Clin. Lab. 2026;72:XXX-XXX

ORIGINAL ARTICLE

Function and Mechanism of TRPV4 in Promoting Vascular Calcification by Regulating RUNX2 through IL-6

Wen-Juan Yang [1, *], Zheng Ma [1, *], Jian-Sheng Pei [2], Shaobin Jia [1, 3]

[*] *These authors contributed equally to this study*
[1] *Heart Centre, General Hospital of Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region, PR China*
[2] *Department of Clinical Medicine, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region, PR China*
[3] *Institute of Cardiovascular Diseases, General Hospital of Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region, PR China*

SUMMARY

Background: **Calcification is an active process where hydroxyapatite crystals deposit in arterial walls or aortic valves, leading to valvular calcification. This study aimed to identify useful molecular markers for vascular calcification and decode the fundamental mechanisms underlying their biological function.**
Methods: **Two published vascular calcification datasets (GSE136593 and GSE159832) from the Gene Expression Omnibus database were downloaded and the data were analyzed using Partek Flow and Qiagen IPA software. We explored the functional expression and pathways of TRPV4 in vascular calcification. A7R5 cells were treated with TRPV4 agonist GSK1016790A (10 nM) and selective TRPV4 antagonist HC067047 (10 μM) for 3 weeks in Dulbecco's modified eagle medium and calcification medium (induced by β-glycerophosphoric acid).**
Results: **The transcriptomes of calcified and non-calcified blood vessels were found to be significantly distinct. Calcified blood vessels consistently increased TRPV4 expression and regulated the expression of inflammatory factors. TRPV4 was up-regulated and strongly correlated with calcification both in human carotid plaque and in mouse carotid artery calcified plaque. TRPV4 promotes osteogenic differentiation and vascular smooth muscle cell calcification induced by β-glycerophosphorus. Western blot and RT-qPCR experiments revealed that TRPV4 promoted smooth muscle cell calcification and osteogenic differentiation by regulating RUNX2 by upregulating IL-6.**
Conclusions: **TRPV4 regulates vascular calcification by activating IL-6 and suggest potential avenues for the development of interventions to prevent or treat vascular calcification.**
(Clin. Lab. 2026;72:xx-xx. DOI: 10.7754/Clin.Lab.2025.250575)

Correspondence:
Shaobin Jia
Institute of Cardiovascular Diseases
General Hospital of Ningxia Medical University
No. 804 Shengli South Street
Yinchuan, 750001
Ningxia Hui Autonomous Region
PR China
Phone: + 86 9516743232
Email: yangwenjuan_ywj@126.com

Manuscript accepted July 2, 2025

KEYWORDS

vascular calcification, TRPV4, RUNX2, IL-6, regulation

LIST OF ABBREVIATIONS

TRPV4 - Transient receptor potential vanilloid 4
RUNX2 - Runt-related transcription factor 2
BMP2 - Bone morphogenetic proteins 2
VSMC - Vascular smooth muscle cells
α-SMA - Alpha smooth muscle Actin
IL-6 - Interleukin 6
IL-1β - Interleukin 1β
TNF-α - Tumor Necrosis Factor alpha

INTRODUCTION

Calcification is an active pathophysiological process characterized by the deposition of calcium phosphate crystals in the form of hydroxyapatite, which occurs in the media or intima of arteries and aortic valve leaflets, contributing to valvular calcification. Vascular calcification associated with inflammation is the hallmark of disease progression and a powerful independent risk marker for adverse coronary events [1]. The mechanisms of atherosclerosis-related vascular calcification (VC) are complicated and poorly understood [2]. Cardiovascular calcification is now recognized as an active process, presenting a potential therapeutic targeting opportunity [3]. Numerous studies have demonstrated that osteogenic differentiation of vascular smooth muscle cells (VSMCs) in response to various local stimuli is a key mechanism in the development of vascular calcification [4,5].

In recent years, it has been reported that bioinformatics analysis is effective in detecting the complex network during the vascular calcification process and in screening biomarkers for vascular calcification diagnosis and prognosis. Gene Expression Omnibus (GEO) is an online database containing millions of gene profiles of various diseases, and the GEO datasets can be used to identify differentially expressed genes (DEGs). Partek Flow is a bioinformatics software package designed to analyze high-throughput genomic data, such as gene expression data obtained from RNA sequencing (RNA-seq). Qiagen Ingenuity Pathway Analysis (IPA) software is a powerful tool for analyzing high-throughput genomic data, including pathway enrichment analysis and comparison analysis of multiple DEGs. In this study, we used Partek Flow and Qiagen IPA for further investigation. Dysregulated genes in vascular calcification samples were examined to screen vital predictors. They were analyzed using bioinformatic tools, Gene Ontology (GO) annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, protein-protein interaction (PPI) network, and gene set enrichment analysis (GSEA). Transient receptor potential cation channel subfamily V member 4 (TRPV4) has the highest expression in terms of vascular calcification. TRPV4 belongs to the TRP superfamily of cation channels. TRPV4, a Ca2+-permeable mechanosensitive cation channel, is ubiquitously expressed in various cell types, such as VSMCs. TRPV4 responds not only to exogenous and endogenous chemical stimuli, but also to physical stimuli, such as mild heat and shear stress [6]. Studies have shown that impairment of TRPV4 channel function is linked to endothelial dysfunction, oxidized LDL-induced macrophage foam cell formation, and vascular diseases [7-11]. Although the data are suggestive, it is unclear if TRPV4 plays a role in vascular calcification. Therefore, we examined the TRPV4 interregulation network using bioinformatic methods and discovered that TRPV4 was up-regulated in vascular calcification tissues.

The binding and regulation of Il-6 by TRPV4 is exciting, as is the functional expression of TRPV4 in vascular calcification. In this study, we demonstrate that TRPV4 is significantly upregulated in vascular calcification and that TRPV4 promotes VSMC calcification and osteogenic differentiation though RUNX2 and RUNX2 by activating IL-6.

MATERIALS AND METHODS

Bioinformatics analysis

We downloaded the vascular calcification datasets GSE136593 and GSE159832 from the GEO database and analyzed the data using Partek Flow and Qiagen IPA. Partek Flow is a bioinformatics software package designed to analyze high-throughput genomic data, such as gene expression data obtained from RNA sequencing (RNA-seq). The Partek Flow methodology for RNA-seq data analysis can be summed up as follows:

Data preprocessing

First, the RNA-seq data are preprocessed to remove any low-quality reads and adapter sequences. Following this, the reads are aligned to a reference genome or transcriptome.

Quantification

Next, gene expression levels are quantified by counting the number of reads that map to each gene. Typically, software such as featureCounts or HTSeq is used for this purpose.

Normalization

The raw gene expression counts are then normalized to account for differences in sequencing depth and composition between samples. This is typically done using methods such as the trimmed mean of M-values (TMM) or the upper quartile (UQ) normalization.

Differential gene expression analysis

The normalized gene expression counts are then employed to determine which genes are differentially expressed between two or more sample groups. Typically, statistical methods such as edgeR or DESeq2 are employed.

Pathway analysis

Finally, a pathway analysis is performed to identify biological pathways where differentially expressed genes are enriched. Typically, this is accomplished with software such as Gene Set Enrichment Analysis (GSEA). Qiagen IPA is a powerful tool for analyzing high-throughput genomic data, including pathway enrichment analysis and comparison analysis of multiple DEGs. Here is a concise description of the IPA methodology for pathway enrichment analysis and comparison analysis of multiple DEGs:

Data preprocessing

The first step is to preprocess the gene expression data obtained from RNA-seq or microarray experiments. This involves normalizing the data and identifying the DEGs between experimental conditions.

Pathway enrichment analysis

IPA permits users to identify biological pathways significantly enriched in the DEGs identified in step 1. IPA accomplishes this by comparing the list of DEGs to its database of manually curated biological pathways and identifying pathways that are overrepresented among DEGs. IPA also offers tools for visualizing pathways and identifying key genes and regulators within each pathway.

Comparison analysis of multiple DEGs

IPA enables users to compare multiple sets of DEGs to identify pathways and biological processes that are differentially regulated across experimental conditions. This is accomplished by performing overlap analysis and creating Venn diagrams to visualize the degree of overlap between different DEG sets. IPA also provides tools for creating heat maps and conducting cluster analysis to identify data patterns.

Human carotid artery specimens

From January 2016 to December 2022, we collected 42 pathological specimens of patients who underwent carotid plaque removal at the General Hospital of Ningxia Medical University. They consisted of 29 calcifications and 13 non-calcifications. The pathology department created paraffin sections. HE staining, Alizarin Red staining, and immunohistochemistry were used to analyze the specimens. The protocol was approved by the Ethics Committee of General Hospital of Ningxia Medical University (KYLL-2022-0494) and all patients signed an informed consent form in accordance with the Declaration of Helsinki.

