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# Roles of Immune and Oxidative Stress-Related Factors in the Diagnosis of Coronary Artery Disease: A Retrospective Study

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## Abstract

**Background:** Coronary artery disease (CAD) is one of the main causes of sudden death, but its exact pathogenesis requires further study. Thus, this study aimed to explore the immune and oxidative stress-related factors in CAD progression and their roles in CAD diagnosis. **Methods:** Bioinformatics analysis was used in this study, and the GSE23561 dataset (training set) we used contained the transcriptome sequencing results of six CAD peripheral blood samples and nine control samples. The data were obtained and analysed by querying the Gene Expression Omnibus database. First, the differentially expressed immune and oxidative stress-related genes (DEIOGs) between the groups were identified. DEIOGs were then analysed based on Gene Ontology annotation and Kyoto Encyclopedia of Genes and Genomes pathway enrichment. A protein–protein interaction (PPI) network for DEIOGs was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins database, and hub genes were identified through the PPI network. Moreover, transcription factors and microRNAs (miRNAs) targeting hub genes were identified to explore the potential regulatory mechanisms of hub genes. The receiver operating characteristic (ROC) curve analysis was constructed to examine the role of hub genes in CAD diagnosis. Finally, the data of GSE23561 (validated set) were used to validate the diagnostic potential of these hub genes. **Results:** Primarily, 66 DEIOGs were identified, which are involved in many important pathways related to CAD, such as the “mitogen-activated protein kinase (MAPK) signalling pathway” and “lipid and atherosclerosis”. A PPI network of DEIOGs was then constructed, and 10 hub genes were identified sequentially. A total of 37 transcription factors and 481 miRNAs that played important roles in hub genes regulation were identified. The ROC curves indicated that five special hub genes (*Fos*, *Il6*, *Jun*, *Mapk3*, and *Mmp9*) could serve as potential diagnostic biomarkers for CAD. **Conclusions:** Bioinformatics analysis technology was used to identify 10 hub DEIOGs that might play a crucial role in CAD progression, and five spe-

cial hub genes (*Fos*, *Il6*, *Jun*, *Mapk3*, and *Mmp9*) could be regarded as potential biomarkers for CAD diagnosis. However, further studies are required to verify the functions of these hub genes.

## Keywords

coronary artery disease; immune; oxidative stress; biomarkers; coronary artery disease diagnosis; bioinformatics analysis technology

## Introduction

Coronary artery disease (CAD) is caused by atherosclerotic plaques, which lead to coronary artery stenosis or even obstruction, leading to myocardial ischaemia, hypoxia, and necrosis [1]. Currently, CAD is one of the most severe diseases worldwide, with a predicted 12 million deaths by the end of 2030. Common risk factors for CAD are hypertension, hyperlipidaemia, diabetes, and smoking. However, they account for only two-thirds of patients with CAD [2]. Therefore, further studies on CAD pathogenesis are necessary.

At present, four hypotheses have been proposed to explain the pathogenesis of CAD: the lipid infiltration theory, endothelial injury theory, platelet aggregation and thrombosis hypothesis, and smooth muscle cell clone theory [3]. Regardless of the theory, infiltration of inflammatory cells and release of inflammatory mediators play important roles in CAD progression [4]. Oxidative stress refers to the imbalance between reactive oxygen species production and antioxidative defences. When patients are under oxidative stress, metabolites, such as reactive oxygen species and reactive nitrogen species, increase, leading to the activation and infiltration of inflammatory cells, such as centrioles, causing structural and functional damage to the cardiovascular system. Therefore, oxidative stress and the inflammatory response may work together and play an important role in CAD progression [5].

Considering CAD pathogenesis, in the present study, we used bioinformatics analysis technology to analyse the transcriptome sequencing results of CAD peripheral blood samples, analyse the abnormal expression of immune- and oxidative stress-related genes in patients with CAD, identify the immune- and oxidative stress-related hub genes that play a key role in CAD progression, and preliminarily explore the role of these hub genes in diagnosing CAD.

