*Original Research* *Feature article*

# Apolipoprotein A-IV of diabetic-foot patients upregulates tumor necrosis factor α expression in microfluidic arterial models

**Jun Ji[1], Xiaoyu Zhao[2], Jiajun Huang[2], Xuanqin Wu[1], Fang Xie[3], Liang Li[1], Tao Wang[1] and Shengli Mi[2]**

[1]Department of Cardiovascular Surgery, University of Chinese Academy of Science Shenzhen Hospital, Shenzhen 518027, China; [2]Bio-manufacturing Engineering Laboratory, Tsinghua Shenzhen International Graduate School, Tsinghua University, Shenzhen 518055 China; [3]Department of Endocrinology, University of Chinese Academy of Science Shenzhen Hospital, Shenzhen 518027, China
Corresponding authors: Tao Wang. Email: 794479116@qq.com; Shengli Mi. Email: mi.shengli@sz.tsinghua.edu.cn

### Impact statement

Diabetic peripheral arterial atherosclerosis is one of the important characteristics of diabetic foot syndrome, but its pathogenesis is different from that of non-diabetic atherosclerosis. Diabetes mellitus glycosylates apolipoprotein (Apo A-IV), which impels its anti-atherosclerosis function in diabetes. The establishment of vascular chip with microfluidic technology can precisely simulate the real hemodynamics, solving many shortcomings of traditional two-dimensional cell culture model and animal experiments. In this study, we successfully construct a microfluidic real arterial chip model to simulate arterial pulse and found that the serum endogenous Apo A-IV from Type 2 diabetes mellitus with diabetic foot (T2DM-F) patients could induce tumor necrosis factor alpha (TNF-α) expression in chip arteries to turn the blood vessels into a pre-atherosclerotic state. This may provide experimental basis to further understand the pathogenesis of diabetic peripheral vascular disease.

### Abstract

Diabetic peripheral arterial atherosclerosis is one of the important characteristics of diabetic foot syndrome. Apolipoprotein (Apo A-IV) participates in various physiological processes, and animal studies have shown that it has roles of anti-atherosclerosis, prevention of platelet aggregation and thrombosis. Apo A-IV glycosylation is closely related to the occurrence and development of diabetic peripheral atherosclerosis. This study aimed to explore the mechanism of diabetic peripheral arterial lesions caused by glycosylated Apo A-IV. Type 2 diabetes mellitus (T2DM) and T2DM with diabetic foot patients (T2DM-F; $n = 45$, 30) were enrolled in this study, and individuals without diabetes ($n = 35$) served as normal controls (NC). In T2DM group, serum Apo A-IV content was higher than those in NC and T2DM-F group, as carboxymethyl lysine (CML) glycosylation of Apo A-IV in mixed serum from T2DM-F group was identified to be more significant than those in two other groups. Within a microfluidic arterial chip model, Apo A-IV from T2DM and T2DM-F group significantly increased transcription and protein levels of tumor necrosis factor alpha (TNF-α) in chip arteries, and CML expression was observed in T2DM-F group, which were associated with increased nuclear receptor subfamily 4 group A member 3 (NR4A3) expression. Recombinant human Apo A-IV could reverse the stimulating effect of serum Apo A-IV from T2DM-F group on TNF-α expression, and NR4A3 blocking peptide downregulated TNF-α expression by inhibiting NR4A3 expression. In the chip arteries, Apo A-IV from T2DM and T2DM-F increased TNF-α expression and turn them into a pre-atherosclerotic state, which might be one of the important mechanisms of glycosylated Apo A-IV to induce diabetic peripheral arterial lesions and eventually lead to diabetic foot.

**Keywords:** Diabetic foot, apolipoprotein A-IV, arterial chip, TNF-α

***Experimental Biology and Medicine*** **2023; 248: 691–701. DOI: 10.1177/15353702221147562**

## Introduction

Diabetic foot is one of the serious chronic complications that lead to disability and death in diabetic patients and defined by the World Health Organization as lower limb infection, ulceration and/or destruction of deep tissue in diabetic patients due to the combination of nerve and various degrees of peripheral arterial lesions. It has been reported that more than 25% of people with diabetes worldwide will develop foot ulcers or gangrene at some point in their lives.[1,2] There are as many as 60 million diabetics in China, and about a quarter of them suffer from diabetic foot. More advanced patients are often associated with widespread infection, higher rates of amputation, and mortality. Traditional treatment methods for diabetic foot include drugs, vascular bypass, and interventional surgery, with poor clinical outcome. At least 30~40% of patients with diabetic foot have to undergo amputation because of poor lower extremity microcirculation and complete occlusion of small blood vessels, which cannot lead to surgical vascular reconstruction.[3,4]

ISSN 1535-3702

Diabetic peripheral arterial atherosclerosis is one of the important characteristics of diabetic foot syndrome.[5]

Apolipoprotein A-IV (Apo A-IV) is a lipid-binding protein that is synthesized primarily in the small intestine and packaged as chylomicron during fat absorption and enters the bloodstream through the thoracic duct. In circulation, Apo A-IV exists in three forms: chylomicron, high-density lipoprotein, and free lipid-free form (about 80%). Apo A-IV participates in various physiological processes of the body, such as promoting lipid absorption and metabolism, maintaining the dynamic balance of glucose, and stimulating appetite. Animal studies have shown that it has roles of anti-atherosclerosis, prevention of platelet aggregation and thrombosis.[6] Apo A-IV glycosylation is closely related to the occurrence and development of diabetic peripheral atherosclerosis. ApoE-deficient mice were prone to atherosclerosis when glycosylated Apo A-IV increased.[7] The glycosylation level of diabetic Apo A-IV correlated with the severity of coronary artery disease, and its atherosclerotic effect was significantly reduced by mutation of major glycosylation sites.[8] Recombinant glycosylated Apo A-IV stimulated inflammatory responses both *in vitro* and *in vivo*.[9] These findings suggested that glycosylation of Apo A-IV protein impels its anti-atherosclerosis function in diabetes.

The organ-on-a-chip is a bionic system that can simulate the main functions of human organs on microfluidic chips by using micromachining technology. Organ chip technology with microfluidic technology, miniaturization, and integration can precisely control multiple system parameters such as chemical concentration gradient and fluid shear stress to simulate the complex structure of human organs, microenvironment, and physiology function, solving many shortcomings of traditional two-dimensional cell culture model and animal experiments. It may become a bionic, efficient, and energy-saving tool for physiological research, disease model, and drug development.[10–12] The establishment of vascular chip is of great significance to study vascular diseases such as atherosclerosis, hemostasis and thrombosis, inflammatory vascular diseases, and tumor transvacuolar metastasis.[13]

In this study, a polydimethylsiloxane (PDMS) microfluidic chip was constructed based on the photolithography principle, and the microfluidic real artery chip platform was constructed by implanting human internal mammary artery segments into a dynamic continuous culture perfusion system. Effect of serum Apo A-IV from diabetes mellitus type 2 (T2DM) patients with diabetic foot (T2DM-F) on chip arteries was observed to explore the mechanism of arterial lesions caused by glycosylated Apo A-IV, and provide evidence for prevention and control of diabetic foot.