Experimental animals

The experimental protocols were approved by the Institutional Ethics Committee for Animal Experiments of Ningxia Medical University in accordance with the animal welfare principles (2021-030). We acquired 20 male *ApoE*$^{-/-}$ 8-week-old mice from the Animal Center of Peking University Health Science Center (Beijing). *ApoE*$^{-/-}$ mice are a well-established model for atherosclerosis. All mice were housed in a controlled environment (25 ± 2°C, 12-hours light-dark cycles) with free access to water and a standard diet of chow. Mice were evenly divided into two treatment groups: the control group (AC group, n = 10) and the high-fat diet group (HF group, n = 10). Mice in the AC group were fed a standard rodent maintenance diet as recommended by the American Institute of Nutrition-93 purified diet (AIN-93G) for 30 weeks, whereas mice in the HF group were fed a control diet containing 1.25% cholesterol (HF) (MD12015, Jiangsu). Random selection and blinding methods were used to select the experimental animal.

Tissue processing

After 30 weeks of feeding, mice were anesthetized with isoflurane (China catalog number 55) inhaled at a concentration of 2%. The mice carotid artery tissue was washed with sterile phosphate-buffered saline (PBS), fixed for 15 minutes with 4% paraformaldehyde, and then embedded in paraffin. Sections cut into 4 mm thickness were stained with Alizarin Red, hematoxylin & eosin (H&E), and immunohistologically analyzed.

HE staining and artery calcification

Carotid artery sections were stained with hematoxylin and eosin, used to evaluate the severity of atherosclerotic calcified lesions in carotid artery cross-sections with a thickness of 10 μm. Calcium deposits in the carotid artery were detected by staining with Alizarin Red (Sigma Aldrich) and incubating for 5 minutes in Alizarin Red solution after washing with distilled water [12, 13]. The sections were dehydrated, and cover-slipped before being examined and photographed under a microscope. Using ImageJ software, the percentage of positively stained surface area in each section was calculated (NIH; Bethesda, MD, USA).

Immunohistochemical staining

Immunohistochemical staining was performed to determine protein expression in paraffinized sections. Carotid paraffin section was deparaffinized in Histo Clear reagent, washed in distilled water, and treated with avidin/biotin blocking kit to inhibit endogenous biotin activity, as per the manufacturer's protocol. The sections were incubated with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity, then blocked with 10% goat serum at room temperature for 1 hour. Next, specimens were incubated overnight at 4°C with primary antibodies TRPV4 (Abcam, DF8624), RUNX2 (Abcam, ab23981), and BMP2 (Absin, abs159224). The sections were incubated with diluted biotinylated secondary antibody (Cell Signaling, 7075) for 1 hour at 37°C, then treated for 20 minutes with horseradish peroxidase-labeled streptavidin solution at 37°C. Finally, the sections were counterstained with hematoxylin and then stained with diaminobenzidine (DAB kit, ZSGB-BIO, Beijing, China).

VSMC culture

Rat aortic smooth muscle cells (A7r5) were purchased from the American Type Culture Collection for in vitro experiments (ATCC, USA). The cells were cultured at 37°C in a 5% CO2-humidified atmosphere and were grown to reach the desired confluence. The growth medium consisted of Dulbecco's modified eagle's medium (DMEM, high glucose, Hyclone), 10% fetal bovine serum (FBS, C2810-0500, BI, USA), 100 mg/mL streptomycin, and 100 unit/mL penicillin. The cells were used between the 4th and 7th passage. The media was replaced daily. For the preparation of an in vitro calcification model, 10 mmol/L β-sodium glycerophosphate was added to the growth medium and the cells were cultured for 3 weeks [13]. DMEM and β-sodium glycerophosphate medium were supplemented with TRPV4 agonist GSK 1016790A (10 nM) and selective TRPV4 antagonist HC067047 (10 μM), respectively [14,15]. GSK 1016790A (10 nM) and HC067047 (10 μM) were dis-

solved in DMSO. The doses and administration schedules were chosen based on previous studies and pilot experiments, and methods for minimizing side effects were also considered [16,17].

In vitro calcification

For calcification staining *in vitro*, the cells were cultured in a medium containing 10 mmol/L β-sodium glycerophosphate. After washing with PBS, cell samples were fixed with paraformaldehyde for 45 minutes at 4°C, then stained for 5 minutes at room temperature with Alizarin Red (Sigma Aldrich), washed with double-distilled water, dehydrated, and cover slipped. The section was microscopically examined and photographed. We used ImageJ software (NIH; Bethesda, MD, USA) to calculate the percentage of positively stained surface area in each section.

Reverse-transcriptase quantitative PCR (RT-qPCR)

Total RNA was extracted from vascular tissues or cells using a Total RNA kit (TIANGEN, Beijing, CHN) as per the manufacturer's instructions. The RNA was used to synthesize cDNA using PrimeScriptTM RT Master Mix kit (Takara, Tokyo, JPN). The mRNA levels of SM-actin, SM MHC, RUNX2, and GAPDH were detected using real-time quantitative PCR with the QuantiNova® SYBR® Green PCR Kit (Qiagen, Hilden, GER) on a 7500 Multicolour Realtime PCR Detection System (ThermoFisher Scientific, CA, USA). GADPH mRNA levels were used for normalization. The relative expression of mRNA was computed using the formula 2-△△CT. The RT-qPCR primers were acquired from Sangon Biotech (Shanghai) Co., Ltd, and following are the particular primers:
TRPV4, 5′-GGAGTCCTGTTCTTCTTTACC-3′ (forward) and
5′-ACCAGCACTGAGTAGATGAA-3′ (reverse);
BMP2, 5′-AAGCGTCAAGCCAAACACAAC-3′ (forward) and
5′-ACATCACTGAAGTCCACATACA-3′ (reverse);
RUNX2, 5′-CCACCACTCACTACCACACG-3′ (forward) and
5′-GGACGCTGACGAAGTACCAT-3′ (reverse);
IL-6, 5′-CAGAGTCATTCAGAGCAATAC-3′ (forward) and
5′-ATGGTCTTGGTCCTTAGCCAA-3′ (reverse);
TNF-α, 5′-ATGGGCTCCCTCTCATCAGT-3′ (forward) and
5′-GCTTGGTGGTTTGCTACGAC-3′ (reverse);
IL-1β, 5′-GCTATGGCAACTGTCCCTGA-3′ (forward) and
5′-GGGCTTGGAAGCAATCCTTAAT-3′ (reverse);
α-SMA, 5′-CCACCAACCCCCAAAGAGAA-3′ (forward) and
5′-GGGCAAAGAACGAGGGATCA-3′ (reverse);
GAPDH, 5′-AGACTGGCAGTGGTTTGCTT-3′ (forward) and
5′-CTCTCTGCATGGTCTCCGTC-3′ (reverse)

Western blot analysis

Cells were lysed with lysis buffer, then centrifuged for 30 minutes at 12,000 rpm, 4°C, to extract total protein from the lysate (KeyGEN BioTECH, Jiangsu, China). The BCA protein assay kit was employed for protein concentration (KeyGEN BioTECH, JiangSu, China). On a 10% SDS-PAGE gel, approximately 40 μg of protein samples were separated and electrophoretically transferred to a nitrocellulose membrane. The membrane was treated with 5% skim milk for 1 hour at room temperature, and the blot was incubated with the following primary antibody overnight at 4°C: anti-SMα-actin (Abcam, ab5694, 1:2,000), TRPV4 (Abcam, DF8624, 1:1,000), antiRUNX2 (Abcam, ab23981, 1:1,000), anti-BMP2 (Absin, abs159224, 1:1,000), anti-IL-1β (Absin, abs135607, 1:500), anti-IL-6 (Rockland, 56166, 1:1,000), anti-TNF-α (Absin, abs110635, 1:1,000), and anti-GAPDH (Cell Signaling, 97166, 1:1,000) antibodies for normalization. The blot membrane was washed with TBST and incubated with HRP-conjugated antibody for 1 hour at room temperature, then detected by ECL test.

Statistical analysis

All experimental results were analyzed using GraphPad Prism 8.0 (USA) and displayed as mean ± standard deviation or median. The *in vitro* data came from a minimum of three separate experiments. All data were evaluated for normality and equal variance. We used the Student's *t*-test to compare differences between two groups, whereas we used one-way or two-way ANOVA for multiple comparisons, followed by a Student-Newman-Keuls test. The Mann-Whitney test was used for non-normally distributed data, $p < 0.05$ was set as the threshold for significance.