## Materials and Methods

### Data Source

Gene expression profile data of patients with CAD were obtained from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). GSE23561 was used as training set, which contained the transcriptome sequencing results of six CAD peripheral blood samples and nine control samples. GSE166780 was used as the validation set, which contained the transcriptome sequencing results of 16 CAD peripheral blood samples and 8 normal coronary artery samples (control samples). CAD diagnosis was mainly based on coronary angiography. The patients with coronary artery stenosis were aligned to the disease group, while the patients without coronary artery stenosis were aligned to the control group. The training set was used to identify hub genes, construct the transcription factor (TF)–gene–miRNA regulatory network, and explore the sensitivity and specificity of hub genes in CAD diagnosis. The validation dataset was used to validate the diagnostic potential of the hub genes. The dataset was obtained using the GPL10775 platform (Human 50K Exonic Evidence-Based Oligonucleotide array). Moreover, 2533 immune-related genes were obtained from the Immport (<http://www.immport.org/>) and Innatedb (<http://www.innatedb.com>) databases, while 436 oxidative stress-related genes were obtained from “GOBP\_RESPONSE\_TO\_OXIDATIVE\_STRESS (GO: 0006979)” in the Gene Ontology (GO) database [6]. **Supplementary Fig. 1** illustrates the study strategy.

### Identification of Differentially Expressed Genes (DEGs)

The “limma” R package (v3.50.1; Matthew E Ritchie, Victoria, Australia) was used to obtain DEGs between the CAD (n = 6) and control (n = 9) groups [7]. The screening standard was set at an adjusted *p*-value (adj. *p*) < 0.05. Volcano and cluster heatmaps were constructed to visualise the DEGs.

### Functional Enrichment of Differentially Expressed Immune and Oxidative Stress-Related Genes (DEIOGs)

The DEIOGs were obtained by assessing overlapping DEGs between CAD and control groups, immune-related

genes, and oxidative stress-related genes. Then, Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were implemented using the “clusterProfiler” (v4.0.5) R package (Tianzhi Wu, Guangzhou, China) [8]. GO annotation was separated into biological processes, cell components, and molecular functions. GO terms and KEGG pathways with adj. *p* < 0.05 were significant.

### Protein–Protein Interaction (PPI) Network Construction and Hub Gene Identification

The proteins encoded by the DEIOGs were used to construct the PPI interaction network using the Search Tool for the Retrieval of Interacting Genes (STRING) database [9]. First, the proteins encoded by the DEIOGs were typed into the database. Then, according to the node degree, the top 10 proteins were identified using CytoHubba (Chia-Hao Chin, Taiwan, China), which is a plugin for the Cytoscape software (v3.9.1; Paul Shannon, Washington, USA) [10]. Genes encoding these proteins were identified as hub genes for CAD. After that, hub gene expression in the CAD and control groups was compared.

### Prediction of the Transcription Factor and microRNA (TF–Gene–miRNA) Regulatory Network

Gene expression is regulated by multiple TFs and microRNAs (miRNAs), all of which form a complex regulatory network. To further explore the transcriptional and post-transcriptional regulation of the top 10 hub genes, the JASPAR (<http://jaspar.genereg.net/>) and miRNet online tools (<https://www.mirnet.ca>) were used to predict the TFs and miRNAs targeting hub genes [11,12]. A TF–gene–miRNA regulatory network was constructed and visualised using Cytoscape software.

### Receiver Operating Characteristic (ROC) Analysis of Hub Genes

ROC analysis was used to examine the sensitivity and specificity of hub genes in CAD diagnosis. The ROC curve was plotted, and the area under the curve (AUC) was calculated by the “pROC” R package (v1.18.0; Xavier Robin, Geneva, Switzerland) [13]. Typically, the AUC of the ROC analysis ranges from 0.5 to 1, with near 1 indicating perfect predictive ability and 0.5 indicating no predictive ability.

### Validated the Diagnostic Potential of Hub Genes

The hub gene expression data from GSE166780 were used to plot ROC curves, which were compared to those of hub genes from GSE23561 to validate the diagnostic potential of the hub genes.