## Materials and methods

### Study population and serum sample collection

Forty-five T2DM and 30 T2DM-F patients were enrolled in this study, who were inpatients from January 2020 to December 2021 in the department of endocrinology, University of Chinese Academy of Science Shenzhen Hospital, Shenzhen, China. Inclusion criteria for T2DM was made according to the diagnostic criteria of American Diabetes Association.[14] Inclusion criteria for T2DM-F was T2DM with clinical manifestations of ischemic lower extremity and local chronic ulcers or gangrene, and computed tomography (CT) angiography imaging of arterial stenosis in the lower extremities (stenosis rate $\geqslant 60\%$); They were accompanied with or without carotid, coronary, and aortic atherosclerotic plaques, and the imaging showed a stenosis rate of $< 60\%$, without clinically relevant symptoms and signs. Exclusion criteria for all participants were: renal insufficiency or renal failure, dialysis, pregnancy, and known or treated malignancies. All subjects gave written informed consent to participate in this study. The study protocol was approved by the ethics committee of University of Chinese Academy of Science Shenzhen Hospital, which conformed to the principles of the Declaration of Helsinki. Thirty-five healthy subjects without evidence of T2DM or T2DM-F were recruited as normal controls (NC) from the annual physical check-up people in the same hospital, who had no history of any cardiovascular or cerebrovascular diseases with normal results of carotid artery ultrasound examination, coronary CT angiography, and blood glucose testing. Three milliliter peripheral venous blood from three groups was collected in sterile tubes without anticoagulants, and kept at room temperature for 30 min, followed by centrifugation at 500 g for 30 min. Then, it was gathered in aseptic frozen tube (Thermo Fisher, USA) and frozen at −80°C until further use. Blood samples were subjected to centrifugation within 1 h of acquisition.

### Detection of serum Apo A-IV by ELISA

Enzyme-linked immunosorbent assay (ELISA, Abcam, USA) was used according to the manufacturer's instruction to detect the serum Apo A-IV. Based on standard curve and concentration range of samples determined by pre-experiment, sample concentration was calculated with dilution of 500 times and triple parallel repeat.

### Isolation, purification and glycosylation identification of Apo A-IV

Apo A-IV were isolated and purified from fasting lipoprotein depleted serum, as previously described.[15] Brief method was summarized in the Supplementary material. Purity and glycosylation identification of the isolated Apo A-IV preparations were determined by immunoblotting with anti-Apo A-IV and carboxymethyl lysine (CML) antibodies (Abcam, R&D, USA), using SuperSignal West Dura Extended Duration Substrate kit (Thermo Fischer, USA).

### Preparation of PDMS microfluidic chip and establishment of dynamic continuous culture perfusion system

The mold casting method was adopted to prepare microfluidic chips. A teflon mold is designed and optimized iteratively according to the requirements of simulating vascular hydrodynamics expected by microfluidic artery chip. The drawing software used for mold design is AutoCAD Version 2020. For fabricating the PDMS microfluidic chip, a polytetrafluoroethylene mold was processed and assembled with steel needle. The prepared PDMS (SYLGARD™ 184 silicone elastomer kit, Dow-Corning, Midland) was poured onto a silica

wafer and put in an oven (2 h, 80°C) to form a 5 mm thick PDMS layer. The mold was put above the cured PDMS layer and PDMS was poured on the mold to construct a chamber for vascular cultivation. A second curing process (1 h, 80°C) was conducted, afterwards the mold was disassembled and the microfluidic chip was acquired, which was thoroughly cleaned with isopropyl alcohol and sterilized with autoclave before use. The dynamic continuous culture perfusion system including infusion tank, microfluidic micro-peristaltic pump, substrate and waste liquid pipe was connected with medical silicone hose (ϕ1 mm, Dongguan Yongle Rubber & Plastic Products Co., Ltd., China) to construct a microfluidic chip platform. The substrate included at least one holding space for accommodating blood vessel, and the accommodating space was connected with at least one pair of liquid inlet and outlet with connecting heads for connecting blood vessel and the hose. The 10–12 mm residual and abandoned normal human internal mammary arteries with smooth lumen surface and a diameter of about 1–1.5 mm obtained during coronary artery bypass of non-diabetic coronary heart disease patients were used for the microfluidic arteriosus chip construction. These arterial segments were repaired at both ends with removal of necrotic tissue and clot, and immediately implanted into cold Dulbecco's Modified Eagle's whole culture medium (DMEM) (Gibco, USA) containing antibiotics after washing with HANKs three times for use. They were inserted and anastomosed to the inlets and outlet in the substrates and ligated with surgical nylon to prevent leakage. All the above operations were performed under completely aseptic condition.

### Continuous arterial microfluidic perfusion culture and experimental group

The base infusion was DMEM medium (Gibco, USA) containing 10% fetal bovine serum (Corning, USA), 1% penicillin, and 1% streptomycin (DMEM whole culture medium). Microfluidic micro-peristaltic pump worked to simulate vascular hydrodynamics and squeezed the hoses to move fluid inside the blood vessels. By adjusting the pulley speed of the microfluidic peristaltic pump, the perfusion amount, perfusion time, and perfusion pressure could be changed in real time. In this experiment, the pump speed was adjusted to approximately 1.8–2.0 rpm to make the intravascular fluid flow rate 30 cm/s (Color Doppler Ultrasonic Diagnostic Instrument s2000. Siemens, German), the mean intravascular pressure 60–70 mmHg (Multi-Parameter Monitor MEC-1000, Mindray, China), and the chip arterial pulse 80 times per minute, which was basically consistent with the arterial pulse rate of normal people. The arterial chip was placed in an incubator with 5% $CO_2$ at 37°C for culture. According to the pre-experimental optimal condition and requirement, the culture time was set as 96 h.

The experiment was divided into two groups: control group (CG, $n = 9$), DMEM whole culture medium plus healthy human serum Apo A-IV and experimental group (EG, $n = 36$), which was further subdivided into the following groups: Experimental group 1 (EG1, $n = 9$): DMEM whole culture medium plus serum Apo A-IV of T2DM patients; Experimental group 2 (EG2, $n = 9$): DMEM whole culture medium plus serum Apo A-IV of T2DM-F patients. Experimental group 3 (EG3, $n = 9$): DMEM whole culture medium plus serum Apo A-IV of T2DM-F patients with recombinant human Apo A-IV (Abcam, USA). Experimental group 4 (EG4, $n = 9$): DMEM whole culture medium plus serum Apo A-IV of T2DM-F patients, before which, 10 μg/mL nuclear receptor subfamily 4 group A member 3 (NR4A3) blocking peptide (Fitzgerald, USA), was added and incubated for 2 h. The total amount of serum Apo A-IV added to the continuous culture perfusion system was 10 μg/mL, and the recombinant human Apo A-IV added to the system was 5 μg/mL according to the average Apo A-IV content of subjects in three groups detected by ELISA. The final perfusion amount of 10 mL in the dynamic continuous culture perfusion system was changed once after 48 h of culture.

### Pathological, immunohistochemical, and apoptosis staining

After the culture, the chip arteries were immediately removed and implanted in 4% paraformaldehyde for fixation, tissue dehydration, wax immersion, embedding, and pathological section. Some sections were taken for LightCycler real-time polymerase chain reaction (PCR; Roche, Switzerland). Hematoxylin and eosin (H&E), immunohistochemical stain, and apoptosis detection were performed on vascular cross-section. To count positive cells accurately, continuous sections along the long axis of blood vessels were taken and one slice was taken from every 20 sections for staining and detection. The section thickness was 4 μm. Main antibodies used in immunohistochemistry included monoclonal mouse anti-α-actin (1:200, Santa Cruz, USA) for smooth muscle cells, anti-intercellular adhesion molecule-1 (ICAM-1, 1:100, Santa Cruz, USA), antitumor necrosis factor-α (TNF-α, 1:100, Abcam, USA), anti-interleukin 8 (IL-8, 1:100, Santa Cruz, USA) anti-proliferating cell nuclear antigen (PCNA, 1:100, Santa Cruz, USA), anti-CML (1:200, Abcam, USA), and anti-NR4A3/NOR-1 (1:200, Abcam, USA) antibodies, which were stained by immunohistochemical streptavidin-peroxidase method (SP, Santa Cruz, USA). Apoptosis was detected by TdT-mediated dUTP Nick-End Labeling (TUNEL, Abcam, USA) with DAB chromogenic and the method was performed according to the manufacturer's instruction. Digital image analysis was performed using the HALO image analysis software v3.3.2541 (Indica Labs, USA). One thousand cells were counted under microscope magnification of 100 times and the number of antibody-reactive positive cells was calculated as the actual amount of expression.

