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Altered Expression of *TXNIP* in the peripheral leukocytes of patients with coronary atherosclerotic heart disease

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Abstract

Background: Coronary atherosclerotic heart disease (CAD) is mainly caused by atherosclerosis, an inflammatory disease characterized by plaque formation in arteries. Reactive oxygen species caused structural damage and dysfunction of arterial endothelial cells. Thioredoxin-interacting protein (*TXNIP*) is the endogenous inhibitor and regulator of thioredoxin, a major cellular antioxidant and antiapoptotic system. In order to explore the role of *TXNIP* in the occurrence and development of CAD, we detected the *TXNIP* expression and discussed its molecular mechanisms in CAD.

Methods: The mRNA levels of *TXNIP* gene in peripheral leucocytes were detected in CAD and healthy controls (CTR) by quantitative real-time polymerase chain reaction. And *TXNIP* proteins were detected by western blotting.

Results: *TXNIP* gene expression levels in patients with unstable angina pectoris (UAP, n = 96) were significantly increased compared with those of CTR (n = 192, $P < .05$). However, the situation is different in acute myocardial infarction (n = 96, $P > .05$). Logistic regression analysis showed that *TXNIP* levels were significantly positive correlated with UAP (OR = 1.728, $P < .05$).

Conclusions: *TXNIP* gene expression in the peripheral leucocytes was increased in patients with UAP, indicating that *TXNIP* in circulating leucocytes may be involved in the pathogenesis of UAP.

Abbreviations: AMI = acute myocardial infarction, AUC = area under the curve, CAD = coronary atherosclerotic heart disease, CTR = healthy controls, HDL-C = high-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein-cholesterol, ROC = receiver operating characteristic, ROS = reactive oxygen species, RT-qPCR = real-time quantitative reverse-transcription polymerase chain reaction, TRX = thioredoxin, TXNIP = thioredoxin-interacting protein, UAP = unstable angina pectoris.

Keywords: coronary atherosclerotic heart disease, gene expression, leucocytes, *TXNIP*

1. Introduction

Coronary atherosclerotic heart disease (CAD) is caused by both genetic and environmental factors. Its incidence and mortality have been increasing in recent years. The identified risk factors include hyperlipidemia, hypertension, cigarettes smoking, and diabetes mellitus.[1–3] However, its clinical implications cannot be exclusively explained by these risk factors, indicating the importance of genetic factors in CAD. From the pathological point of view, CAD is mainly caused by atherosclerosis, an inflammatory disease characterized by plaque formation. Previous studies indicated that macrophages and lymphocytes played an important role in plaque formation and progression. Recent studies suggested neutrophils also played an important role in the development of atherosclerosis.[4–7]

Reactive oxygen species (ROS) are produced in the process of inflammation, which makes oxidative modification of low-density lipoprotein and causes structural damage and dysfunction of endothelial cells. ROS can also directly damage the vascular endothelium, resulting in vascular remodeling. Thioredoxin-interacting protein (*TXNIP*), also known as vitamin D3 upregulated protein 1, is an endogenous inhibitor and regulator of thioredoxin (*TRX*), the major cellular antioxidant and antiapoptotic system.[8,9] *TXNIP* could bind *TRX* to regulate its expression and antioxidant activity negatively, playing an important role in the regulation of redox reactions in vivo.[10–12] And *TXNIP* gene expression could be induced by many stress factors, such as peroxide, heat shock, and starvation. In addition, nitric oxide, insulin, and transcription factor *FOXO1* could inhibit the expression of the *TXNIP*.[13,14]

In this study, we detected the *TXNIP* expression in peripheral leucocytes of patients with CAD and healthy controls (CTR) to explore the role of *TXNIP* in the occurrence and development of CAD. The mRNA levels were examined by real-time quantitative reverse-transcription polymerase chain reaction (RT-qPCR). Protein levels were examined by western blotting. The molecular mechanisms of *TXNIP* in CAD were discussed.

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All the authors declare no conflict of interest.