## Statistical Analysis

Open databases and R (v4.1.0; Robert Gentleman and Ross Ihaka, Auckland, New Zealand) were used to analyse and visualise the data. Boxplots were plotted in the ggplot2 (v3.3.5; Hadley Wickham, Houston, USA) package. Heat maps were drawn using the Pheatmap (v1.0.12; Raivo Kolde, Tartu, ESTONIA) package. Venn diagrams were drawn using the Ggvenn (v0.1.9; Linlin Yan, Nanjing, China) package. Differences between two groups were analysed using the Wilcoxon test. A  $p$ -value  $< 0.05$  was considered significant unless otherwise stated.

## Results

### DEG Identification

In the GSE23561 dataset used in this study, the peripheral blood samples of CAD were compared with the control samples to obtain 9485 DEGs, of which 9159 genes were significantly upregulated, and 326 genes were significantly downregulated. Fig. 1A shows a volcano map of the DEGs. The top 10 upregulated and downregulated DEGs (sorted by  $\log_2FC$ ) were identified, and a cluster heatmap was constructed (Fig. 1B). The top 10 upregulated DEGs were *Muc2*, *Cog7*, *Hbg1*, *Lgi4*, *Slc9a3*, *Hba1*, *Adamts13*, *Hba2*, *Hbb*, and *Znf500*. The top 10 downregulated DEGs were *Krtap6-3*, *Slc1a5*, *Pcgf6*, *Pam*, *Plcb3*, *Nudc*, *P2ry11*, *Fam78a*, *Parp10*, and *Ndufb7*.

### Functional enrichment Analysis of DEIOGs

A total of 66 overlapping immune- and oxidative stress-related genes from the DEGs were identified and retained as DEIOGs for subsequent analysis (Fig. 1C). To obtain deeper insight into the biological roles of these DEIOGs, GO annotation and KEGG pathway enrichment analyses were performed (Supplementary Table 1). The GO annotation showed that: (1) The top 5 significantly enriched GO biological process terms were “response to oxidative stress”, “cellular response to chemical stress”, “cellular response to oxidative stress”, “response to reactive oxygen species”, and “cellular response to reactive oxygen species”. (2) For GO-cell component analysis, the top 5 significantly enriched terms were “membrane raft”, “membrane microdomain”, “focal adhesion”, “plasma membrane raft”, and “caveola”. (3) For GO-molecular function analysis, these DEIOGs were markedly enriched in “RNA polymerase II-specific DNA-binding transcription factor binding”, “DNA-binding transcription factor binding”, “growth factor receptor binding”, “heme binding”, and “tetrapyrrole binding” (Fig. 1D). In addition, in the KEGG enrichment analysis, the markedly enriched pathways of these DEIOGs were “mitogen-activated protein kinase (MAPK) signalling pathway”, “lipid and atherosclerosis”, “fluid shear stress

and atherosclerosis”, “hepatitis B”, and “Kaposi sarcoma-associated herpesvirus infection” (Fig. 1E).

### PPI Network Construction and Hub Gene Identification

The proteins encoded by these DEIOGs were used to construct the PPI network based on the data from the STRING database analysis (Fig. 2A). According to the node degree of the PPI network, 10 proteins were identified: CASP3, EGFR, FOS, IL-10, IL-6, JUN, MAPK3, MMP9, SRC, and TP53 (Fig. 2B). There was a close correlation among all these proteins, and the genes encoding them were identified as hub genes. The expression of these hub genes was significantly different between the CAD and control groups (Fig. 2C,D and Table 1).

**Table 1. Comparison of the expression of hub genes between groups.**

Group	<i>Casp3</i>	<i>Egfr</i>	<i>Fos</i>	<i>Il10</i>	<i>Il6</i>
CAD vs. Control ( $p$ -value)	0.025	0.029	0.025	0.005	0.024
Group	<i>Jun</i>	<i>Mapk3</i>	<i>Mmp9</i>	<i>Src</i>	<i>Tp53</i>
CAD vs. Control ( $p$ -value)	0.020	0.026	0.020	0.011	0.021

