stability of atherosclerotic plaques.

1. Introduction

The main pathology of coronary heart disease (CHD) is coronary atherosclerosis, characterized by a dynamic change in the stability of atherosclerotic plaques[1]. Unstable plaques are easily eroded or ruptured, leading to thrombosis, that results in complete or subtotal occlusion of coronary arteries clinically named as acute coronary syndrome (ACS), which is the main cause of death in patients with CHD[2]. Therefore, the early detection of vulnerable populations is crucial for the prevention of clinical events in CHD patients. However, biomarkers, that could be used in clinical settings and which would aid in predicting the future course of plaques have not been identified.

Urotensin II (Ull) is the most potent vasoactive substance[3] and its binding to the G protein-coupled receptor GPR14 (also named as UT) produces a variety of biological effects and is involved in multiple cardiovascular diseases, such as essential hypertension, coronary heart disease, congestive heart failure, diabetes mellitus and renal failure[3-5]. In addition, recent evidence suggested Ull contributes to the development of atherosclerotic cardiovascular diseases [6-9]. For example, Ull induces monocytes chemotaxis and contributes to the recruitment of monocyte expressing the UT receptor to the atherosclerotic lesions[10]. This event promotes the accumulation of inflammatory cells in the plaque and accelerates foam cells formation[11, 12], which suggests that Ull is involved in the development of atherosclerotic plaques. However, the reported data regarding the level of circulating Ull in ACS patients is inconsistent. Al Kindi H et al. found that Ull plasma level was elevated in ACS patients, immediately after

clinical presentation[13]. On the contrary, Babińska M et al. and Joyal D et al. found that Ull plasma level was significantly decreased in ACS[14, 15]. Therefore, Ull dynamic change is inconsistent and ambiguous and may be due to the different techniques of Ull detection and sets of patients.

In this study, we first observed the Ull plasma level in ACS patients' admission and follow-up. Furthermore, we verified this dynamic change in a rabbit animal model of atherosclerotic that is vulnerable to plaques formation and analyzed the correlation with plaques stability. Our results showed that Ull plasma level was increased in the process of atherosclerotic plaque formation, but it dramatically decreases when the plaque is unstable. Thus, in ACS patients with ST-segment elevation myocardial infarction (STEMI), a significant decrease in Ull level may predict the future course of plaques.

2. Materials And Methods

2.1.Study Subjects

One hundred and thirty-five consecutive ACS patients, without age limit, were admitted to the cardiology department and enrolled in the study. Patients with chronic stable coronary heart disease, other cardiovascular diseases (including chronic heart failure, congenital heart disease, valvular disease and cardiomyopathy), other systemic diseases (Including chronic liver and kidney insufficiency, hematological diseases, digestive system diseases, connective tissue diseases, neurological diseases, neoplastic diseases, severe infections, trauma, pregnant or lactation patients) were excluded. For the follow up, the ACS patients who received standard secondary prophylactic treatment of coronary heart disease were discharged after 3 months if they had no acute ischemic attack, or are clinically stable which represents plaque stability. All the other patients received standard secondary prophylaxis for coronary heart disease, including statins, after discharge. A total of 48 healthy nursing and care worker volunteers working in the cardiology department served as healthy controls. The study complied with the Declaration of Helsinki and was approved by the ethics committee of Xuanwu Hospital Capital Medical University [2016] 010 and a written informed consent was obtained from patients and healthy controls.

2.2.Ull Measurement

The baseline data of the patients were recorded and Ull level was immediately measured on admission and after 3 months follow-up by Radioimmunoassay (RIA) as previously described [16]. Briefly, venous blood was collected into tubes containing EDTA and aprotinin. The blood samples were immediately centrifuged for 15min (2000g at 4°C), and the supernatants were stored at -70 °C until measurement. For the Ull immunoreactivity assay, the samples displaced the traces parallel to standards curves and the cross-reactivity between human and rabbit Ull was 100%. No cross-reactivity was found with human and rabbit angiotensin II, brain natriuretic peptide, or endotoxin. The Ull intra- and inter-assay coefficients of variation for blood samples were <10%. The content of high-sensitivity C-reactive protein (hs-CRP) was determined by a high-sensitive enzymeimmunoassay kit for C-reactive protein (hs-CRP EIA Kit, Beijing Yonghan Xinggang Biotechnology Co., Ltd.).