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2. Materials and methods

2.1. Patients and controls

From February 2015 to April 2016, a total of 192 patients with CAD, consisting of 96 with unstable angina pectoris (UAP) and 96 with acute myocardial infarction (AMI), were recruited from Department of Cardiology, Jining No. 1 People's Hospital, Jining, Shandong Province, P.R.China. All patients were diagnosed with angiography. A total of 192 age- and sex-matched CTR, with no history of CAD and cerebrovascular diseases, were recruited from Health and Physical Examination Center at the same time. In addition, when selecting CTR, individuals with family history of CAD and other heart diseases, as well as cerebrovascular diseases, were excluded from the study. All of the subjects were coded, and the authors could not identify a single subject. This study was approved by the Human Ethic Committee of Jining No. 1 People's Hospital, and informed consents were obtained. Total cholesterol, high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and triglyceride were determined with an ADVIA 2400 automated analyzer (Siemens Healthcare Diagnostics, Erlangen, Germany) in the Laboratory of Experimental Medicine.

2.2. Samples collection

After an overnight fast, peripheral venous blood (5 mL) of the CAD and CTR was collected into Elhylene Diamine Tetraacetic Two Potassium Salt (EDTA-K2) anticoagulant tube. The leucocytes were isolated by density gradient centrifugation with Human Leukocyte Isolation System (LTS-1078, Haoyang Biological, Tianjin, China), according to the manufacturer's protocol.

2.3. Real-time quantitative reverse-transcription polymerase chain reaction

Total RNA of the leucocytes was isolated using TRIzol reagent (15596026, Thermo Fisher Scientific) following the manufacturer's protocol. Quality control of the RNA was completed by Nanodrop 2000 (Thermo scientific). cDNA was reverse-transcribed from 1.5 μg of DNase-treated total RNA with RevertAid H Minus First Strand cDNA Synthesis Kit (K1632, Thermo Fisher Scientific) following the manufacturer's protocol. RT-qPCR was performed to detect the mRNA of *TXNIP* using SYBR Premix Ex Taq (RR420A, Takara, China) by Applied Biosystems 7500 Real-Time PCR System, following the operation instructions of reagent kits and instruments. The PCR system consisted of amplification primers *TXNIP*-F: 5'-GCC ACA CTT ACC TTG CCA AT-3'; *TXNIP*-R: 5'-TTG GAT CCA GGA ACG CTA AC-3'; *β-actin*-F: 5' GGA CTT CGA GCA AGA GAT GG-3'; and *β-actin*-R: 5'-AGC ACT GTG TTG GCG TAC AG-3'. The RT-qPCR reaction was set up in a reaction volume of 20 μL containing 10 μL SYBR Premix, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 2 μL cDNA, and 7 μL nuclease-free water. All samples were performed in triplicate. The amplification was run with an initial denaturation for 30 s at 95°C, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 34 s. The relative expression was calculated with the following equation: relative expression $= 2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = \Delta Ct_{(TXNIP)} - \Delta Ct_{(\beta\text{-}actin)}$.[15,16] *β-Actin* is the reference gene for normalization.

2.4. Western blotting

Proteins of leucocytes were prepared with lysis buffer, containing Triton X-100, sodium deoxycholate, sodium dodecyl sulfate (SDS), EDTA, and protease inhibitor cocktail (Roche, Mannheim, Germany).[17] Proteins levels were determined with western blotting. First, the cellular proteins (30 μg) were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After separation, the proteins were transferred onto a nitrocellulose membrane. After the blocking with 5% nonfat milk powder, the membrane was first incubated with the rabbit anti-*TXNIP* polyclonal antibodies[18,19] (1:1000, Proteintech, 18243-1-AP, or mouse antihuman *β-actin* antibodies (1:5000, Proteintech, 66009-1-Ig, USA) and then with the secondary antibodies (goat antirabbit IgG-HRP or goat antimouse IgG-HRP). Then the immunoblots were detected using western blotting luminol reagent (sc-2048, Santa Cruz Biotechnologies) according to the instructions of the manufacturer. Immunoblot signals were measured and analyzed by using the Gel Imaging System (Tanon 3500, China) and normalized to the *β-actin* signals. All the samples were repeated 3 times.

2.5. Statistical analysis

Data were expressed as means ± standard deviation. Comparisons of means between groups were made by Student *t* test and comparisons of prevalence by chi-square test. Logistic regression analyses were performed to detect the correlation between *TXNIP* and CAD. Receiver operating characteristic (ROC) curves were used to compare the sensitivity and specificity. All statistical analyses were performed with SPSS v13.0. $P < .05$ was considered statistically significant.