RESULTS

Transcriptomes of calcified and non-calcified blood vessels in diseases differ significantly

To identify diagnostically useful biomarkers for vascular calcification and decode the fundamental biological mechanisms underlying their function, we downloaded the published vascular calcification datasets GSE136 593 and GSE159832 from the GEO database and analyzed the data using Partek Flow. For GSE136593, PCA analysis revealed significant differences in the transcriptional profiles of calcified and non-calcified groups (Figure 1A). Differential gene analysis revealed 1,362 upregulated and 445 downregulated genes using the criteria $|logFC| > 1$ and FDR $p < 0.05$ when compared to the non-calcified group, with no changes in 19,230 genes (Figure 1B). The genes with differential expression between the two groups were depicted using a heatmap (Figure 1C). For GSE159832, the transcriptomes of intimal calcification, medial calcification, and non-calcification were distinct (Figure 1D). Differential gene analysis revealed 970 upregulated and 410 down

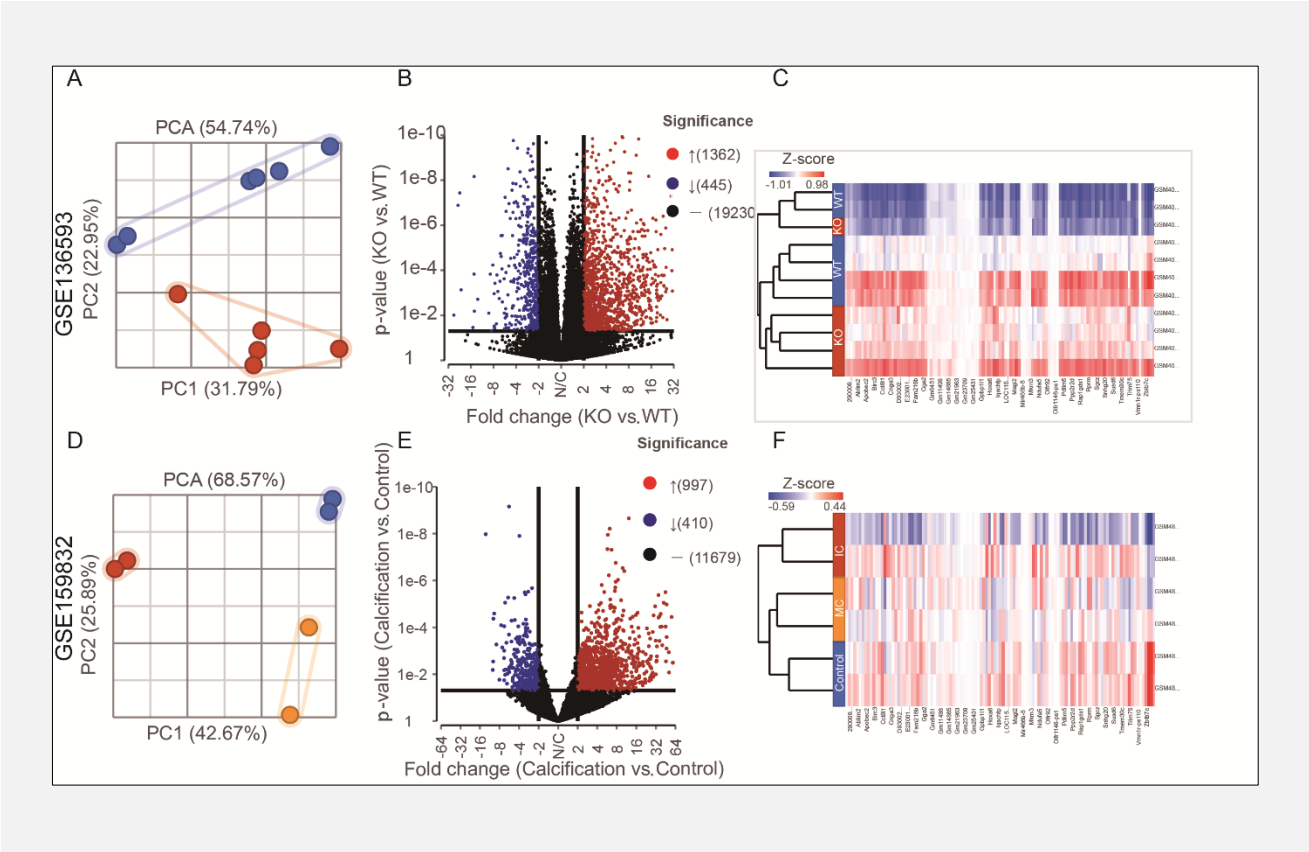

Figure 1. The transcriptomes of calcified and non-calcified blood vessels are significantly different.

A) For GSE136593, PCA analysis showed significant differences in transcriptional profiles between calcified and non-calcified groups. B) Differential gene analysis showed 1,362 upregulated genes and 445 downregulated genes under the criteria of |logFC| > 1 and FDR p < 0.05 when compared to the non-calcified group, with 19,230 genes showing no change. C) Heatmap displays the differentially expressed genes between the two groups. D) For GSE159832, the transcriptomes of intimal calcification, medial calcification, and non-calcification are different. E) Differential gene analysis revealed 970 upregulated genes and 410 downregulated genes under the criteria of |logFC| > 1 and FDR p < 0.05 when compared to the non-calcified group, with 11,679 genes showing no change. F) Heatmap displayed the differentially expressed genes between the three groups.

regulated genes based on the criteria |logFC| > 1 and FDR p < 0.05 when compared to the non-calcified group, with 11,679 genes showing no change (Figure 1E). Heatmap displayed the differentially expressed genes between the three groups (Figure 1F).

Calcified blood vessels consistently increased TRPV4 expression and controlled the expression of inflammatory factors

We analyzed data to determine the biological characteristics of vascular calcification. TRPV4 had the most significant and consistent upregulation in calcification and non-calcification groups (Z score > 2) (Figure 2A); TRPV4 had statistically significant differential upregulation in calcification and non-calcification groups (-logB-H p-value > 1.3) (Figure 2B); in the calcification model, TRPV4 may regulate the inflammatory pathway by activating IL-6 (Figure 2C-2E). In comparison to non-calcification, the HIF-1α signaling pathway was consistently and significantly activated in calcification, as determined by an IPA analysis. The samples were analyzed using the Z score > 2 and a criteria of (-logB-H p > 1.3).

HIF-1α signaling pathway was significantly and consistently activated in calcification compared to non-calcification

IPA comparative analysis showed that differentially activated signaling pathways between the calcification and non-calcification groups were analyzed using the Z score > 2 criterion (Figure 3A); differentially activated signaling pathways between the calcification and non-calcification groups were analyzed using the criterion of (-logB-H p > 1.3) (Figure 3B).