2.3. Animal study and atherosclerotic vulnerable plaque model

Thirty-two male Japanese white rabbits (1.5 -2.0kg) were provided by the Laboratory Animal Center of Capital Medical University. The rabbits were randomly divided into four groups: Control: common diet; HFD: high fat diet (containing 5% lard, 1.5% cholesterol and 5% egg yolk powder) alone; HFD + BI: high fat diet + balloon injury; and HFD + BI +*P53*: high fat diet + balloon injury + gene transfection. The rabbits were individually housed in metal cages in an air-conditioned rooms under a 12 h light/12 h dark cycle. Water was allowed ad libitum, and 100 g/day food was provided. No adverse events were observed. The rabbits were anesthetized with 1:1 Zoletil 50 and ketamine mixture (1ml/kg) given intramuscularly, and the right femoral artery was exposed. A 3F Fogarty balloon catheter (American Baxter Healthcare Corporation) was introduced through the right femoral artery and proximally advanced 25 cm into the iliac artery and abdominal aorta. The balloon was inflated with saline to distend the abdominal artery and then was pulled back to the femoral artery, a step which was repeated three times. Eight weeks after artery injury, the rabbits in HFD + BI were transfected with 0.5×10^{13} pfu/L of a recombinant human *P53* adenovirus gene (Shenzhen Sebano Gene Technology Co., Ltd. China). The atherosclerotic vulnerable plaque model was replicated as previously described[17]. The blood was weekly collected from the auricular vein and Ull plasma level was determined by RIA. The animal experiments were approved by the Laboratory Animal Administration Committee of Capital Medical University (Ethics lot number AEEI-2015-001) and carried out according to the Guidelines for Animal Experimentation of Capital Medical University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 2011). All sections of this report adhere to the ARRIVE Guidelines for reporting animal research[18]. A completed ARRIVE guidelines checklist is included in Additional file 1.

2.4. Histopathology, immunohistochemistry and plaque stability evaluation

At the 9th week, the animals were injected intramuscularly with a 1:1 mixture of Zoletil 50 and Ketamine (1ml/kg). The arteries were quickly dissected out and rinsed with cool PBS, fixed in 4% paraformaldehyde and embedded in paraffin. All animals were euthanized once arterial sample collection was complete by injecting a certain amount of (20-50 ml) air into the ear vein. The paraffin sections (5 μ m) were placed on microscope slides, deparaffinized, and then stained for histological analysis. For histological analysis, artery sections (5 μ m) were stained with hematoxylin and eosin (H&E), Verhoeff-van Gieson (VVG), Sirius red, Oil-red O and immunohistochemistry with Mac-2, α -SMA, Ull antibody as previously described[19]. The area of positive staining for lipids, collagen, vascular smooth muscle cells (VSMCs) and Macrophages was expressed as a percentage of staining area divided by plaque area at least at 10 high power fields (400x), The plaque vulnerability index was calculated as (macrophage staining% + lipid staining%) / (SMCs% + collagen fiber%)[20]. All quantifications were performed using ImageJ software (NIH, Bethesda, MD, USA).

2.5. Quantitative real-time PCR analysis

Total RNA was extracted by the Trizol reagent method (Invitrogen) and Total RNA (1 µg) were reverse-transcribed to generate cDNA with GoScript™ Reverse Transcription System (Promega, USA). The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the iCyclerIQsystem (Bio-Rad,USA) and as previously described[21] and using the following primers: Ull-Forward primer 5'-CTTCAGCTTCCCCTGCCC-3', Ull-Reward primer 5'-GACCTCGCACCCACAAAAC-3'.Ull expression was determined as the relative expression of the gene of interest to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

2.6.Statistical analysis

All values are expressed as the mean ± standard deviation. The statistical analyses were performed with two-tailed unpaired Student's t test when comparing only two groups and with one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. To analyse dynamic changes of plasma Ull levels in different groups of rabbits, two-way repeated measures ANOVA followed by Tukey's post-hoc test was performed. All analyses were carried out using the GraphPad Prism 7 Software. *p* values < 0.05 were considered statistically significant.

3. Results

3.1.Patient characteristics

The characteristics of ACS subjects are summarized in Table 1. The healthy control group is mainly young women with a low incidence of atherosclerosis. Low-density lipoprotein (LDL) levels in ACS patients, at admission, were significantly higher than that at follow-up (2.66 ± 0.81 mmol/L, 1.75 ± 0.48 mmol/L, $p < 0.05$); while, high-density lipoprotein (HDL) levels (1.19 ± 0.31 mmol/L, 1.22 ± 0.31 mmol/L, $p > 0.05$) and triglyceride (TG) levels (2.02 ± 1.60 mmol/L, 1.74 ± 1.30 mmol/L, $p > 0.05$) had no significant change. The change trend of LDL, HDL and TG levels in the UAP, STEMI and NSTEMI subgroups, at admission and follow-up, were similar to those in ACS patients and as a whole.