3. Results

3.1. Clinical characteristics

The clinical characteristics are summarized in Table 1. CTR are age- and sex-matched with patients. Compared with CTR, patients with AMI had a higher smoking rate ($P < .05$). The HDL-C levels in patients with UAP and AMI were lower than CTR respectively ($P < .05$). No differences for triglycerides, total cholesterol, and LDL-C were detected between patients and CTR.

3.2. Real-time quantitative reverse-transcription polymerase chain reaction

TXNIP mRNAs were examined with RT-qPCR (Fig. 1). *TXNIP* expression levels in patients with UAP were significantly increased compared with CTR ($P < .05$). However, the situation

Table 1

Clinical characteristics.

	CTR	UAP	AMI
Male (%)	73.96	63.54	64.58
Hypertension (%)	31.25	40.63	37.50
Smoking (%)	12.50	18.75	72.40*
Diabetes (%)	26.04	37.50	36.46
Age	62.75 ± 5.85	58.88 ± 11.11	60.50 ± 8.16
HDL-C (mM)†	1.44 ± 0.39	1.15 ± 0.33	0.99 ± 0.26
LDL-C (mM)	3.00 ± 0.97	2.81 ± 0.42	2.76 ± 0.51
Triglycerides (mM)	1.26 ± 0.72	1.56 ± 0.62	1.22 ± 0.47
Total cholesterol (mM)	5.14 ± 1.01	4.36 ± 0.46	4.27 ± 0.69

AMI = acute myocardial infarction, CTR = healthy controls, HDL-C = high-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein-cholesterol, UAP = unstable angina pectoris.

* $P < .05$, AMI vs CTR.

† $P < .05$, UAP vs CTR, AMI vs CTR.

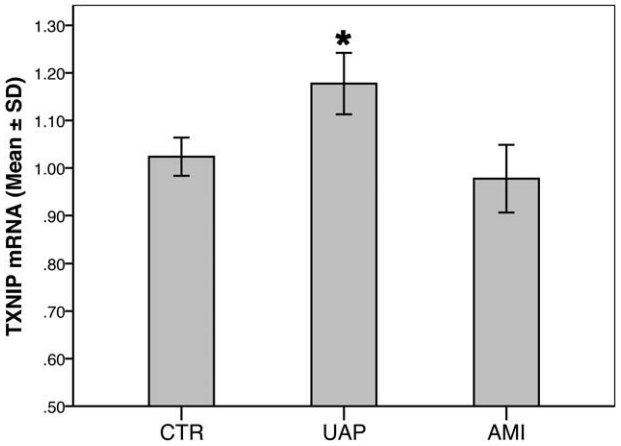

Figure 1. The mRNA expression levels of *TXNIP* gene of peripheral leucocytes in patients and healthy controls. The mRNA levels of *TXNIP* gene were normalized to *β-actin*, which was used as an internal control. Data were expressed as means ± SD. $^{*}P < .05$. AMI = acute myocardial infarction, CTR = healthy controls, SD = standard deviation, UAP = unstable angina pectoris.

is different in AMI. *TXNIP* gene expression levels in patients with AMI were decreased, but there was no statistical significance ($P > .05$).

Further multivariate logistic repression analysis between UAP and *TXNIP* mRNA levels, smoking, hypertension, and diabetes was carried out. And the results showed that *TXNIP* mRNA levels were positively associated with UAP (OR = 1.728, $P < .05$). It is important to point out that *TXNIP* expression levels were redefined in the multivariate logistic repression analysis.[20] *TXNIP* mRNA relative expression <1.00 was defined as the low expression, and ">1.00" were defined as high expression.

3.3. Western blotting

To detect *TXNIP* protein levels of the leucocytes, western blotting was performed. The results are shown in Figure 2. As expected, the protein levels were consistent with the results of RT-qPCR. The protein levels of *TXNIP* in UAP were significantly increased compared with CTR ($P < .05$).