### LightCycler real-time PCR

According to the instruction of RNeasy formalin-fixed, paraffin-embedded (FFPE) Kit (Qiagen, Germany), total RNA was purified from formalin-fixed, paraffin-embedded tissues, followed by complementary DNA (cDNA) synthesis with random primers using RevertAid H. Minus M-MLV reverse transcriptase (Thermo Fischer, USA). Negative control samples (no first-strand synthesis) were prepared by performing reverse transcription reaction in the absence of reverse transcriptase. Relative expression of IL-8, TNF-α, ICAM-1, and NR4A3 were quantitated by quantitative

**Table 1.** Baseline characteristics of study population.

| | NC ($n=35$) | T2DM ($n=45$) | T2DM-F ($n=30$) |
|---|---|---|---|
| Male patients (%) | 68.57 | 68.89 | 63.33 |
| Age (years) | 54.23 ± 6.96 | 51.18 ± 7.72 | 57.50 ± 7.93** |
| BMI (kg/m$^2$) | 22.45 ± 1.29 | 23.85 ± 2.88 | 23.32 ± 1.70 |
| Cigarette smoking (%) | 45.71 | 51.11 | 30.00 |
| Hypertension (%) | 5.71 | 33.33* | 68.57*/** |
| Fasting blood glucose (mmol/L) | 5.44 ± 0.40 | 13.7 ± 3.64* | 12.81 ± 2.39* |
| HbA1c (%) | 4.93 ± 0.48 | 10.5 ± 2.02* | 9.15 ± 1.89*/** |
| Blood uric acid (mmol/L) | 288.63 ± 77.14 | 368.29 ± 86.09* | 339.24 ± 73.92* |
| Serum creatinine (mmol/L) | 70.01 ± 8.01 | 71.17 ± 23.43 | 79.83 ± 19.87* |
| Triglyceride (mmol/L) | 0.98 ± 0.24 | 2.94 ± 2.04* | 2.35 ± 1.38* |
| Total cholesterol (mmol/L) | 3.83 ± 0.61 | 5.24 ± 1.22* | 4.83 ± 1.29* |
| HDL cholesterol (mmol/L) | 1.35 ± 0.24 | 1.02 ± 0.21* | 0.94 ± 0.15* |
| LDL cholesterol (mmol/L) | 2.44 ± 0.43 | 2.89 ± 0.71* | 2.59 ± 0.81 |
| hsCRP (mg/L) | 3.38 ± 1.38 | 7.33 ± 11.42* | 25.62 ± 31.37*/** |
| Serum Apo A-IV (μg/mL) | 4.18 ± 1.06 | 5.54 ± 1.34* | 4.79 ± 1.47** |
| Antidiabetic medication | | | |
| Metformin (%) | – | 42.22 | 33.33 |
| Glimepiride (%) | – | 17.78 | 3.33 |
| α-glucosidases (%) | – | 13.33 | 10.00 |
| Insulin therapy (%) | – | 20.00 | 26.67 |

NC: normal controls; T2DM: diabetes mellitus type 2; T2DM-F: T2DM with diabetic foot patients; BMI: body mass index; HbA1c: glycosylated hemoglobin A1c; HDL: high-density lipoprotein; LDL: low-density lipoprotein; hsCRP: high-sensitivity C-reactive protein.
Data are mean ± standard deviation (SD) or number (percentage).
*$p < 0.05$, versus NC.
**$p < 0.05$, versus T2DM.

real-time PCR using a LightCycler 480 Instrument (Roche, Switzerland). Real-time PCR primer information is available on request. The final results were calculated as ratios of the relative transcript levels of the target genes to the relative amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Compared with the control group, equal or more than twofold relative expression level of target genes in experimental groups and $p < 0.05$ were considered as differential expression. The detailed method was described in the Supplementary material.

### Statistical analysis

Statistical Package for the Social Sciences (SPSS), version 22.0 software (SPSS, USA) was used for data analysis. Results are expressed as mean ± SD. Differences in variables between groups were determined by unpaired *t*-tests or by Chi-square tests. Two-sided $p < 0.05$ were considered as statistical significance.

## Results

### Study population

All 110 subjects including T2DM patients, T2DM-F patients, and NC were enrolled in this study. As shown in Table 1, the three groups were gender-matched, and there was no significant difference of age between T2DM or T2DM-F group with the NC group, as the age of patients with T2DM-F was higher than that of the T2DM group. Clinical examination and laboratory test results showed that the prevalence of hypertension, fasting blood glucose, glycosylated hemoglobin A1c, blood uric acid, triglyceride, total cholesterol, low-density lipoprotein and high-sensitivity C-reactive protein (hsCRP) in T2DM and T2DM-F groups were higher than those in control group, but high-density lipoprotein was lower in them. It was noteworthy that hsCRP of T2DM-F group is significantly higher than that of T2DM group. The serum creatinine content in T2DM-F group was slightly higher than that in NC group but still within normal range. Meanwhile, ischemic wounds or ulcers might be located on the skin surfaces of the dorsal and lateral feet. There was ischemia and even gangrene at the end of the plantar foot. Noninvasive ultrasonography and even CT angiography revealed local luminal stenosis and flow slowing or occlusion of peripheral arteries. Local vascular stenosis lesions most commonly involved the dorsalis pedis and anterior tibial arteries. In severe cases, the vascular lesions were multiple and distributed along arterial branches (Figure 1).

### Serum Apo A-IV content by ELISA

Serum free Apo A-IV content was determined by ELISA and results of three groups were shown in Table 1, in which, serum Apo A-IV concentration in T2DM group was significantly higher than that in NC group and T2DM-F group.

### Identified results of isolated and glycosylated Apo A-IV

Immunoblotting method was used to identify the purification and glycosylation of isolated Apo A-IV protein from serum. Obvious and clear bands formed by Apo A-IV separated from mixed samples of the three groups were observed in electrophoresis. The CML glycosylation status of Apo