Table 1
Characteristics of patients with acute coronary syndrome and healthy controls

variable	Patients (n = 135)	Controls(n = 48)
Age (range, years)	61.59 ± 11.75(31–89)	36.94 ± 8.57(21–53)
Gender M/F, (%)	96/39(71.1/28.9)	4/44(8.3/91.7)
UAP(%)	7(5.2)	N/A
NSTEMI(%)	22(16.3)	N/A
STEMI(%)	106(78.5)	N/A
Previous myocardial infarction(%)	17(12.6)	None
Arterial hypertension(%)	84(62.2)	1(2.1)
Diabetes mellitus(%)	46(34.1)	None
Ejection fraction	56.98 ± 11.49	N/A
NT-proBNP(pg/ml)	1150.51 ± 2869.18	N/A
Peak value of cTNI(ng/ml)	18.87 ± 19.38	N/A
hs-CRP(mg/L)	5.72 ± 5.50	N/A
LDL-C(mmol/L)	2.66 ± 0.81	N/A
HDL-C (mmol/L)	1.19 ± 0.31	N/A
TG (mmol/L)	2.02 ± 1.60	N/A
Blood pressure (mmHg)	133 ± 25/77 ± 17	120 ± 11/75 ± 7
Statin treatment (%)	135 (100)	N/A
PCI treatment (%)	86(63.7)	N/A
Primary PCI (%)	41(30.4)	N/A
Selective PCI (%)	45(33.3)	N/A
CABG (%)	7(6.19)	N/A

Data is mean ± SD or number (%). ACS, acute coronary syndrome; UAP, unstable angina pectoris; NSTEMI, non-ST-segment elevation myocardial infarction; STEMI, ST segment elevation myocardial infarction; EF, ejection fraction; NT-proBNP, N-terminal B-type natriuretic peptide; cTNI: troponin I; hs-CRP, high-sensitivity C reactive protein; LDL-C, Low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triacylglycerol; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting. N/A, not applicable.

3.2.the Content Of Uii In Plasma Of Acs Patients

Compared with the healthy control subjects, Ull plasma level in ACS patients was higher (Fig. 1A). Then, we divided ACS patients into three groups (UAP, NSTEMI and STEMI) and found that Ull was significantly lower, especially in patients with STEMI, compared with the UAP and NSTEMI group (Fig. 1B). Interestingly, Ull was higher again in ACS patients after discharge for three months (Fig. 1C).

3.3. The content of Ull and hs-CRP in rabbits with a vulnerable plaque model

To observe Ull dynamic change, a vulnerable plaque model was duplicated in rabbits fed with high fat diet (HFD) + balloon injury (BI) + *P53* transfection. The result showed that HFD + BI significantly increased the content of Ull (Fig. 2A). However, it was interesting to observe that Ull content was immediately decreased after *P53* transfection and again increased significantly after *P53* transfection for 1 week (Fig. 2A). Because serum hs-CRP is a sensitive indicator of inflammation, which is closely related to the progress of plaque formation, we also measured the content of hs-CRP in the rabbits. The result showed that HFD + BI had a significantly increased hs-CRP content, which did not decrease after *P53* transfection (Fig. 2B).

3.4. histological Change Of Plaque

In order to verify the success of the atherosclerotic vulnerable plaque model, HE and Elastin staining were detected in the rabbits iliac and abdominal aortic arteries. Compared with the Control group, HFD induced artery thickness and disorder of elastic fibers, and BI or BI + *P53* treatment further aggravated this change and showed a large number of inflammatory cell infiltration, thinning of fibrous cap and intra-plaque bleeding (Fig. 3). Then, we further measured the stability of plaques by the vulnerable index. As shown in Fig. 4, the vulnerability index was significantly higher in the HFD + BI + *P53* group (Fig. 4). These results demonstrated that *P53* transfection of the plaques changed the composition and vulnerability of the abdominal artery plaque.

3.5. Ull Expression And Distribution

Compared with the Control group, the HFD + BI and HFD + BI + *P53* treatment increased Ull mRNA expression, and in the HFD alone, it did not induce Ull expression in plaques (Fig. 5A).

Immunohistochemical staining revealed that Ull expression (green) was seen to be localized mainly in the endothelial cell, foam cell and smooth muscle cell-rich compartment of the plaque and the artery tissue of HFD + BI and HFD + BI + *P53* had a strong Ull protein expression, which was mainly concentrated in cytoplasm, but a little Ull expression was found in the Control and HFD (Fig. 5B).

4. Discussion

In this study, we have identified that Ull can be a clinically practicable biomarker of atherosclerotic plaque instability for AMI. In AMI patients, the plasma Ull level was low at admission (representing plaque

instability) and significantly elevated in the post-treatment 3 months follow-up (representing plaque stability). At the same time, Ull plasma level in STEMI patients was significantly lower than that in NSTEMI patients on admission. Most ACS result from the loss of integrity of the protective covering over an atherosclerotic plaque. This occurs with plaque rupture when the fibrous cap overlying the plaque gets disrupted or with erosion when the endothelial lining of the plaque is disturbed. Compared with non-ST-elevation ACS, in acute STEMI, much higher percent of cases are due to plaque rupture. This may mean that plaques in STEMI are more unstable and therefore Ull levels are lower. Age and sex are uncontrollable risk factors in the development of atherosclerosis[22]. With the increase of age, the degree of atherosclerosis is also progressing, and compared with men, the incidence of premenopausal women is significantly lower than that of men. Therefore, the younger and mainly female people were selected as the healthy control group in this study. One of the limitation of this study is that the sample size of UAP was small, so it may not be able to explain the actual situation of Ull level in UAP subgroup patients.