3.4. Receiver operating characteristic curves

In addition, we compared the sensitivity and specificity of the RT-qPCR and western blot using the ROC curves (CTR and UAP). The area under the curve (AUC) for the level of *TXNIP* mRNA was significantly higher than *TXNIP* protein (Fig. 3. $AUC_{mRNA} = 0.791$, $AUC_{Protein} = 0.656$, $P < .05$). This may be related to the sensitivity of the 2 experimental methods themselves.

4. Discussion

Atherosclerosis is a subacute inflammation characterized by infiltration of macrophages and T lymphocytes.[21] Multiple factors leads to cardiovascular clinical events, such as inflammatory response, oxidative stress, apoptosis, vascular remodeling, plaque stress, and blood flow shear stress.[22] *TXNIP*, an inhibitor of antioxidant *TRX*, is a regulator of metabolism of glucose and lipid in vivo.[23,24] *TXNIP* negatively regulates the expression of *JNK*, *P38*, and *VCAM1*; increases vascular inflammation; and accelerates the process of atherosclerosis.[25] Overexpression of *TXNIP* increases the level of ROS in cells, reduces the interaction between *TRX* and proliferation-related genes, and leads to more

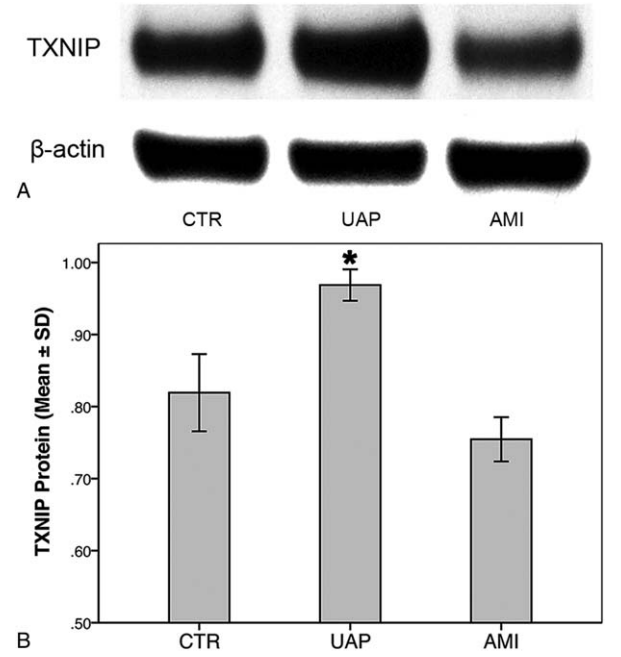

Figure 2. Thioredoxin-interacting protein (*TXNIP*) protein levels of peripheral leucocytes in patients with coronary artery disease and healthy controls. A, Western blotting of TXNIP and *β-actin* protein. B, Relative levels of *TXNIP* protein normalized to *β-actin*. *β-Actin* was used as an internal control. Data were expressed as means ± SD. $^{*}P < .05$. AMI = acute myocardial infarction, CTR = healthy controls, SD = standard deviation, UAP = unstable angina pectoris.

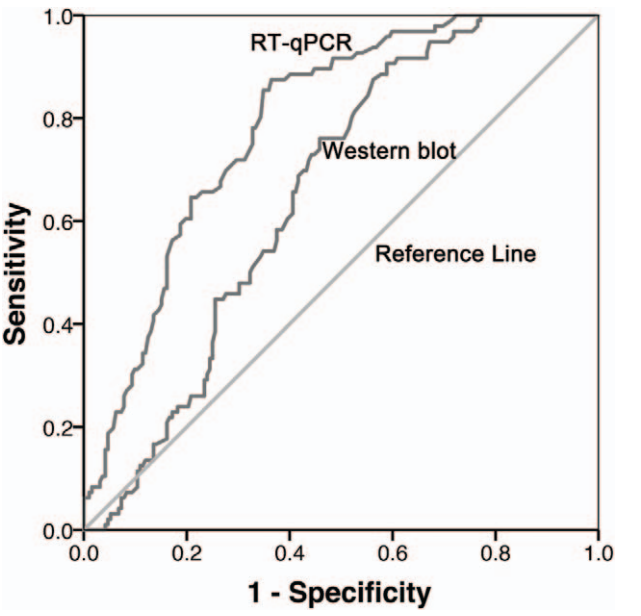

Figure 3. The sensitivity and specificity of the RT-qPCR and western blot were compared using the receiver operating characteristic (ROC) curves. Area under the curve (AUC) for the level of thioredoxin interacting protein (TXNIP) mRNA was significantly higher than protein levels (AUCmRNA = 0.791, AUCProtein = 0.656, $P < .05$). RT-qPCR = real-time quantitative reverse-transcription polymerase chain reaction.

sensitive to oxidative stress.[9,26] In this study, the results showed that *TXNIP* expression levels in UAP were significantly different from that in CTR, indicating that *TXNIP* may play an important role in CAD.