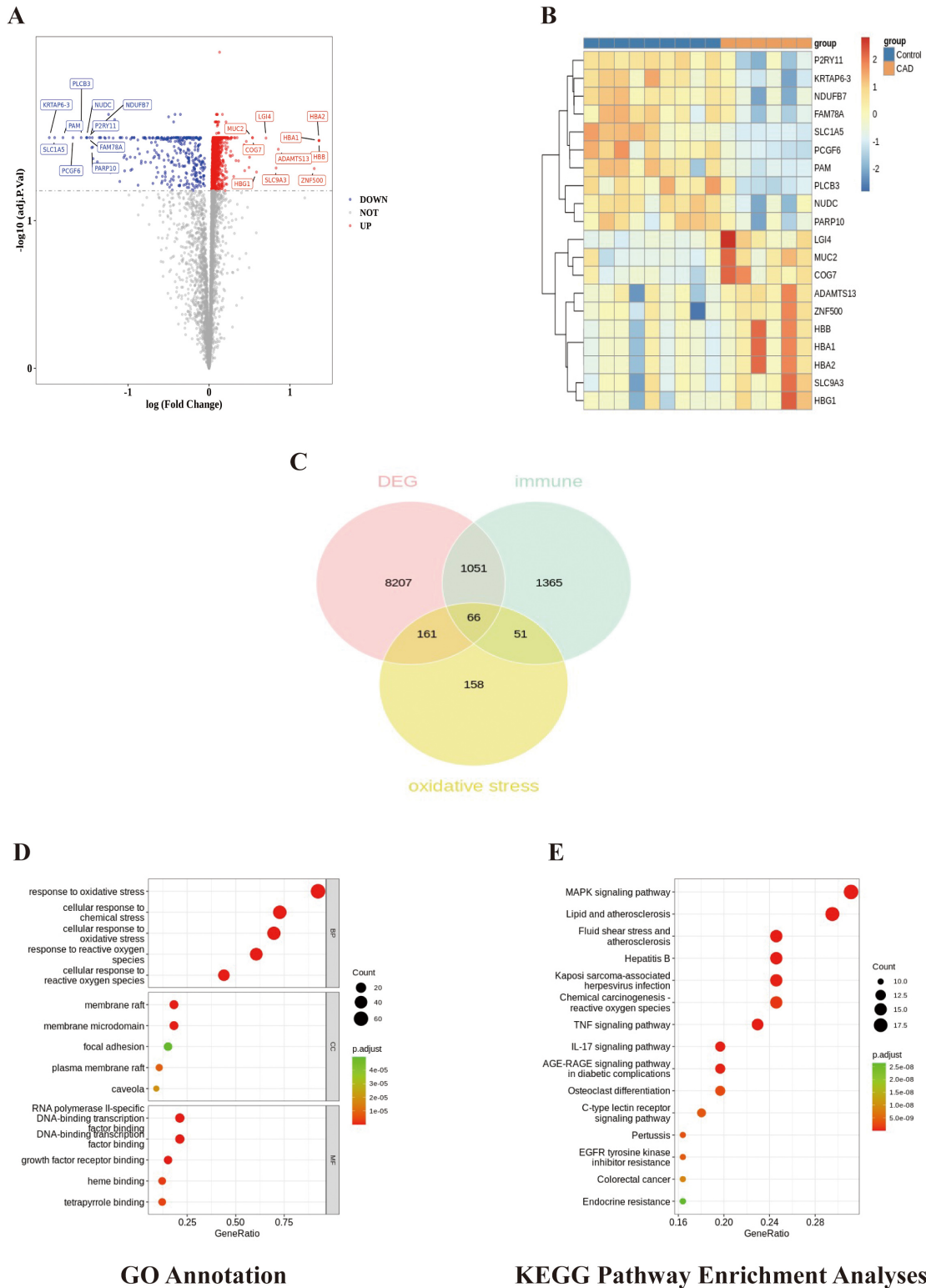
CAD, coronary artery disease.