Studies have found that Ull does play a role in the development of atherosclerosis, which promotes the occurrence and progression of atherosclerosis[23, 24]. Meanwhile, Ull stimulates endothelial and smooth muscle cell proliferation, inhibits endothelial cell apoptosis, increases collagen 1 and decreases MMP-1 expression[25–29]. Taken together, all these effects are expected to favor cell and matrix-enriched plaques, suggesting that Ull might be protective against plaque rupture. Several studies have shown that ACS patients have significantly lower Ull plasma levels than those with stable coronary heart disease[15, 30]. A low Ull level, after acute myocardial infarction, is associated with negative adverse events, such as death, heart failure, myocardial infarction, or emergency revascularization[31]. Based on the above basic and clinical studies, Ull may play an important role in the stability of plaques and its low expression may predict the instability of plaques and the occurrence of clinical events. In order to verify the relationship between the dynamic changes of plasma Ull and plaque stability, we established a rabbit atherosclerotic vulnerable plaque model. Rabbits are a suitable model for atherosclerosis research because of their rapid development of hyperlipidemia and atherosclerosis due to their sensitivity to dietary fat and cholesterol. In addition, rabbit atherosclerotic lesions resemble those observed in humans ranging from early stage lesions (fatty streaks) to complicated lesions (fibrous plaques). Finally, rabbits have lipoprotein profiles that are more similar to humans than those of mice[32]. The resulting atherosclerotic plaques were incubated transluminally with recombinant adenovirus carrying a *P53* transgene which have been demonstrated to induce remodeling in atherosclerotic plaque destabilization[33]. The destabilization of local preexisting atherosclerotic plaques was induced by Intra-arterial injection of the vectors used in this study, which characterized by a large number of inflammatory cell infiltration, thinning of fibrous cap and intra-plaque bleeding (Fig. 3). Interestingly, One day after transfection, Plasma Ull level decreased significantly. however, plasma Ull levels increased again one week later, though the morphological manifestations of plaques were still unstable. Moreover, The level of plasma Ull was positively correlated with the expression of Ull mRNA in plaques (Figure2 and 5). Apoptosis of plaque cells induced by *P53* gene, which is believed to be primarily responsible for the development of the destabilized cap phenotype through a proportional decrease in collagen production by cap VSMCs, are known to be extremely rapid, occurring within several hours after transfection[34]. So the concomitant decrease of plasma Ull level 24

hours after transfection suggests that Ull may be an important mediator of plaque destabilization. The upregulation of Ull expression in plaque and increase of Ull level in plasma one week after transfection may be related to the inherent resistance to injury and the activation of repair potential of plaques induced by plaque remodeling after transfection. A similar phenomenon was found in clinical studies, in which plasma Ull levels increased after acute myocardial infarction (Plaque instability or rupture) or percutaneous coronary intervention procedure (Plaque injury) and the clinical prognosis was positively correlated with the increase of plasma Ull level[16, 35]. Anyway, this study showed that the change of plasma Ull level was positively correlated with the expression of Ull in atherosclerotic plaque and Ull was involved in the modulation of plaque stability/instability. The transformation of a stable plaque to an unstable one involves complex mechanisms, including cell apoptosis and extracellular matrix degradation that promote plaque destabilization and rupture. The effect of U-II is therefore related to its ability to modulate mechanisms involved in plaque stability/instability which needs to be further explored. We have provided evidence for a potential role of Ull effects in the destabilization and rupture of atherosclerotic plaques. Using this model,we further confirmed that HFD + BI increased the Ull level in rabbits and more importantly that Ull level was significantly decreased after *P53* transfection and which can lead to of plaque instability.

Our results showed that Ull was significantly decreased in ACS patients with STEMI, which indicated adverse clinical events and this result was further verified in a rabbit vulnerable plaque model. In conclusion, the level of plasma Ull was significantly decreased in ACS with STEMI and may serve as a reliable biological marker to reflect the progression and stability of atherosclerotic plaque.

Abbreviations

CHD

coronary heart disease

ACS

acute coronary syndrome

Ull

urotensin II

UT

urotensin II receptor

UAP

unstable angina pectoris

STEMI

ST-segment elevation myocardial infarction

NSTEMI

non-ST-segment elevation myocardial infarction

AMI

acute myocardial infarction

RIA

radioimmunoassay

Hs-CRP

high sensitivity C reactive protein

qRT-PCR

quantitative real-time polymerase chain reaction

HFD

high fat diet

BI

balloon injury

VSMCs

vascular smooth muscle cells

LDL

low density lipoprotein

HDL

high density lipoprotein

TG

triglycerin

MMP-1

matrix metalloproteinase 1

Declarations

Ethics approval and consent to participate

The study complied with the Declaration of Helsinki and was approved by the ethics committee of Xuanwu Hospital Capital Medical University [LYS [2016] 010] and a written informed consent was obtained from patients and healthy controls.