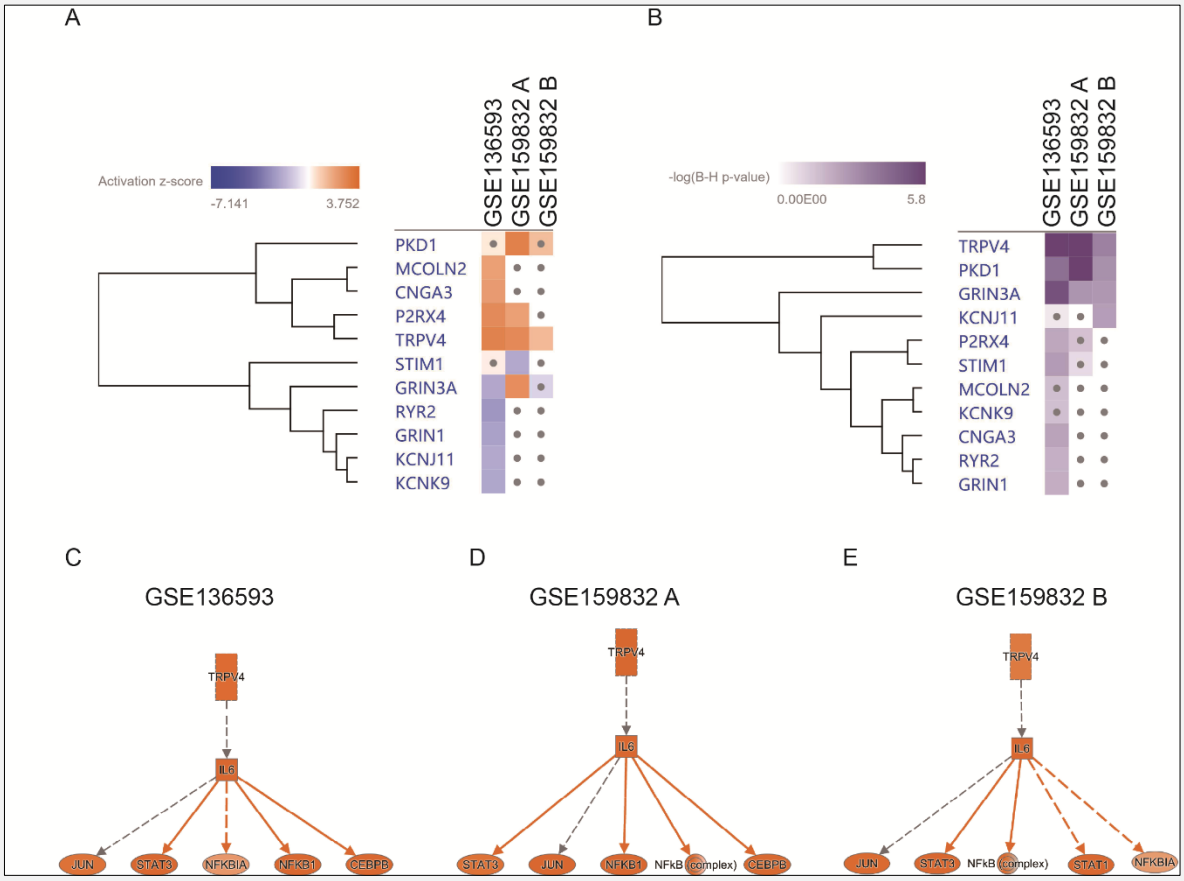

Figure 2. Calcified blood vessels consistently upregulated TRPV4 expression and regulated the expression of inflammatory factors.

A) IPA summary analysis revealed that the TRPV4 gene had the most significant and consistent upregulation in calcification and non-calcification groups (Z score > 2). B) TRPV4 gene had statistically significant differential upregulation in calcification and non-calcification groups (-logB-H p > 1.3). C - E) In the calcification model, TRPV4 may regulate the inflammatory pathway by activating IL6.

TRPV4 is upregulated and strongly associated with calcification in human carotid arteries

To identify calcified blood vessels with increased TRPV4 expression, we initially detected TRPV4 expression in human carotid arteries. A total of 42 pathological specimens were collected from patients undergoing carotid plaque stripping (statistics of patients from January 2016 to December 2022 in Ningxia Medical University Hospital). HE and alizarin red staining were used to evaluate vascular calcification in human carotid arteries (Figure 4A). The alizarin red staining demonstrated that the alizarin red staining positive area in the calcification group (n = 29) was significantly greater than that in the non-calcification group (n = 13) (Figure 4B) (**** $p < 0.0001$). Using immunohistochemistry analysis (Figure 4C), we determined that the level of TRPV4 in the calcification group was significantly higher than that in the non-calcification group (Figure 4D). Also, we observed an increase in RUNX2 (Figure 4E) and a decrease in α-SMA expression in the calcification group (Figure 4F).

In addition, correlation analysis revealed a positive relationship between TRPV4 and vascular calcification markers (Figure 4G, 4H).

TRPV4 is upregulated in calcification plaque in mice carotid artery

To investigate the effects of TRPV4 on vascular calcification *in vivo*, we created a calcification model in ApoE-/- mice which were fed a high-fat diet for 30 weeks. Based on HE and Alizarin Red staining, high fat diet-fed mice showed a significant increase in calcification area after 30 weeks compared to ApoE-/- mice fed a control diet (Figure 5A, 5B). Immunohistochemical

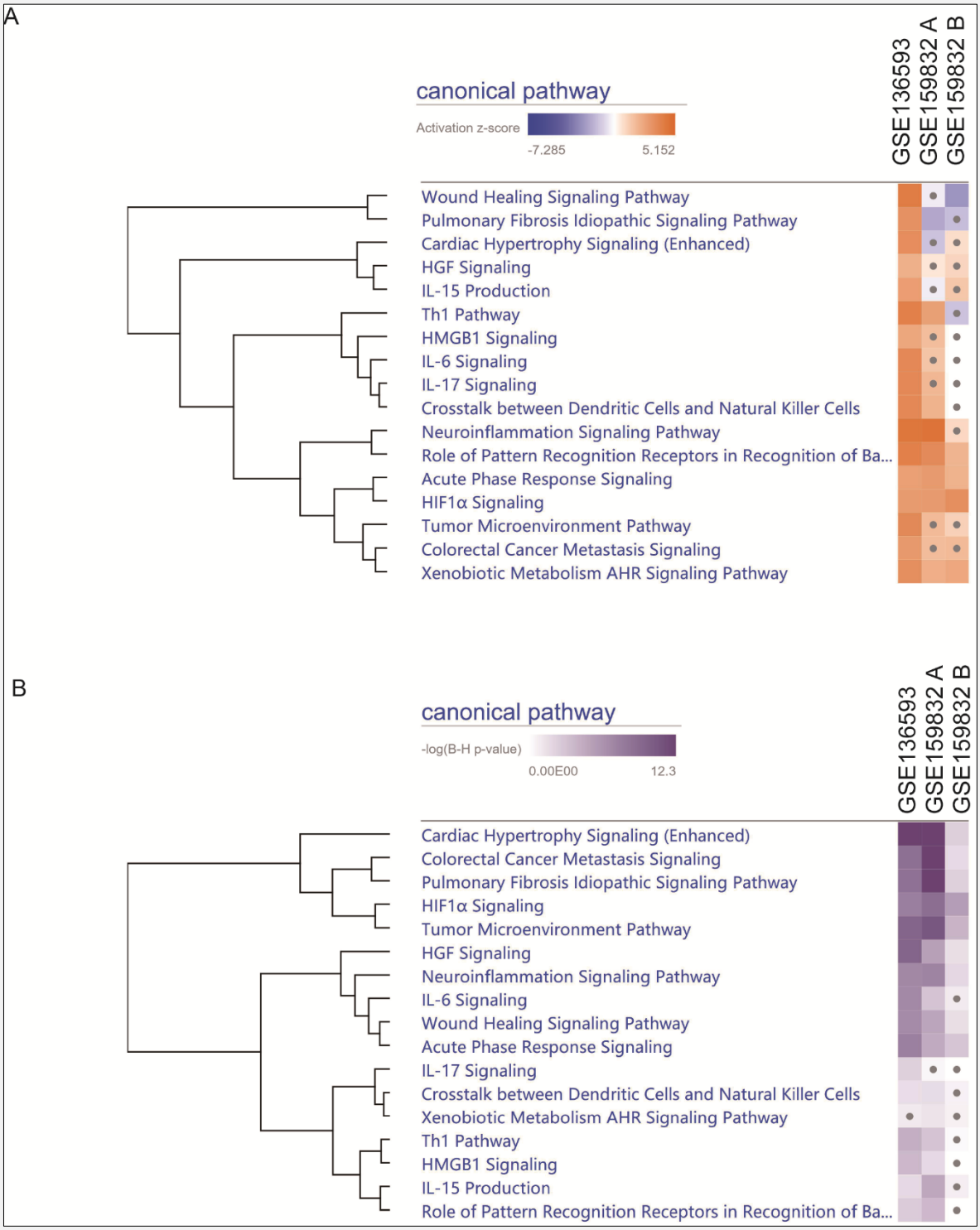

Figure 3. IPA comparative analysis showed that HIF-1α signaling pathway was significantly and consistently activated in the calcification compared to non-calcification groups.

A) The differentially activated signaling pathways between the calcification and non-calcification groups were analyzed using a criterion of Z score > 2. B) The differentially activated signaling pathways between the calcification and non-calcification groups were analyzed using a criterion of (-logB-H P > 1.3).