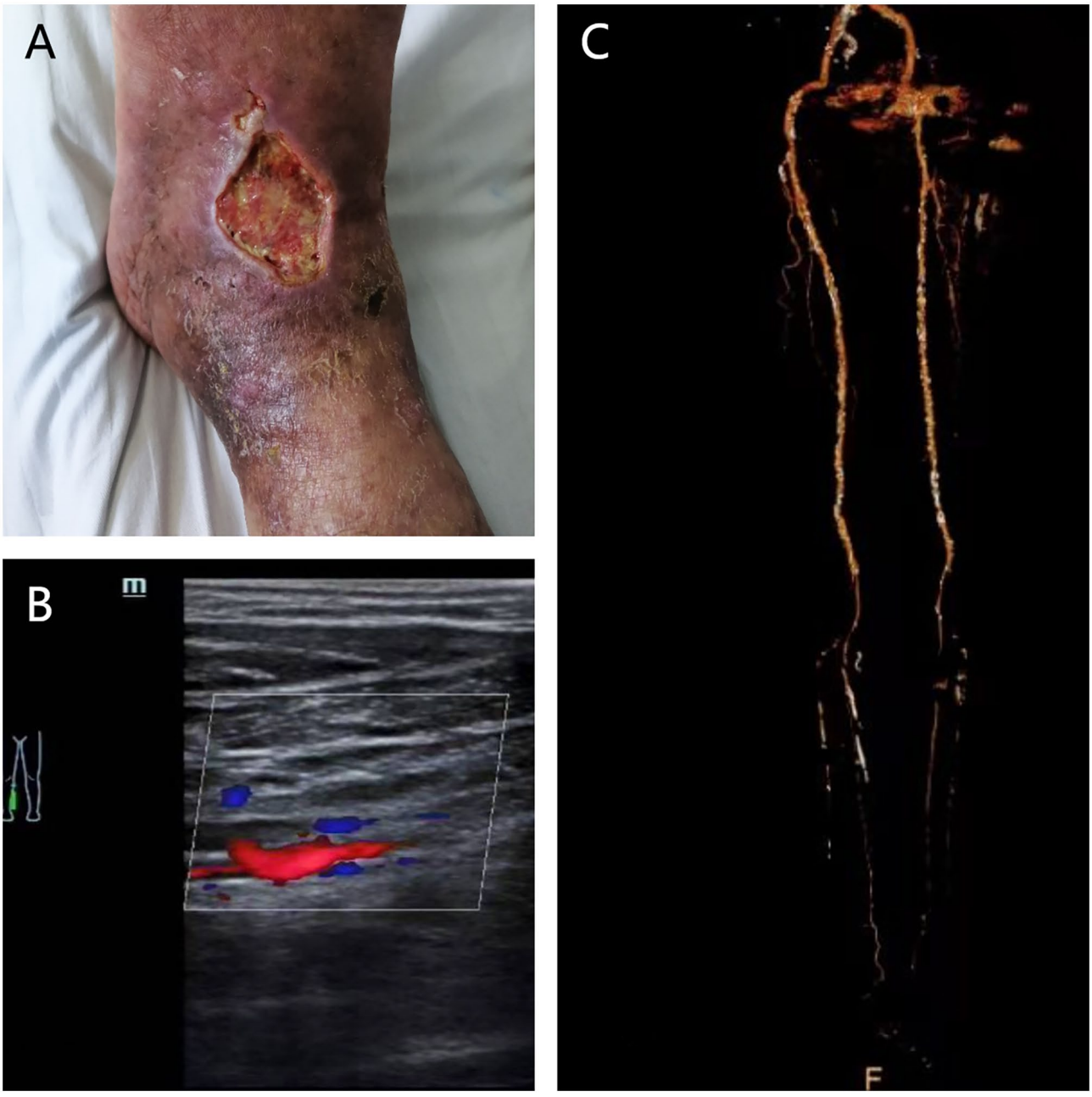


**Figure 1.** Clinical features of diabetic foot patients. (A) Local chronic ulcer with inflammatory exudate on the back of left foot of a 65-year-old male T2DM-F patient. (B) Color ultrasonic image displayed occlusion of the right anterior tibial artery of a 61-year-old male T2DM-F patient with skin ulceration on the inside of the ankle of the right lower extremity. (C) CT angiography showed multiple arterial occlusions in both lower limbs of a 69-year-old female T2DM-F patient with skin ulceration on the back of left foot.

A-IV isolated from the three groups was detected by anti-CML antibody, indicating that the CML glycosylation of Apo A-IV in diabetic foot group was more significant (Figure 2).

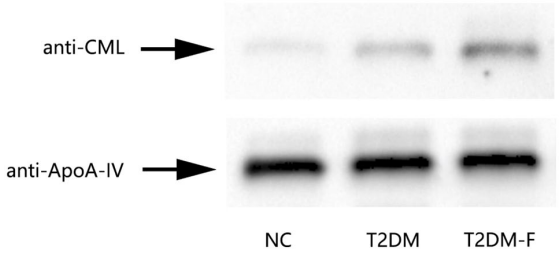


**Figure 2.** Total Apo A-IV protein and glycosylated Apo A-IV protein in serum from T2DM patients with and without diabetic foot. Serum Apo A-IV proteins isolated by fast protein liquid chromatography (FPLC), were mixed with equal column in the same group, and detected by western blot using anti-Apo A-IV and anti-CML antibody.

### Microfluidic artery chip construction and vascular morphology

The microfluidic arterial chip platform was constructed by implanting human internal mammary artery segments into the dynamic continuous culture perfusion system. After culturing for 96 h, the blood vessels were removed for histopathological examination. Morphology and immunohistochemistry showed clear layer structure of the artery,

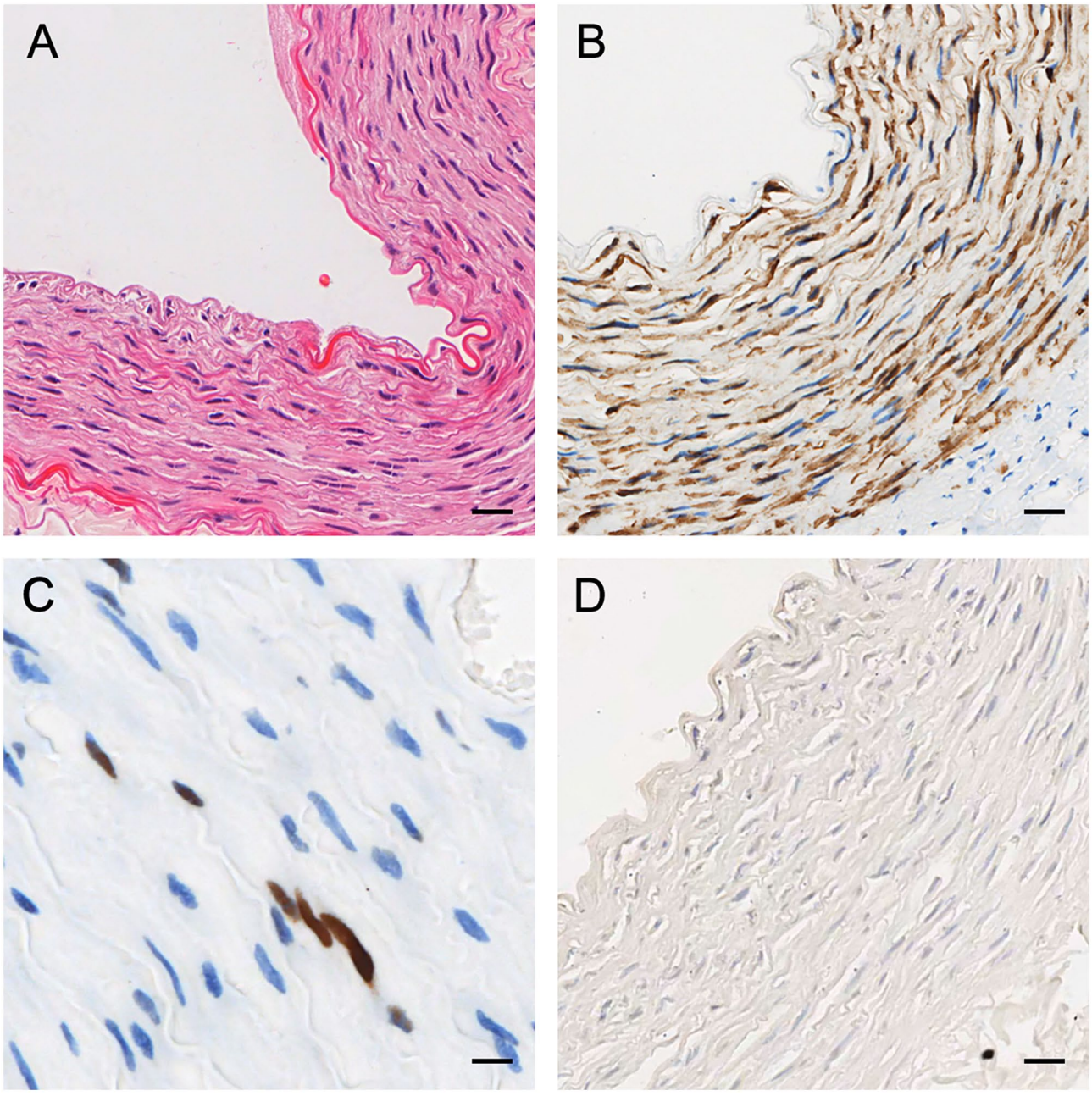


**Figure 3.** Histopathology of chip arteries after serum Apo A-IV perfusion. (A) Morphology and immunohistochemistry showed clear layer structure of the artery, intact vascular endothelial cells, and non-hyperplasia intima. Smooth muscle cells in vascular middle layer were arranged orderly. Scale bar, 20 μm. (B) All the smooth muscle cells in chip vascular medium showed α-actin-positive by immunohistochemical staining. Scale bar, 20 μm. (C) Some medium smooth muscle cells showed PCNA-positive by immunohistochemical staining. Scale bar, 10 μm. (D) Most of the arteries showed TUNEL-negative in apoptosis assay. Scale bar, 20 μm.