This study conformed to the Guide for the Care and Use of Laboratory Animals and was approved by the Laboratory Animal Administration Committee of Capital Medical University (Ethics lot number AEEI-2015-001) The methods carried out in the experiment were in accordance with the approved guidelines.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author's contributions

CLY conceived the study; CLY, HXW drafted the manuscript; CLY, LJG, XL, GNL, JGX, MCY, HHY and HXW set the experiments, acquired the analyzed data; CLY, XL, LJG and HXW supervised data analysis and interpreted the results; All authors provided critical comments on the manuscript.

All authors read and approved the final manuscript.

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Figures

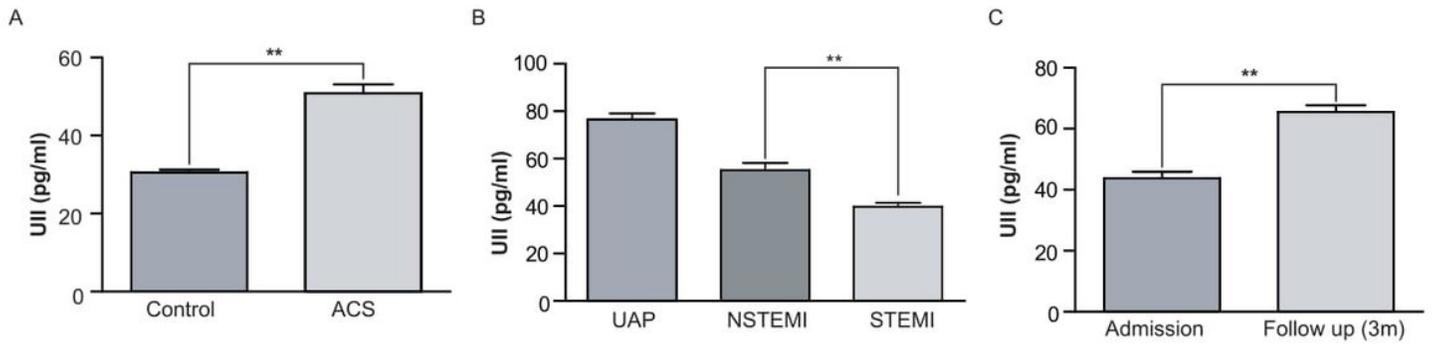


Figure 1

Figure 1

Detection of plasma Ull in ACS patients. (A) The plasma was collected from patients and healthy people and the level of circulating Ull was measured by the RIA method (ACS n = 135, healthy control n = 48); (B) ACS patients were further divided into UAP (n = 7), NSTEMI (n = 22) and STEMI (n = 106); (C) The plasma was collected from patients 3 months after discharge. The data are expressed as mean \pm SD; Statistical analysis were performed using unpaired Student's t test in figure A and C, **p < 0.01 vs Control, **p < 0.01 vs admission ; Statistical comparisons in figure B were made using one-way ANOVA followed by the Tukey's multiple comparison test, **p < 0.01 vs NSTEMI; UAP: unstable angina pectoris; NSTEMI: non-ST-segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction.