Accumulating evidences suggest that neutrophils and macrophages may be involved in the initiation and progression of atherosclerosis by mediating inflammation process.[27–29] Neutrophils are detected in atherosclerotic lesions in patients with CAD.[30] In this study, we found that the *TXNIP* gene expression levels were significantly increased in the peripheral leucocytes of patients with UAP, indicating that they may be implicated in the pathogenesis of UAP. However, the results in AMI were inconsistent with UAP, and we speculated that there might be 3 reasons: first, high smoking rate in patients with AMI, which is a high risk factor for AMI.[31,32] In this study, the percentage of smokers in AMI is much higher than that of UAP and CTR. Second, CAD is a slow developing disease under the combined action of many factors, involving a variety of pathological processes, and the expression level of a single gene may be changed by other pathological factors. Third, most of our subjects had a history of primary hospital visits and began to use certain drugs. Studies have shown that these drugs for the treatment of CAD have an impact on the expression of TRX. Haendeler et al[33] found that statins could enhance the redox activity of *TRX* and inhibit the expression of *TXNIP* gene.

In addition, there were some defects or limitations in the present study. Firstly, the number of patients analyzed in this study was relatively small, which could lead to some statistical errors. Because of the small sample size, we could not analyze whether the CAD risk factors such as smoking influencing the expression of *TXNIP* gene in subgroups of patients (AMI and UAP). Secondly, on the choice of the control group, the control population was not validated by angiography, which resulted in asymptomatic coronary heart disease likely to be included in CTR.

In conclusion, we found that *TXNIP* gene expression levels are significantly increased in leucocytes of patients with UAP, but not in AMI. It should be noted that there may be some errors, as a result of high smoking rate and the drug treatment before hospitalization. Further analysis of *TXNIP* will be continued to verify its role in CAD. These data would shed light on our understandings of CAD pathogenesis and development of potential novel therapies.