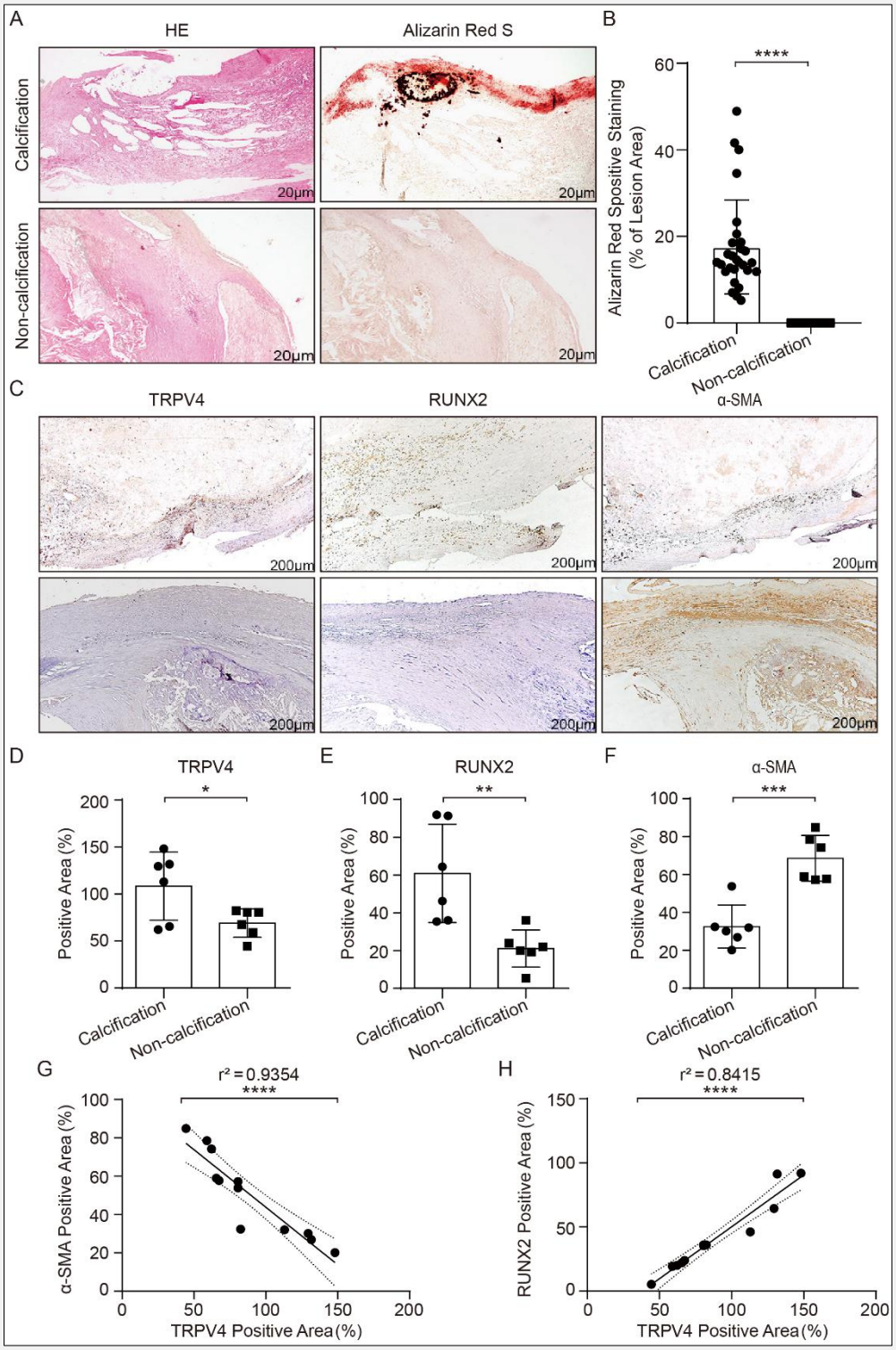

Figure 4. TRPV4 is upregulated and strongly associated with calcification in human carotid artery biopsy.

A) The representative vascular calcification plaque by HE and Alizarin Red staining of human carotid arteries. B) Statistical analysis of calcification area by Alizarin Red staining (calcification group n = 29, non-calcification group n = 13). C) Representative immunohistochemistry staining of TRPV4, RUNX2, and α-SMA in carotid arteries (n = 6). D - F) Comparison of TRPV4, RUNX2, and α-SMA between the calcification group and non-calcification group. G - H) Correlation analysis of TRPV4 and vascular calcification biomarkers RUNX2 and α-SMA in human carotid arteries (calcification group n = 6, non-calcification group n = 6).

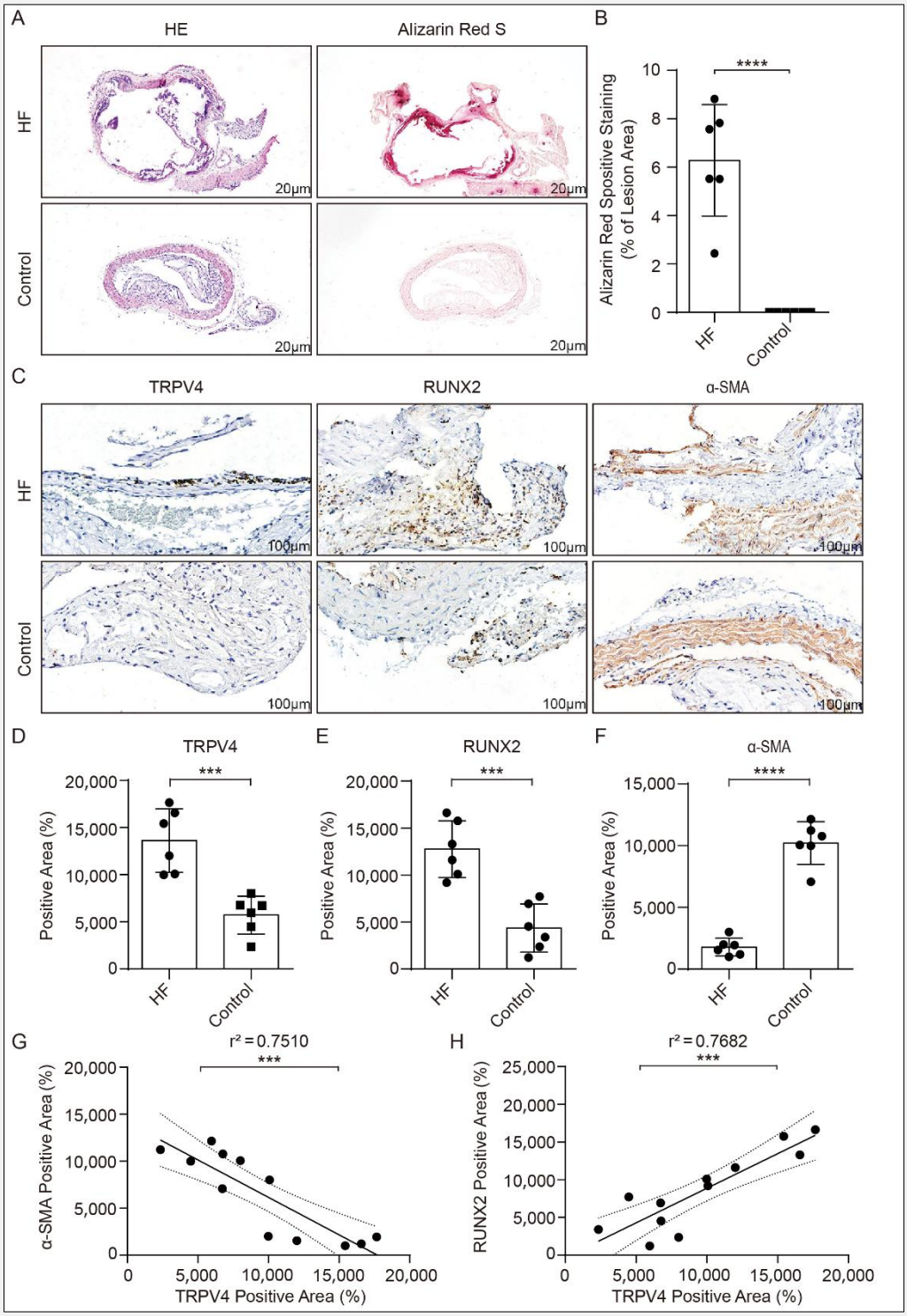

Figure 5. TRPV4 is upregulated in calcification plaque in mice carotid arteries.

A) Representative vascular calcification plaque by HE and Alizarin Red staining in mice carotid arteries. B) Statistical analysis of calcification area by Alizarin Red staining (n = 6). C) Representative immunohistochemistry staining of TRPV4, RUNX2, and α-SMA in mice carotid arteries (n = 6). D - F) Comparison of TRPV4, RUNX2, and α-SMA between the calcification group and non-calcification group. G - H) Correlation analysis of TRPV4 and vascular calcification marker RUNX2 and α-SMA in mice carotid arteries (calcification group n = 6, non-calcification group n = 6).