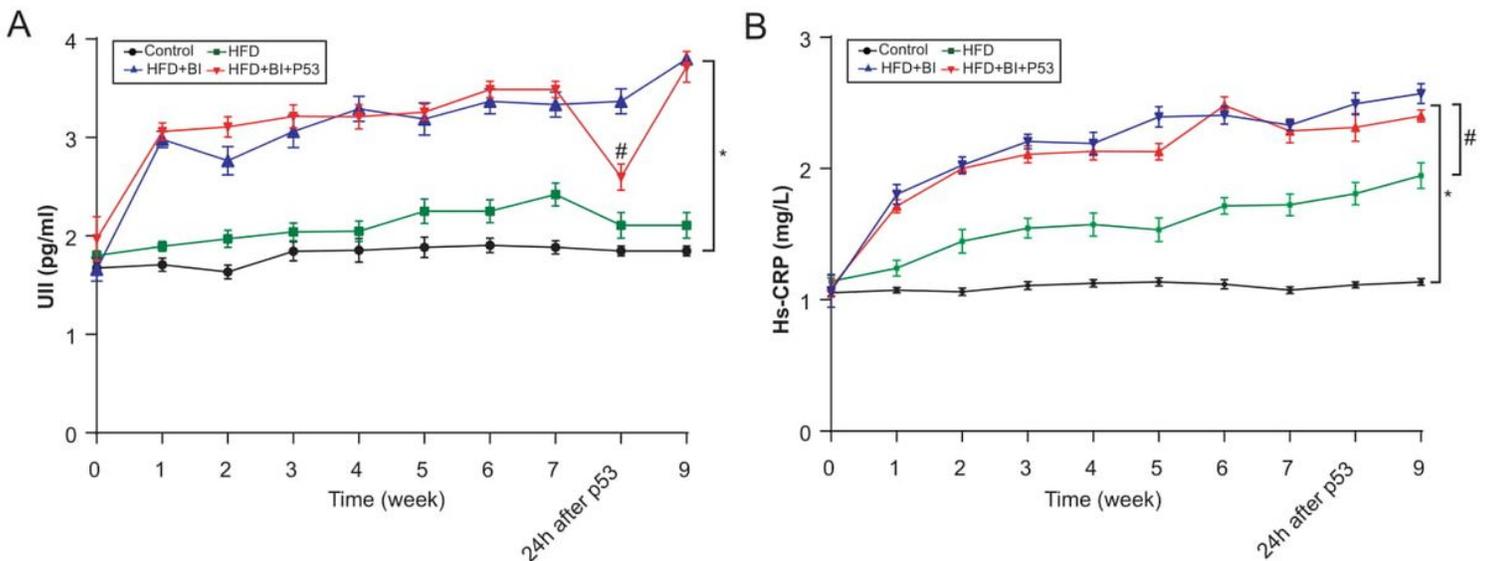


Figure 2

Figure 2

Detection of plasma Ull in rabbits with a vulnerable plaque model. The rabbit atherosclerotic vulnerable plaque model was replicated with HFD + BI + P53. (A) The plasma was collected from rabbits at different times (0, 1, 2, 3, 4, 5,6,7,8 and 9weeks) (n = 8-10 per group) and the level of circulating Ull was measured

by the RIA method. (B) The level of circulating hs-CRP was measured using the EIA kit. The data are expressed as mean \pm SD, Statistical comparisons were made using RM two-way ANOVA followed by the Tukey's multiple comparison test; * $p < 0.05$ vs Control, # $p < 0.05$ vs HFD (B), # $p < 0.05$ vs before transfection (A).