intact vascular endothelial cells, and non-hyperplasia intima. Smooth muscle cells in vascular middle layer were arranged orderly, and showed positive reaction of α-actin (Figure 3(A) and (B)). Some medium smooth muscle cells showed PCNA-positive, indicating that they still maintained the ability to multiply (Figure 3(C)). TUNEL staining was performed to observe vascular apoptosis and injury in microfluidic dynamic culture. Except a few weak positive cells, most of the arteries showed TUNEL-negative (Figure 3(D)), which indicated that we had successfully constructed the microfluidic artery chip model.

### Effect of Apo A-IV on microfluidic vascular biology

*Results of LightCycler real-time PCR.* The melting curve of PCR for each sample had a single peak that indicated the good specificity for PCR amplification (Figure 4(A) and (B)). The relative expression of the target genes was calculated as ratios of the relative transcript levels of the target genes versus that of GAPDH. Of all relative expressions of target genes (including IL-8, TNF-α, ICAM-1, and NR4A3) examined, only TNF-α and NR4A3 were equal or more than twice as expressed relative to control group. The fold changes with

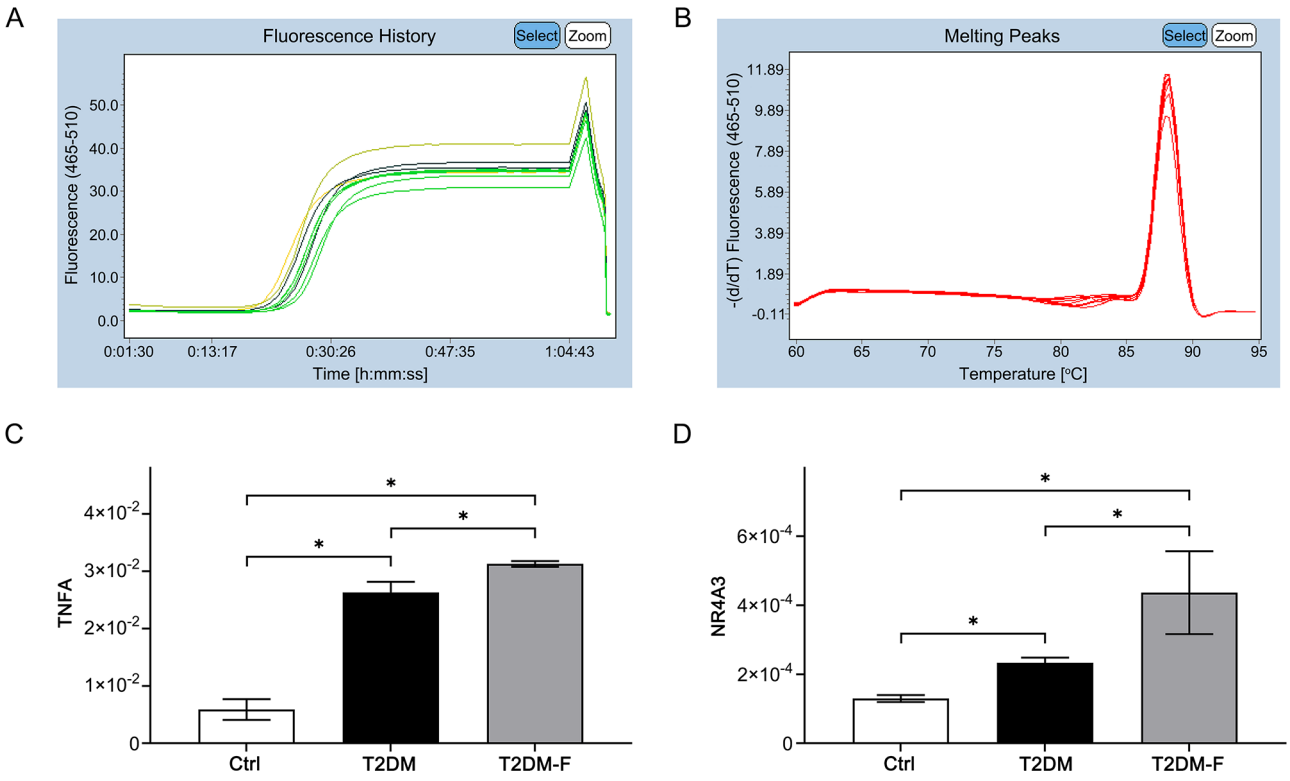


**Figure 4.** Expressions of TNF-α and NR4A3. Relative TNF-α and NR4A3 mRNA expression were determined by real-time PCR using GAPDH as an endogenous reference gene. Normalized TNF-α and NR4A3 value are derived from ratios of TNF-α/GAPDH and NR4A3/GAPDH expressions. (A, B) Concentration and integrity of total mRNA isolated from FFPE tissue were evaluated by quantitative and melting curves using specific primers for GAPDH. (C, D) Expression levels of TNF-α and NR4A3 mRNA in vascular tissue from T2DM patients with or without diabetic foot. Data for control group (Open bar), T2DM group (black bar), and T2DM-F (shaded bars) are shown as mean ± SD. *$p < 0.05$ by Student's *t*-test.

TNF-α in T2DM and T2DM-F groups versus the NC group were 4.48 and 5.30, and with NR4A3 were only 3.36 in T2DM-F group versus NC group (all $p < 0.05$), indicating that plasma Apo A-IV perfusion could upregulate the TNF-α gene expression of microfluidic arteries in T2DM and T2DM-F groups, but increase in NR4A3 gene expression was observed in T2DM-F group (Figure 4(C) and (D)).

### Immunohistochemistry results

In this study, ICAM-1, TNF-α, and IL-8 were selected as indicators of inflammatory response and the effect of serum Apo A-IV perfusion on expressions of these proteins in chip vessels was observed in each group. The results showed that only expression of TNF-α was significantly increased after serum Apo A-IV perfusion in T2DM and T2DM-F group, which was associated with increased NR4A3 antigen expression (Figure 5(A) and (B)). As shown in Table 2, recombinant human Apo A-IV could reverse the stimulating effect of serum Apo A-IV of T2DM-F patients on expression of TNF-α in chip arteries and NR4A3 blocking peptide could downregulate expression of TNF-α in chip arteries by inhibiting NR4A3 expression, although expressions of ICAM-1 and IL-8 were not detected. As one of the glycosylated forms of Apo A-IV, anti-CML staining could reflect whether or not Apo A-IV was glycosylated by CML and its degree. It was found that CML expression in chip arteries was significantly positive in the Apo A-IV infusion group of T2DM-F (Figure 5(C)), while negative in the T2DM and NC groups (results not shown).