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References

[1] Cubukcu A, Murray I, Anderson S. What's the risk? Assessment of patients with stable chest pain. Echo Res Pract 2015;2:41–8.
[2] Task Force M, Montalescot G, Sechtem U, et al. 2013 ESC guidelines on the management of stable coronary artery disease: the Task Force on the management of stable coronary artery disease of the European Society of Cardiology. Eur Heart J 2013;34:2949–3003.
[3] Li Z, Cheng J, Wang L, et al. Analysis of high risk factors and characteristics of coronary artery in premenopausal women with coronary artery disease. Int J Clin Exp Med 2015;8:16488–95.
[4] Drechsler M, Megens RT, Van Zandvoort M, et al. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. Circulation 2010; 122:1837–45.
[5] Goldmann BU, Rudolph V, Rudolph TK, et al. Neutrophil activation precedes myocardial injury in patients with acute myocardial infarction. Free Radic Biol Med 2009;47:79–83.
[6] Frodermann V, Nahrendorf M. Neutrophil-macrophage cross-talk in acute myocardial infarction. Eur Heart J 2017;38:198–200.
[7] Ma Y, Yabluchanskiy A, Iyer RP, et al. Temporal neutrophil polarization following myocardial infarction. Cardiovasc Res 2016;110:51–61.
[8] Mohamed IN, Hafez SS, Fairaq A, et al. Thioredoxin-interacting protein is required for endothelial NLRP3 inflammasome activation and cell death in a rat model of high-fat diet. Diabetologia 2014;57: 413–23.
[9] Devi TS, Hosoya K, Terasaki T, et al. Critical role of TXNIP in oxidative stress, DNA damage and retinal pericyte apoptosis under high glucose: implications for diabetic retinopathy. Exp Cell Res 2013; 319:1001–12.
[10] Singh LP, Perrone L. Thioredoxin interacting protein (TXNIP) and pathogenesis of diabetic retinopathy. J Clin Exp Ophthalmol 2013; 4:287.
[11] Spindel ON, Burke RM, Yan C, et al. Thioredoxin-interacting protein is a biomechanical regulator of Src activity: key role in endothelial cell stress fiber formation. Circ Res 2014;114:1125–32.
[12] Zhou R, Tardivel A, Thorens B, et al. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat Immunol 2010; 11:136–40.
[13] Shaked M, Ketzinel-Gilad M, Ariav Y, et al. Insulin counteracts glucotoxic effects by suppressing thioredoxin-interacting protein production in INS-1E beta cells and in Psammomys obesus pancreatic islets. Diabetologia 2009;52:636–44.
[14] De Candia P, Blekhman R, Chabot AE, et al. A combination of genomic approaches reveals the role of FOXO1a in regulating an oxidative stress response pathway. PLoS One 2008;3:e1670.
[15] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 2008;3:1101–8.
[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) Method. Methods 2001;25:402–8.
[17] Huang J, Xu J, Pang S, et al. Age-related decrease of the LAMP-2 gene expression in human leukocytes. Clin Biochem 2012;45:1229–32.
[18] Zeng H, Gu H, Chen C, et al. ChREBP promotes the differentiation of leukemia-initiating cells to inhibit leukemogenesis through the TXNIP/RUNX1 pathways. Oncotarget 2016;7:38347–58.
[19] Ji S, Qin Y, Liang C, et al. FBW7 (F-box and WD repeat domain-containing 7) negatively regulates glucose metabolism by targeting the c-Myc/TXNIP (thioredoxin-binding protein) axis in pancreatic cancer. Clin Cancer Res 2016;22:3950–60.
[20] Li L, Wang W, Zhang R, et al. High expression of LAMP2 predicts poor prognosis in patients with esophageal squamous cell carcinoma. Cancer Biomark 2017;19:305–11.
[21] Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nat Rev Cardiol 2009;6:399–409.
[22] Waxman S, Ishibashi F, Muller JE. Detection and treatment of vulnerable plaques and vulnerable patients: novel approaches to prevention of coronary events. Circulation 2006;114:2390–411.
[23] Yoshioka J, Chutkow WA, Lee S, et al. Deletion of thioredoxin-interacting protein in mice impairs mitochondrial function but protects the myocardium from ischemia-reperfusion injury. J Clin Invest 2012; 122:267–79.
[24] Yoshioka J, Lee RT. Thioredoxin-interacting protein and myocardial mitochondrial function in ischemia-reperfusion injury. Trends Cardiovasc Med 2014;24:75–80.
[25] Yamawaki H, Pan S, Lee RT, et al. Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. J Clin Invest 2005;115:733–8.
[26] Junn E, Han SH, Im JY, et al. Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. J Immunol 2000;164:6287–95.
[27] Baetta R, Corsini A. Role of polymorphonuclear neutrophils in atherosclerosis: current state and future perspectives. Atherosclerosis 2010;210:1–3.
[28] Schrijvers DM, De Meyer GR, Herman AG, et al. Phagocytosis in atherosclerosis: molecular mechanisms and implications for plaque progression and stability. Cardiovasc Res 2007;73:470–80.
[29] Paulsson J, Dadfar E, Held C, et al. Activation of peripheral and in vivo transmigrated neutrophils in patients with stable coronary artery disease. Atherosclerosis 2007;192:328–34.

[30] Naruko T, Ueda M, Haze K, et al. Neutrophil infiltration of culprit lesions in acute coronary syndromes. Circulation 2002;106: 2894–900.
[31] Traina MI, Almahmeed W, Edris A, et al. Coronary heart disease in the Middle East and North Africa: current status and future goals. Curr Atheroscler Rep 2017;19:24.
[32] Drummond CA, Brewster PS, He W, et al. Cigarette smoking and cardio-renal events in patients with atherosclerotic renal artery stenosis. PLoS One 2017;12:e0173562.
[33] Haendeler J, Hoffmann J, Zeiher AM, et al. Antioxidant effects of statins via S-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. Circulation 2004;110:856–61.