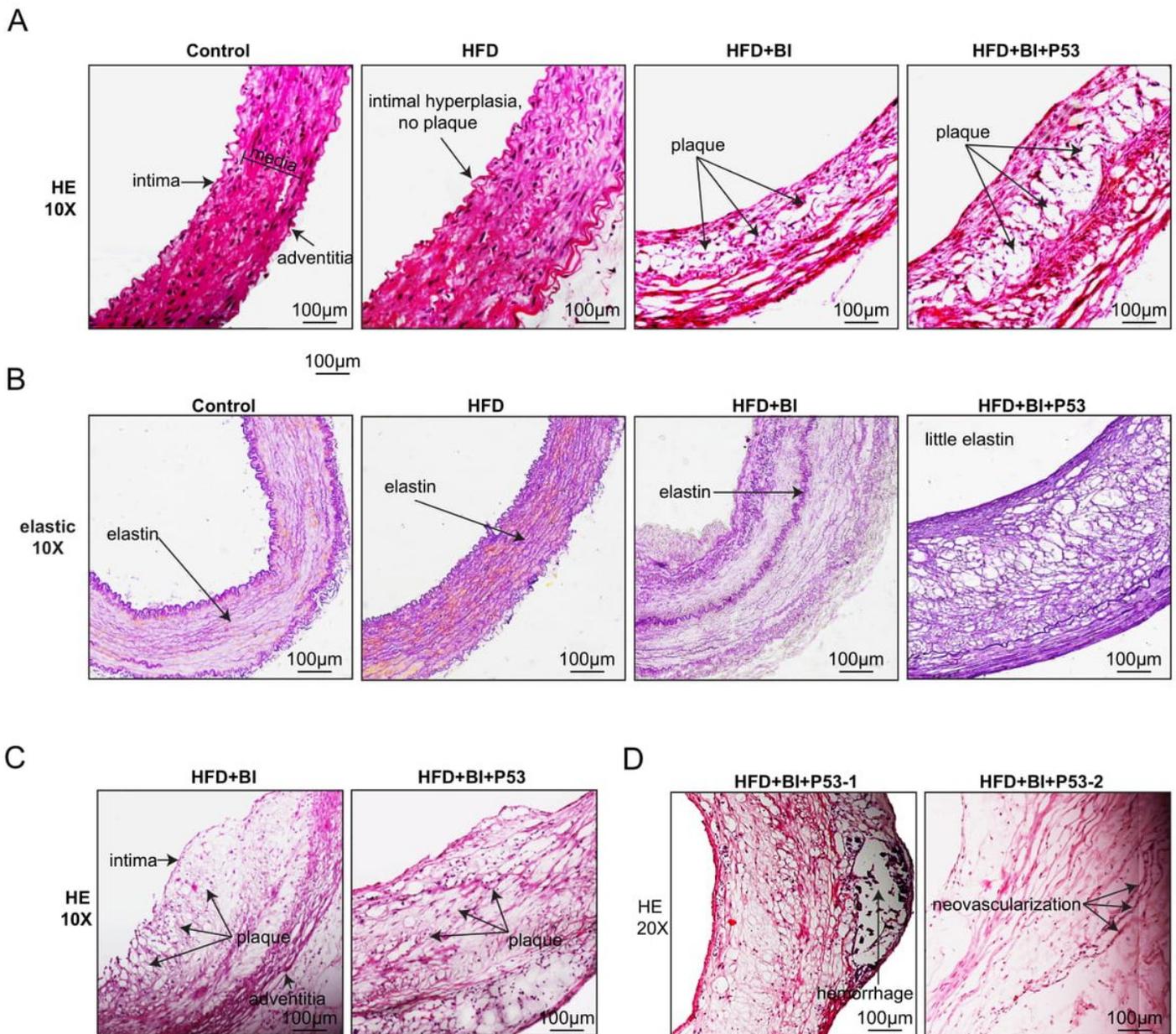


Figure 3

Figure 3

Elevation of a vulnerable plaque model. (A) The right iliac artery and abdominal aorta were stained with HE; (B) The right iliac artery and abdominal aorta were stained with Elastin; (C) After transfection, the number of SMCs decreased, the fibrous cap thinned and a large number of foam cells formed, compared

with HFD + BI (as shown with the arrow); (D) Hemorrhage (shown with long arrows) and neovascularization (shown with short arrows) can be seen in plaques.

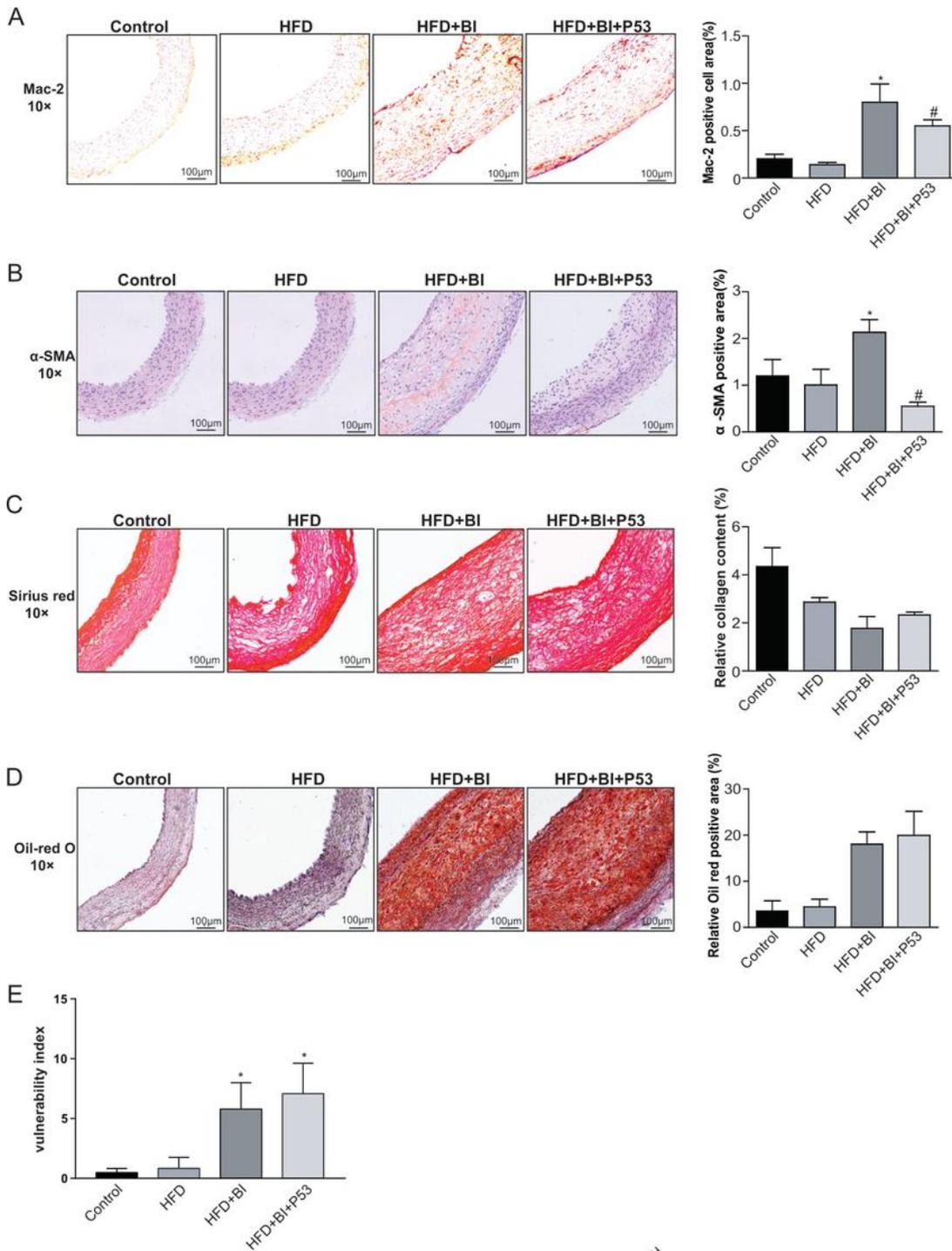


Figure 4

Figure 4

Plaque vulnerability index determination (A) The macrophages were detected by Mac-2 staining; (B) SMCs actin staining was detected by α-SMA; (C) The Collagen fibers were detected by Sirius red staining; (D) Fats were detected by oil red O staining; (E) Determination of plaque vulnerability index in each group.