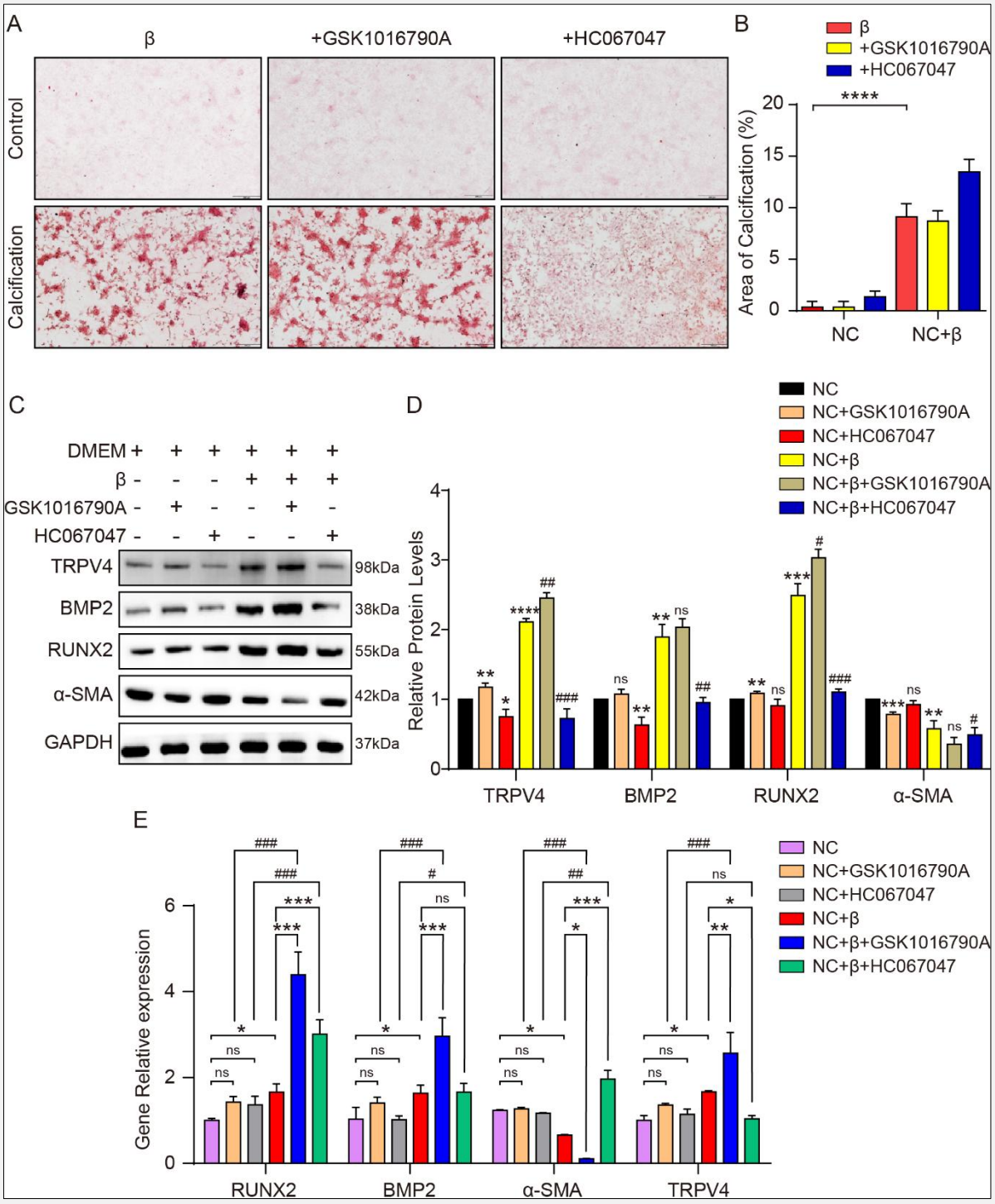

Figure 6. TRPV4 promoted β-GP-induced VSMC calcification and osteogenic differentiation.

A) Representative images of Alizarin Red staining of A7r5 VSMC with a TRPV4 agonist GSK1016790A (10 nM) and a selective TRPV4 antagonist HC067047 (10 µM) in DMEM and osteogenic medium (β-GP-induced). B) Statistical analysis of calcifying area of Alizarin Red staining with GSK1016790A (10 nM) and HC067047 (10 µM) in DMEM and osteogenic medium (β-GP-induced). C) Representative western blot analysis of TRPV4, BMP2, RUNX2, and α-Sma. D) Relative protein expression and quantification of TRPV4, BMP2, RUNX2, and α-Sma in A7r5 VSMCs with GSK1016790A (10 nM) and HC067047 (10 µM) in DMEM and osteogenic medium (n = 3). E) qPCR analysis of RUNX2, BMP2, α-Sma, and TRPV4 in VSMCs; VSMC genes expression at control conditions (first bar in each group) is defined as 1 (n = 3, * p < 0.01 relative to control conditions).

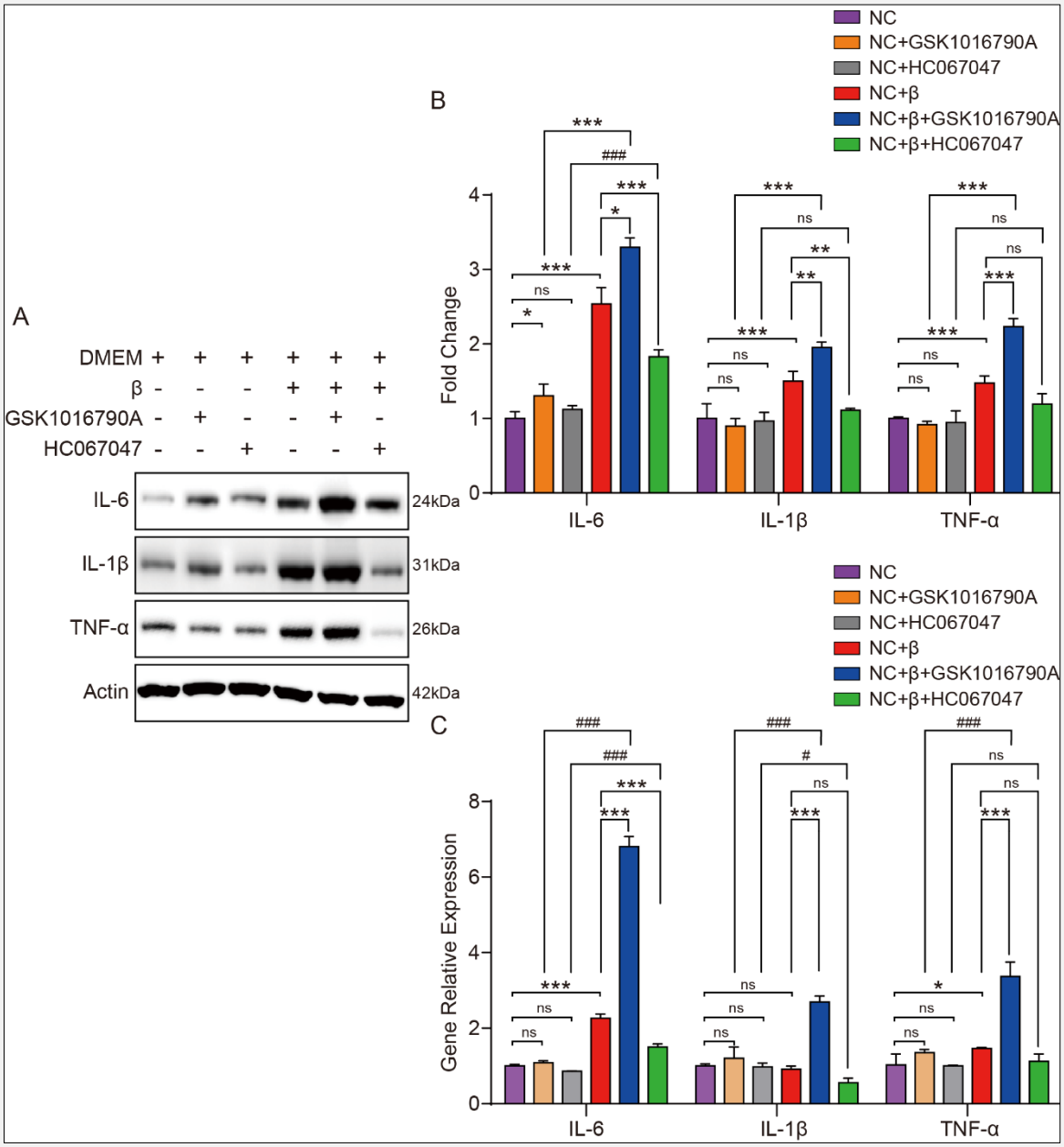

Figure 7. TRPV4 promoted β-GP-induced VSMC calcification by activation of inflammation.