## Discussion

Diabetic foot syndrome is a major complication of diabetes mellitus. The two most important risk factors are peripheral neuropathy and vascular disease. According to epidemiological data, solely neuropathy account for about 50% of the cases of diabetic foot syndrome. Peripheral arterial occlusive disease on its own is accountable for just 15% of the cases, whereas in 35%, foot ulcerations develop as a combination of both neuropathy and angiopathy, which means that about 50% of patients with diabetic foot are related to peripheral arterial disease.[16] Among the patients with T2DM-F enrolled in this study, arterial stenotic rate in the lower extremities was equal or more than 60% according to the results of ultrasound and/or CT angiography findings. This setting of subjects ensured that the occurrence of T2DM-F was indeed associated with peripheral vascular disease. The age of patients with T2DM-F was higher than that of the T2DM group because of the longer course of diabetes with poor glycemic control and the characteristics of onset age. Clinical examination and laboratory test results showed that the prevalence of hypertension, fasting blood glucose, glycosylated hemoglobin A1c, blood uric acid, triglyceride, total cholesterol, low-density lipoprotein, and hsCRP in T2DM and T2DM-F groups were higher than

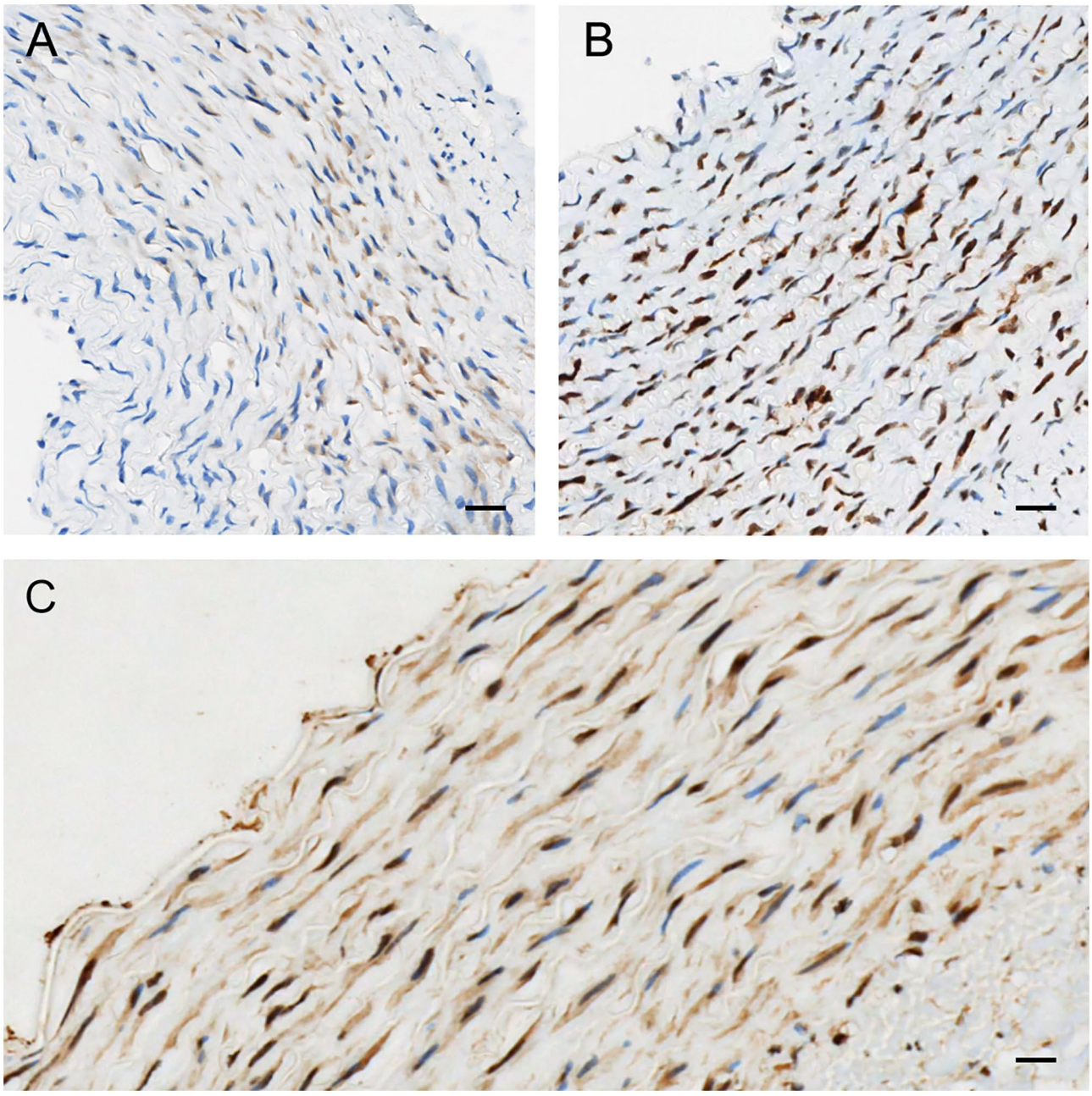


**Figure 5.** Expression of TNF-α, NR4A3, and CML in chip arteries stimulated with serum Apo A-IV from T2DM-F group. (A) Increased TNF-α expression in vascular walls after serum Apo A-IV perfusion from T2DM-F group by immunohistochemical staining. Scale bar, 20 μm. (B) Increased NR4A3 antigen expression after serum Apo A-IV perfusion from T2DM-F group by immunohistochemical staining. Scale bar, 20 μm. (C) Positive CML expression of chip vascular after serum Apo A-IV perfusion from T2DM-F group by immunohistochemical staining. Scale bar, 10 μm.

**Table 2.** Effect of Apo A-IV on expression of TNF-α in microfluidic vessels.

| Group | *n* | TNF-α | NR4A3 |
|---|---|---|---|
| CG | 9 | 1.89 ± 1.26 | 71.31 ± 27.72 |
| EG | 36 | | |
| EG1 | 9 | 123.09 ± 30.44* | 329.32 ± 81.93* |
| EG2 | 9 | 848.07 ± 154.29* | 936.33 ± 27.27* |
| EG3 | 9 | 491.31 ± 68.96** | |
| EG4 | 9 | 55.78 ± 31.26** | 4.62 ± 2.93** |

Apo A-IV: apolipoprotein A-IV; TNF-α: antitumor necrosis factor-α; NR4A3: antinuclear receptor subfamily 4 group A member 3; CG: control group; EG: experimental group. The number of antibody-reactive positive cells under microscope magnification of 100 times.
*$p < 0.05$, versus CG.
**$p < 0.05$, versus EG2.

those in NC group, but high-density lipoprotein was lower in them. Low HDL indirectly reflected a reduced ability of these patients to resist atherosclerosis and predisposition to vascular lesions. It was noteworthy that hsCRP of T2DM-F group is significantly higher than that of T2DM group, indicating local infection of T2DM-F lesions, which is one of the important characteristics of T2DM-F patients different from T2DM patients. Based on their long-term history of diabetes, laboratory characteristics, imaging evidence of peripheral artery stenosis or occlusion, and chronic foot skin ulceration with local chronic infection or ischemic necrosis or gangrene of the toe, the clinical diagnosis of T2DM-F could be established and inclusion criteria for T2DM-F were met.