The data are expressed as mean \pm SD, Statistical comparisons were made using one-way ANOVA followed by the Tukey's multiple comparison test; * $p < 0.05$ vs Control or HFD, # $p < 0.05$ vs control or HFD

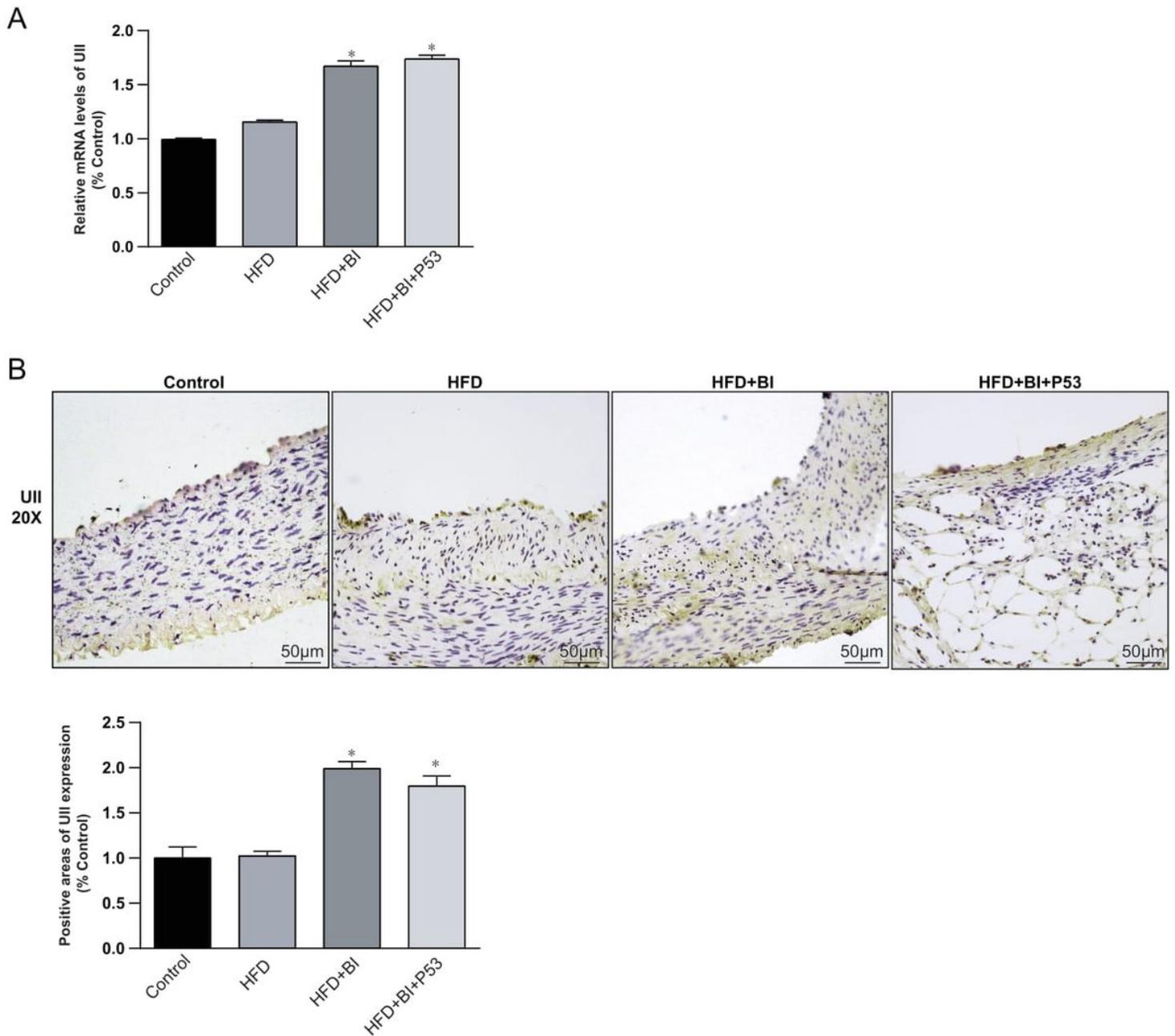


Figure 5

Figure 5

Ull1 expression in rabbits with a vulnerable plaque model. (A)Ull1 mRNA was detected by qPCR; (B) Ull1 expression and distribution were detected by Immunohistochemical staining of Ull1 in iliac artery and abdominal aorta. The data are expressed as mean \pm SD, Comparisons between the groups were performed using a one-way ANOVA, the Tukey's test; * $p < 0.05$ vs Control or HFD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [additionalfile1ARRIVEChecklist.docx](#)