A) Representative western blot analysis of IL-6, IL-1β, and TNF-α. B) Relative protein expression and quantification of IL-6, IL-1β, and TNF-α in A7r5 VSMCs with GSK1016790A (10 nM) and HC067047 (10 μM) in DMEM and osteogenic medium (n = 3). C) qPCR analysis of IL-6, IL-1β, and TNF-α in VSMC, VSMC gene expression at control conditions (first bar in each group) is defined as 1 (n = 3, * p < 0.01 relatives to control conditions).

staining (Figure 5C) revealed that TRPV4 was highly expressed in the calcified region of the carotid artery of mice compared to the non-calcified region (Figure 5D). In addition, compared to the non-calcification group, the calcification group demonstrated a higher expression of RUNX2 (Figure 5E) and a lower expression of α-SMA (Figure 5F) in the carotid artery of mice. In mice carotid artery, relative analysis revealed that TRPV4 was positively correlated with RUNX2 (Figure 5G), but negatively correlated with α-SMA (Figure 5H).

These findings imply that TRPV4 may play a crucial role in vascular calcification.

TRPV4 promotes β-glycerophosphoric acid-induced VSMC calcification and osteogenic differentiation

To determine whether TRPV4 increases vascular calcification in vitro, we treated A7r5 VSMC for 3 weeks with TRPV4 agonist GSK1016790A (10 nM) and selective TRPV4 antagonist HC067047 (10 μM) in DMEM and osteogenic medium (induced by β-glycerophosphoric acid) for 3 weeks (Figure 6A). Alizarin Red staining demonstrated that GSK1016790A could significantly increase the calcification area of A7R5 VSMCS, whereas HC067047 could significantly decrease the calcification area (**** $p < 0.0001$) (Figure 6B). The results confirmed that GSK1016790A suppressed the expression and mRNA level of smooth muscle marker genes alpha smooth muscle actin (α-SMA), while elevating the expression and mRNA level of bone-associated markers, RUNX2 and BMP2. Expression and mRNA levels of TRPV4, BMP2, and RUNX2 were downregulated by treatment, whereas expression and mRNA levels of α-Sma were upregulated in contrast to the control (Figure 6C, 6D, 6E), indicating that TRPV4 promotes osteogenic differentiation and VSMC calcification induced by β-glycerophosphoric acid.

TRPV4 promotes β-glycerophosphoric acid-induced VSMC calcification and osteogenic differentiation by activation of inflammation

To further confirm the role of TRPV4 in mediating the inflammatory pathways, we measured the levels of pro-inflammatory factors using western blot (Figure 7A) and PCR. GSK1016790A increased the protein expression and mRNA level of IL-6, IL-1β and TNF-α significantly, while HC067047 reduced the protein expression and mRNA level of IL-6 (Figure 7B, 7C). These findings suggest that TRPV4 may mediate the inflammatory pathways and that TRPV4 inhibition may have an anti-inflammatory effect.

DISCUSSION

In this study, we downloaded published vascular calcification datasets GSE136593 and GSE159832 from the GEO database and analyzed them using Partek Flow and Qiagen IPA software. Using Partek Flow analysis, we discovered significant differences in transcriptional profiles between calcified and non-calcified groups. Intimal calcification, medial calcification, and non-calcification have distinct transcriptomes. We then analyzed the data using the Qiagen IPA software. The TRPV4 gene had the most significant and consistent upregulation in calcification and non-calcification groups, as determined by an IPA summary analysis. In the calcification model, we discovered that TRPV4 may regulate inflammatory pathway by activating IL6, which may be the most important factor in the pathogenesis of vascular calcification. Moreover, IPA comparative analysis revealed that the HIF-1α signaling pathway was consistently and significantly activated in calcification relative to non-calcification. TRPV4 emerged as our primary concern after careful consideration and was investigated using *in vivo* and in vitro methods.

TRPV4 proteins are ubiquitously expressed in numerous cell types including VSMCs, and TRPV4 activity is known to be regulated by both mechanical and soluble factors [18]. Previous research demonstrated that the TRPV4 channel plays a pivotal role in the maintenance of cardiovascular homeostasis and promotes vascular smooth muscle cell proliferation and migration [19,20, 7]. Recent research shows that TRPV4 plays a protective role against atherosclerosis by inhibiting monocyte adhesion to endothelial cells and activating eNOS in endothelial cells [21,22]. Previous research has shown that the TRPV4 channel plays a crucial role in the maintenance of cardiovascular homeostasis and promotes vascular smooth muscle cell proliferation and migration [7, 19,20]. In contrast, impairment of TRPV4 channels has been linked to endothelial dysfunction, reduced macrophage foam cell generation, and vascular diseases [8,9, 11,23]. TRPV4 channels play a crucial role in the *Porphyromonas gingivalis* lipopolysaccharide-induced exacerbation of macrophage foam cell generation by modulating oxLDL uptake [24]. In this study, IPA summary analysis revealed that the TRPV4 gene had the most consistent and significant upregulation in both the calcification and non-calcification groups. For further confirmation, human carotid artery biopsies revealed that TRPV4 is upregulated and strongly associated with calcification. Immunohistochemistry analysis revealed increased RUNX2 and decreased α-SMA expression in the calcification group. Also, correlation analysis revealed a positive relationship between TRPV4 and vascular calcification markers. As anticipated, an *in vivo* experiment revealed that mice who were fed a high-fat diet had a higher level of TRPV4 expression in the calcified region compared to the non-calcified region. Meanwhile, comparative analysis revealed that TRPV4 was positively correlated with the biomarker RUNX2 and negatively correlated with α-SMA in the mice carotid artery. These findings imply that TRPV4 plays a crucial role in vascular calcification. Importantly, we confirmed in vitro that GSK1016790A can significantly increase the calcification area of A7R5 VSMCs by targeting TRPV4, while HC067047 can significantly decrease the calcification area. GSK1016790A simultaneously decreased the level of the smooth muscle marker gene α-SMA while increasing the level of the bone-associated markers RUNX2 and BMP (Figure 2D, 2E). Our findings indicate that TRPV4 promotes VSMC calcification and osteogenic differentiation induced by β-glycerophosphoric acid. Our hypothesis is supported by these results.

Vascular calcification accompanied by inflammation is a hallmark of disease progression and a strong independent risk marker for adverse coronary events [25-28].

Phenotypic shifts of arterial endothelial cells and VSMCs promote pathogenic inflammation [29,30]. TRPV4 channels are sensitized by alterations in biomechanical stimuli [1,10,31,32,33]. Scheraga et al. recently demonstrated that TRPV4 activation is necessary for LPS-induced macrophage phagocytosis and stimulation of inflammatory cytokines 33TRPV4, as a potential mediator of inflammatory/proatherogenic responses associated with the pathogenesis of periodontitis-induced atherosclerosis [34]. By regulating the release of IL-1β and IL-6, P2X7 contributes to the development of hyperalgesia in CCD mediated by TRPV4 [35]. Research has also identified IL-1 and IL-6 expression as critical cytokines [36].

CONCLUSION

The above studies indicate a link between TRPV4 and inflammation. In this study, IPA summary analysis indicated TRPV4 may regulate the inflammatory pathway by activating IL-6 in the calcification model. We observed the expression and mRNA level of IL-6, IL-1β, and TNF-α in response to the application of GSK1016 790A and HC067047. GSK1016790A significantly increased IL-6, IL-1β, and TNF-α protein expression and mRNA levels, while HC067047 significantly decreased the protein expression and mRNA level of IL-6, however there was a less obvious decrease in IL-1β and TNF-α levels. The results of our study suggest that the activation of TRPV4 promotes osteogenic differentiation of VSMCs, contributing to an increased vascular calcification in atherosclerosis. Furthermore, we uncovered the connection between TRPV4 and IL-6 in VSMCs, and the crucial role that they play in VSMC calcification. Consequently, our findings suggest that TRPV4 may mediate the inflammatory pathways through IL-6, and TRPV4 inhibition may have an anti-calcification effect.
In conclusion, we found that TRPV4 promotes VSMC osteogenic differentiation and vascular calcification in atherosclerosis. TRPV4 promotes β-glycerophosphoric acid-induced VSMC calcification and osteogenic differentiation via Il-6 activation. To determine the role of TRPV4 in regulating atherosclerotic vascular calcification *in vivo*, additional studies with SMC-specific TRPV4 knockout mice are warranted. However, a crucial molecular mechanism by which TRPV4 induces VSMC calcification by activating Il-6 has been uncovered. This provides new targets for vascular calcification management.

Source of Funds:
This work was financially supported by grants from the Natural Science Foundation of Ningxia (No. 2023AAC 03672).

Declaration of Interest:
The authors declare that they have no competing interests.