Keeping a normal content of circulating Apo A-IV is necessary to maintain its roles of anti-atherosclerosis, prevention of platelet aggregation and thrombosis.[17] In this study, the serum free Apo A-IV content in T2DM group was significantly higher than that in NC group and T2DM-F group, which implied a protective role of Apo A-IV in the pathophysiological mechanism of T2DM and T2DM-F and a physiological feedback increase during those procedures.[6] Compared with patients with T2DM, a larger amount of Apo A-IV in patients with T2DM-F was consumed during the course of the disease, which resulted in relative insufficiency of free Apo A-IV in serum and lead to an aggravated condition. Three possible mechanisms have been suggested for the anti-atherosclerotic action of Apo A-IV. One is the role of Apo A-IV in promoting reverse cholesterol transport and the other are the anti-oxidant and anti-inflammatory activities of Apo A-IV.[18–20] In this study, we observed the expressions of inflammatory cytokines including TNF-α, ICAM-1, and IL-8 in chip arteries after a perfusion of serum Apo A-IV from T2DM and T2DM-F patients at the transcription and the protein levels and found that TNF-α expression significantly increased, which was associated with increased NR4A3 antigen expression. Recombinant human Apo A-IV could reverse the stimulating effect of serum Apo A-IV of diabetic foot patients on TNF-α expression, as NR4A3 blocking peptide downregulated TNF-α expression in chip arteries by inhibiting NR4A3 expression, suggesting that the serum Apo A-IV of diabetic foot patients might promote TNF-α expression in chip arteries by stimulating NR4A3 in T2DM-F patients. These observations were consistent with other studies that had found that recombinant glycosylated Apo A-IV could have stimulated inflammatory responses both *in vitro* and *in vivo* through stimulating NR4A3 expression of vascular endothelial cells and were correlated with the severity of the atherosclerotic disease.[8,21] NR4A3 expression was significantly elevated in atherosclerotic plaques from T2DM patients compared with non-atherosclerotic arteries.[8] Indeed, hyperglycemia in T2DM patients caused the glycosylation of serum proteins including Apo A-IV, which might induce increased expression of inflammatory cytokines in local arterial wall by stimulating the NR4A3 receptor and mediate the pro-inflammatory reaction and atherogenesis.[8,22,23] Apo A-IV glycosylation weakened the anti-atherosclerosis function of Apo A-IV in patients with T2DM-F.

There were strong evidences for the role of TNF-α in the development of atherosclerosis.[24] TNF-α downregulated expression of macrophage scavenger receptor via transcriptional and post-transcriptional processes and upregulated expression of several adhesion molecules that are critical in extravasation of monocytes via nuclear factor kappa B (NF-κB) pathway.[25] Therefore, TNF-α is a powerful pro-inflammatory agent that orchestrated the production of a pro-inflammatory cytokine cascade. TNF-α has been proposed as a central player in activation and recruitment of inflammatory cell, and a critical role in the development of many chronic inflammatory diseases and atherosclerosis.[26] In this study, serum Apo A-IV induced increased TNF-α expression in microfluidic chip arteries in T2DM and T2DM-F patients to promote the blood vessels into a pre-atherosclerotic state, which might be one of the important mechanisms of glycosylated Apo A-IV to induce atherosclerosis and eventually lead to diabetic foot. Although the induction of TNF-α expression by glycosylated Apo A-IV was observed in this study, expression of other inflammatory cytokines such as ICAM-1 and IL-8 was not increased at the transcription and protein levels, due to the possible reason mentioned above that TNF was a cytokine involved in the early reaction of inflammation. In this experiment, the culture time of chip arteries was 96 h, and we did observe the trend of increased expression of these two cytokines at the transcriptional level. Perhaps over time, increased expression of these inflammatory cytokines was evident.

Glycosylation of Apo A-IV protein mainly included carbamidomethyl cysteine (a fixed modification), oxidized methionine, N-acetylation, and Nε-CML (variable modifications). In T2DM, CML modifications *in vivo* predominated in advanced glycation end products (AGEs), which was commonly found in diabetic tissues including CML–protein adducts (the predominant AGEs present *in vivo*).[8,27] Immunohistochemistry of the coronary arteries from diabetic patients with severe coronary artery disease (CAD) demonstrated CML and Apo A-IV co-localization in atherosclerotic plaques.[8] In this study, we also found that Apo A-IV from diabetic foot patients could increase the expression of CML in chip arteries, which demonstrated the possibility that this Apo A-IV was glycosylated by CML. Indeed, we found that Apo A-IV from T2DM-F group was obviously glycosylated with CML using immunoblotting. CML-adducts could activate endothelial cells, vascular smooth muscle cells both *in vitro* and *in vivo*, and mononuclear phagocytes to induce expressions of a range of pro-inflammatory molecules and activation of NF-κB.[28] Using liquid chromatography–mass spectrometry, Dai *et al*.[8] has verified glycosylation of Apo A-IV protein and identified 19 CML modification sites in T2DM patients with CAD. Of these sites, K65, K123, K189, and K361 have the highest frequencies in diabetic patients, suggesting that CML modification of these sites results in Apo A-IV dysfunction.

Previous studies on vascular diseases were mainly carried out through animal experiments and *in vitro* culture of vascular endothelial cells and smooth muscle cells. After all, animal results apply differently to human. The environment of single cell two-dimensional culture *in vitro* is far from the environment of body tissue and organ cells. Based on microfluidic chip technology, Zheng *et al*.[29] created a three-dimensional (3D) microvascular network with type I collagen as the scaffold on a chip, and studied the interaction

between human umbilical vein endothelial cells and vascular pericytes and angiogenesis by adding growth factors such as vascular endothelial growth factor (VEGF), and introduced chemical stimuli such as Phorbol ester to mimic the response of blood vessels to inflammation, showing that chemical stimulation could cause endothelial cells to return to a pre-thrombotic state. In another study, a 3D simulation vessel with hemodynamic characteristics was constructed by the flow control chip technology to study the process of thrombosis and its risk factors, providing a platform for the future *in vitro* study of vascular diseases and the theoretical basis for clinical application of anticoagulant therapy.[30] In this study, we constructed a true arterial chip model by microfluidic perfusion using human internal mammary arteries. It had several advantages. It maintained the structural integrity of the vascular tissue and avoided mechanical or enzymatic damage to the cells during cell culture that might affect their function. Microfluidic continuous dynamic perfusion culture completely simulated arterial blood flow, blood flow velocity, blood pressure, and periodic arterial beats under physiological conditions, which was relatively real *in vivo* conditions. In this 96-h culturing system, tissue structure of these arteries was intact, and smooth muscle cells in vascular middle layer were arranged orderly with little apoptosis, as some cells still maintained their ability to multiply. With successful construction of microfluidic real arterial chip, arterial lesions could be dynamically observed in simulating arterial pulse during the whole process, which will provide the basis for the future study of the pathogenesis of vascular diseases and drug application.

## Conclusions

A microfluidic real arterial chip model was successfully constructed. Based on it, we found that the serum Apo A-IV from T2DM and T2DM-F patients could induce TNF-α expression in chip arteries to turn the blood vessels into a pre-atherosclerotic state, which might be one of the important mechanisms of glycosylated Apo A-IV to induce peripheral arterial atherosclerotic lesions and eventually lead to T2DM-F.

### AUTHORS' CONTRIBUTIONS

All authors participated in the experimental design, interpretation of the results, and review of the article; JJ and XZ were involved in the experimentation and wrote the article; JH served as experimental instruction and performed data analysis; XW, FX, and LL participated in the selection of experimental subjects and the analysis and arrangement of clinical data; TW and SM were responsible for the funding, design, and checking of the thesis, and were fully responsible for the authenticity of the thesis content, reliability of the data, credibility of the conclusion, compliance with legal norms, academic norms and ethical norms, and approved the final version of the article.

### DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### ETHICAL APPROVAL

The study was approved by the Medical Ethics Committee, University of Chinese Academy of Sciences Shenzhen Hospital, which conformed to the principles of the Declaration of Helsinki. All subjects gave written informed consent to participate in this study.

### FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Sanming Project of Medicine in Shenzhen (Shen Wei Ji Fa 2018 No.9), and the 2020 Basic Research Program of Shenzhen Science and Technology Innovation Commission (JCYJ20190813143221901).