References:

1. Adapala RK, Thoppil RJ, Luther DJ, et al. TRPV4 channels mediate cardiac fibroblast differentiation by integrating mechanical and soluble signals. J Mol Cell Cardiol 2013;54:45-52. (PMID: 23142541)

2. Voelkl J, Lang F, Eckardt KU, et al. Signaling pathways involved in vascular smooth muscle cell calcification during hyperphosphatemia. Cell Mol Life Sci 2019;76(11):2077-91. (PMID: 30887097)

3. Rogers MA, Aikawa E. Cardiovascular calcification: artificial intelligence and big data accelerate mechanistic discovery. Nat Rev Cardiol 2019;16(5):261-74. (PMID: 30531869)

4. Byon CH, Sun Y, Chen J, et al. Runx2-upregulated receptor activator of nuclear factor kappaB ligand in calcifying smooth muscle cells promotes migration and osteoclastic differentiation of macrophages. Arterioscl Throm Vas Biol 2011;31(6):1387-96. (PMID: 21454810)

5. Yang L, Dai R, Wu H, et al. Unspliced XBP1 Counteracts beta-Catenin to Inhibit Vascular Calcification. Circ Res 2022;130(2): 213-29. (PMID: 34870453)

6. Song X, Sun Z, Chen G, et al. Matrix stiffening induces endothelial dysfunction via the TRPV4/microRNA-6740/endothelin-1 mechanotransduction pathway. Acta Biomater 2019;100:52-60. (PMID: 31606530)

7. Gupta N, Goswami R, Alharbi MO, Biswas D, Rahaman SO. TRPV4 is a regulator in P. gingivalis lipopolysaccharide-induced exacerbation of macrophage foam cell formation. Physiol Rep 2019;7(7):e14069. (PMID: 30980509)

8. Zhang DX, Mendoza SA, Bubolz AH, et al. Transient receptor potential vanilloid type 4-deficient mice exhibit impaired endothelium-dependent relaxation induced by acetylcholine in vitro and *in vivo*. Hypertension 2009;53(3):532-8. (PMID: 19188524)

9. Ye L, Kleiner S, Wu J, et al. TRPV4 is a regulator of adipose oxidative metabolism, inflammation, and energy homeostasis. Cell 2012;151(1):96-110. (PMID: 23021218)

10. Goswami R, Cohen J, Sharma S, et al. TRPV4 ION Channel Is Associated with Scleroderma. J Invest Dermatol 2017;137(4): 962-5. (PMID: 27889423)

11. Du J, Wang X, Li J, et al. Increasing TRPV4 expression restores flow-induced dilation impaired in mesenteric arteries with aging. Sci Rep 2016;6:22780. (PMID: 26947561)

12. Yang Y, Sun Y, Chen J, et al. AKT-independent activation of p38 MAP kinase promotes vascular calcification. Redox Biol 2018; 16:97-103. (PMID: 29495001)

13. Deng L, Huang L, Sun Y, Heath JM, Wu H, Chen Y. Inhibition of FOXO1/3 promotes vascular calcification. Arterioscl Throm Vas Biol 2015;35(1):175-83. (PMID: 25378413)

14. Jin M, Wu Z, Chen L, et al. Determinants of TRPV4 activity following selective activation by small molecule agonist GSK1016790A. PloS One 2011;6(2):e16713. (PMID: 21339821)

15. Dahan D, Ducret T, Quignard JF, Marthan R, Savineau JP, Esteve E. Implication of the ryanodine receptor in TRPV4-induced calcium response in pulmonary arterial smooth muscle cells from normoxic and chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 2012;303(9):L824-33. (PMID: 22962011)

16. Greenland P, Yano Y, Lloyd-Jones DM. Coronary Calcium Score and Cardiovascular Risk in Elderly Populations-Reply. JAMA Cardiol 2018;3(2):180-1. (PMID: 29188289)

17. Hutcheson JD, Goettsch C. Cardiovascular Calcification Heterogeneity in Chronic Kidney Disease. Circ Res 2023;132(8):993-1012. (PMID: 37053279)

18. Benjamin EJ, Blaha MJ, Chiuve SE, et al. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation 2017;135(10):e146-603. (PMID: 28122885)

19. Planas-Rigol E, Terrades-Garcia N, Corbera-Bellalta M, et al. Endothelin-1 promotes vascular smooth muscle cell migration across the artery wall: a mechanism contributing to vascular remodelling and intimal hyperplasia in giant-cell arteritis. Ann Rheum Dis 2017;76(9):1624-34. (PMID: 28606962)

20. Huetsch JC, Walker J, Undem C, et al. Rho kinase and Na(+)/H(+) exchanger mediate endothelin-1-induced pulmonary arterial smooth muscle cell proliferation and migration. Physiol Rep 2018;6(9):e13698. (PMID: 29756391)

21. Xu S, Liu B, Yin M, et al. A novel TRPV4-specific agonist inhibits monocyte adhesion and atherosclerosis. Oncotarget 2016; 7(25):37622-35. (PMID: 27191895)

22. Wang X, Wang Y, Huo H, et al. Transient Receptor Vanilloid Subtype 4-Mediated Ca(2+) Influx Promotes Glomerular Endothelial Inflammation in Sepsis-Associated Acute Kidney Injury. Lab Invest 2023;103(6):100126. (PMID: 36889540)

23. Goswami R, Merth M, Sharma S, et al. TRPV4 calcium-permeable channel is a novel regulator of oxidized LDL-induced macrophage foam cell formation. Free Radical Bio Med 2017;110: 142-50. (PMID: 28602913)

24. Gupta N, Goswami R, Alharbi MO, Biswas D, Rahaman SO. TRPV4 is a regulator in P. gingivalis lipopolysaccharide-induced exacerbation of macrophage foam cell formation. Physiol Rep 2019;7(7):e14069. (PMID: 30980509)

25. Skenteris NT, Seime T, Witasp A, et al. Osteomodulin attenuates smooth muscle cell osteogenic transition in vascular calcification. Clin Transl Med 2022;12(2):e682. (PMID: 35184400)

26. Liu SF, Nambiar VN, Li Q, Kucherenko MM, Knosalla C, Kuebler WM. Pulmonary hypertension: Linking inflammation and pulmonary arterial stiffening. Front Immunol 2022;13: 959209. (PMID: 36275740)

27. Turner ME, Bartoli-Leonard F, Aikawa E. Small particles with large impact: Insights into the unresolved roles of innate immunity in extracellular vesicle-mediated cardiovascular calcification. Immunol Rev 2022;312(1):20-37. (PMID: 36089771)

28. Dzobo KE, Kraaijenhof JM, Stroes E, Nurmohamed NS, Kroon J. Lipoprotein(a): An underestimated inflammatory mastermind. Atherosclerosis 2022;349:101-9. (PMID: 35606070)

29. Niu Y, Zhang W, Yang Z, et al. Association of plasma osteoprotegerin levels with the severity of lower extremity arterial disease in patients with type 2 diabetes. BMC Cardiovasc Disord 2015; 15:86. (PMID: 26260869)

30. Fukuoka K, Sawabe A, Sugimoto T, et al. Inhibitory actions of several natural products on proliferation of rat vascular smooth muscle cells induced by Hsp60 from Chlamydia pneumoniae J138. J Agr Food Chem 2004;52(20):6326-9. (PMID: 15453708)

31. Liedtke W, Tobin DM, Bargmann CI, Friedman JM. Mammalian TRPV4 (VR-OAC) directs behavioral responses to osmotic and mechanical stimuli in Caenorhabditis elegans. P Natl Acad Sci USA 2003;100 Suppl 2(Suppl 2):14531-6. (PMID: 14581619)

32. Liedtke W, Friedman JM. Abnormal osmotic regulation in trpv4-/- mice. P Natl Acad Sci USA 2003;100(23):13698-703. (PMID: 14581612)

33. Sharma S, Goswami R, Merth M, et al. TRPV4 ion channel is a novel regulator of dermal myofibroblast differentiation. Am J Physiol Cell Physiol 2017;312(5):C562-72. (PMID: 28249987)

34. Scheraga RG, Abraham S, Niese KA, et al. TRPV4 Mechanosensitive Ion Channel Regulates Lipopolysaccharide-Stimulated Macrophage Phagocytosis. J Immunol 2016;196(1):428-36. (PMID: 26597012)

35. Yang C, Si M, Zhou J. Silencing TRPV4 partially reverses the neurotoxic effects caused by excess Ketamine. J Toxicol Sci 2021;46(2):69-81. (PMID: 33536391)

36. Boltana S, Sanhueza N, Donoso A, et al. The expression of TRPV channels, prostaglandin E2 and pro-inflammatory cytokines during behavioural fever in fish. Brain Behav Immun 2018;71:169-81. (PMID: 29574261)