### ORCID ID

Tao Wang https://orcid.org/0000-0002-1013-1843

### SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

### REFERENCES

1. Pérez-Panero AJ, Ruiz-Muñoz M, Cuesta-Vargas AI, Ruiz-Muñoz M. Prevention, assessment, diagnosis and management of diabetic foot based on clinical practice guidelines: a systematic review. *Medicine* 2019;**98**:e16877
2. Volmer-Thole M, Lobmann R. Neuropathy and diabetic foot syndrome. *Int J Mol Sci* 2016;**17**:917–27
3. Du F, Ma J, Gong H, Bista R, Zha P, Ren Y, Gao Y, Chen D, Ran X, Wang C. Microbial infection and antibiotic susceptibility of diabetic foot ulcer in China: literature review. *Front Endocrinol* 2022;**13**:881659–68
4. Xu Z, Ran X. Diabetic foot care in China: challenges and strategy. *Lancet Diabetes Endocrinol* 2016;**4**:297–8
5. Kaur R, Kaur M, Singh J. Endothelial dysfunction and platelet hyperactivity in type 2 diabetes mellitus: molecular insights and therapeutic strategies. *Cardiovasc Diabetol* 2018;**17**:121–37
6. Qu J, Ko CW, Tso P, Bhargava A. Apolipoprotein A-IV: a multifunctional protein involved in protection against atherosclerosis and diabetes. *Cells* 2019;**8**:319–36
7. Ostos MA, Conconi M, Vergnes L, Baroukh N, Ribalta J, Girona J, Caillaud JM, Ochoa A, Zakin MM. Antioxidative and antiatherosclerotic effects of human apolipoprotein A-IV in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2001;**21**:1023–8
8. Dai Y, Shen Y, Li QR, Ding FH, Wang XQ, Liu HJ, Yan XX, Wang LJ, Yang K, Wang HB, Chen QJ, Shen WF, Zhang RY, Lu L. Glycated apolipoprotein A-IV induces atherogenesis in patients with CAD in type 2 diabetes. *J Am Coll Cardiol* 2017;**70**:2006–19
9. Roula D, Theiler A, Luschnig P, Sturm GJ, Tomazic PV, Marsche G, Heinemann A, Sturm EM. Apolipoprotein A-IV acts as an endogenous anti-inflammatory protein and is reduced in treatment-naive allergic patients and allergen-challenged mice. *Allergy* 2020;**75**:392–402
10. Low LA, Mummery C, Berridge BR, Austin CP, Tagle DA. Organs-on-chips: into the next decade. *Nat Rev Drug Discov* 2021;**20**:345–61
11. Sontheimer-Phelps A, Hassell BA, Ingber DE. Modelling cancer in microfluidic human organs-on-chips. *Nat Rev Cancer* 2019;**19**:65–81
12. Tajeddin A, Mustafaoglu N. Design and fabrication of organ-on-chips: promises and challenges. *Micromachines* 2021;**12**:1443–75
13. Osaki T, Sivathanu V, Kamm RD. Vascularized microfluidic organ-chips for drug screening, disease models and tissue engineering. *Curr Opin Biotechnol* 2018;**52**:116–23
14. American Diabetes Association. Standards of medical care in diabetes — 2012. *Diabetes Care* 2012;**35**:S11–63.

15. Steinmetz A, Clavey V, Vu-Dac N, Kaffarnik H, Fruchart JC. Purification of human apolipoprotein A-IV by fast protein liquid chromatography. *J Chromatogr* 1989;**487**:154–60
16. Reardon R, Simring D, Kim B, Mortensen J, Williams D, Leslie A. The diabetic foot ulcer. *Aust J Gen Pract* 2020;**49**:250–5
17. Peng J, Li XP. Apolipoprotein A-IV: a potential therapeutic target for atherosclerosis. *Prostaglandins Other Lipid Mediat* 2018;**139**:87–92
18. Shimabukuro Okuda L, Tallada Iborra R, Ramos Pinto P, Fabres Machado U, Corrêa-Giannella ML, Pickford R, Woods T, Brimble MA, Rye KA, Passarelli M. Advanced glycated apoA-IV loses its ability to prevent the LPS-induced reduction in cholesterol efflux-related gene expression in macrophages. *Mediators Inflamm* 2020;**2020**:6515401
19. Vowinkel T, Mori M, Krieglstein CF, Russell J, Saijo F, Bharwani S, Turnage RH, Davidson WS, Tso P, Granger DN, Kalogeris TJ. Apolipoprotein A-IV inhibits experimental colitis. *J Clin Invest* 2004;**114**:260–9
20. Heinecke JW. Lipoprotein oxidation in cardiovascular disease: chief culprit or innocent bystander? *J Exp Med* 2006;**203**:813–6
21. Martí-Pàmies I, Cañes L, Alonso J, Rodríguez C, Martínez-González J. The nuclear receptor NOR-1/NR4A3 regulates the multifunctional glycoprotein vitronectin in human vascular smooth muscle cells. *FASEB J* 2017;**31**:4588–99
22. Pirri D, Fragiadaki M, Evans PC. Diabetic atherosclerosis: is there a role for the hypoxia-inducible factors? *Biosci Rep* 2020;**40**:BSR20200026
23. Yuan T, Yang T, Chen H, Fu D, Hu Y, Wang J, Yuan Q, Yu H, Xu W, Xie X. New insights into oxidative stress and inflammation during diabetes mellitus-accelerated atherosclerosis. *Redox Biol* 2019;**20**:247–60
24. Hopkins PN. Molecular biology of atherosclerosis. *Physiol Rev* 2013;**93**: 1317–542
25. Gao W, Liu H, Yuan J, Wu C, Huang D, Ma Y, Zhu J, Ma L, Guo J, Shi H, Zou Y, Ge J. Exosomes derived from mature dendritic cells increase endothelial inflammation and atherosclerosis via membrane TNF-alpha mediated NF-kappaB pathway. *J Cell Mol Med* 2016;**20**:2318–27
26. Munjal A, Khandia R. Atherosclerosis: orchestrating cells and biomolecules involved in its activation and inhibition. *Adv Protein Chem Struct Biol* 2020;**120**:85–122
27. Gutierrez-Mariscal FM, Cardelo MP, de la Cruz S, Alcala-Diaz JF, Roncero-Ramos I, Guler I, Vals-Delgado C, López-Moreno A, Luque RM, Delgado-Lista J, Perez-Martinez P, Yubero-Serrano EM, Lopez-Miranda J. Reduction in circulating advanced glycation end products by Mediterranean diet is associated with increased likelihood of type 2 diabetes remission in patients with coronary heart disease: from the cordioprev study. *Mol Nutr Food Res* 2021;**65**:e1901290
28. Miyahara Y, Ikeda S, Muroya T, Yasuoka C, Urata Y, Horiuchi S, Kohno S, Kondo T. Nepsilon-(carboxymethyl)lysine induces gamma-glutamylcysteine synthetase in RAW264.7 cells. *Biochem Biophys Res Commun* 2002;**296**:32–40
29. Zheng Y, Chen J, Craven M, Choi NW, Totorica S, Diaz-Santana A, Kermani P, Hempstead B, Fischbach-Teschl C, Lopez JA, Stroock AD. In vitro microvessels for the study of angiogenesis and thrombosis. *Proc Natl Acad Sci U S A* 2012;**109**:9342–7
30. Costa PF, Albers HJ, Linssen JEA, Middelkamp HHT, van der Hout L, Passier R, van den Berg A, Malda J, van der Meer AD. Mimicking arterial thrombosis in a 3D-printed microfluidic in vitro vascular model based on computed tomography angiography data. *Lab Chip* 2017;**17**: 2785–92

*(Received September 14, 2022, Accepted November 21, 2022)*