### TF-Gene-miRNA Regulatory Network

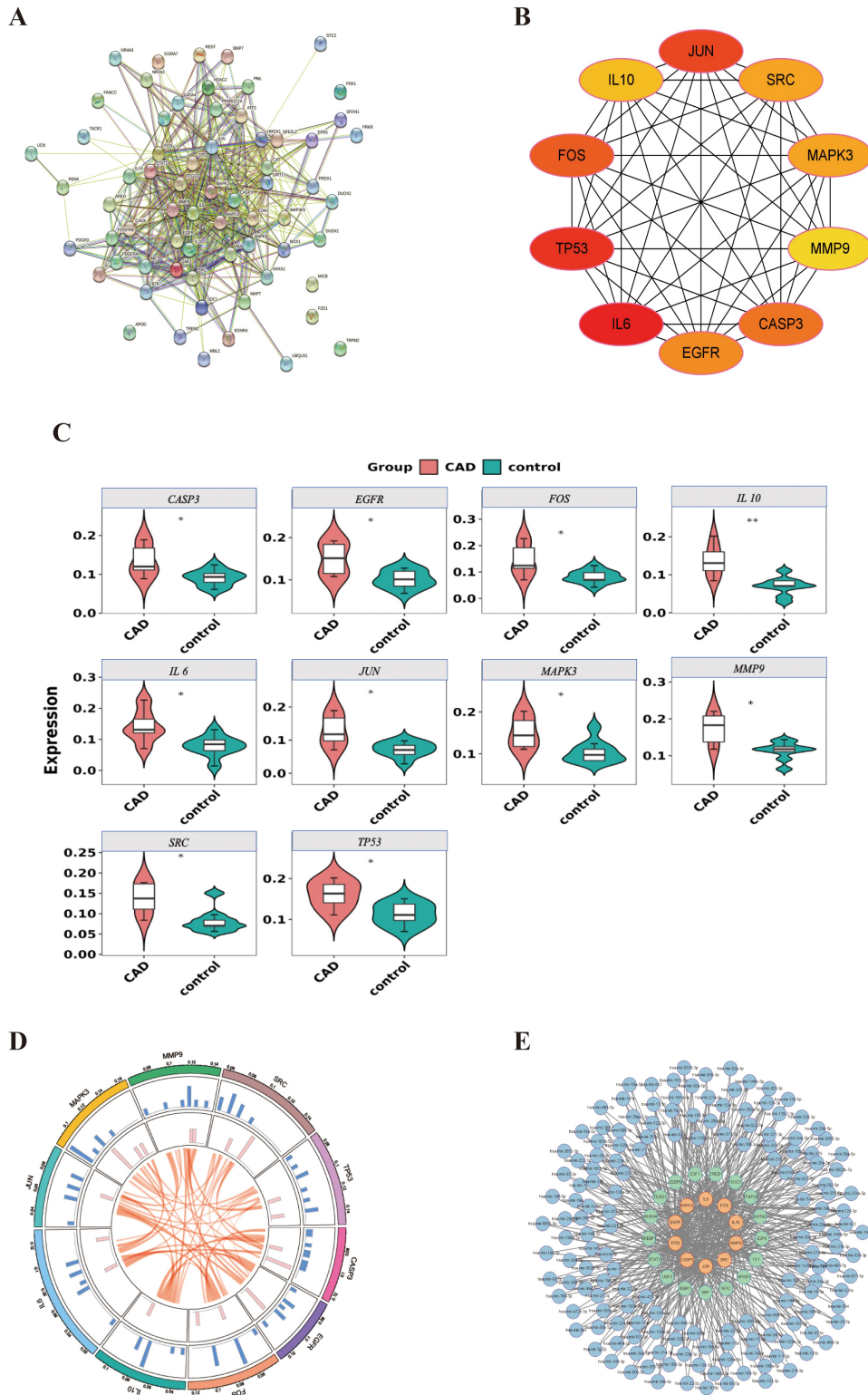
To further explore the expression regulatory network of hub genes, the JASPAR and miRNet online tools were used to construct a TF-gene-miRNA regulatory network composed of 37 TFs, 481 miRNAs, and 10 hub genes (Fig. 2E and Supplementary Table 2). A sub-regulatory network of a single hub gene was also constructed to show the regulatory networks of different hub genes (Supplementary Fig. 2). The expression of these 10 hub genes was found to be regulated not only by different TFs and miRNAs but also by each other. In addition, among these 10 hub genes, *Tp53* was the gene most targeted by both TFs and miRNAs.

### Function of Hub Genes in CAD Diagnosis

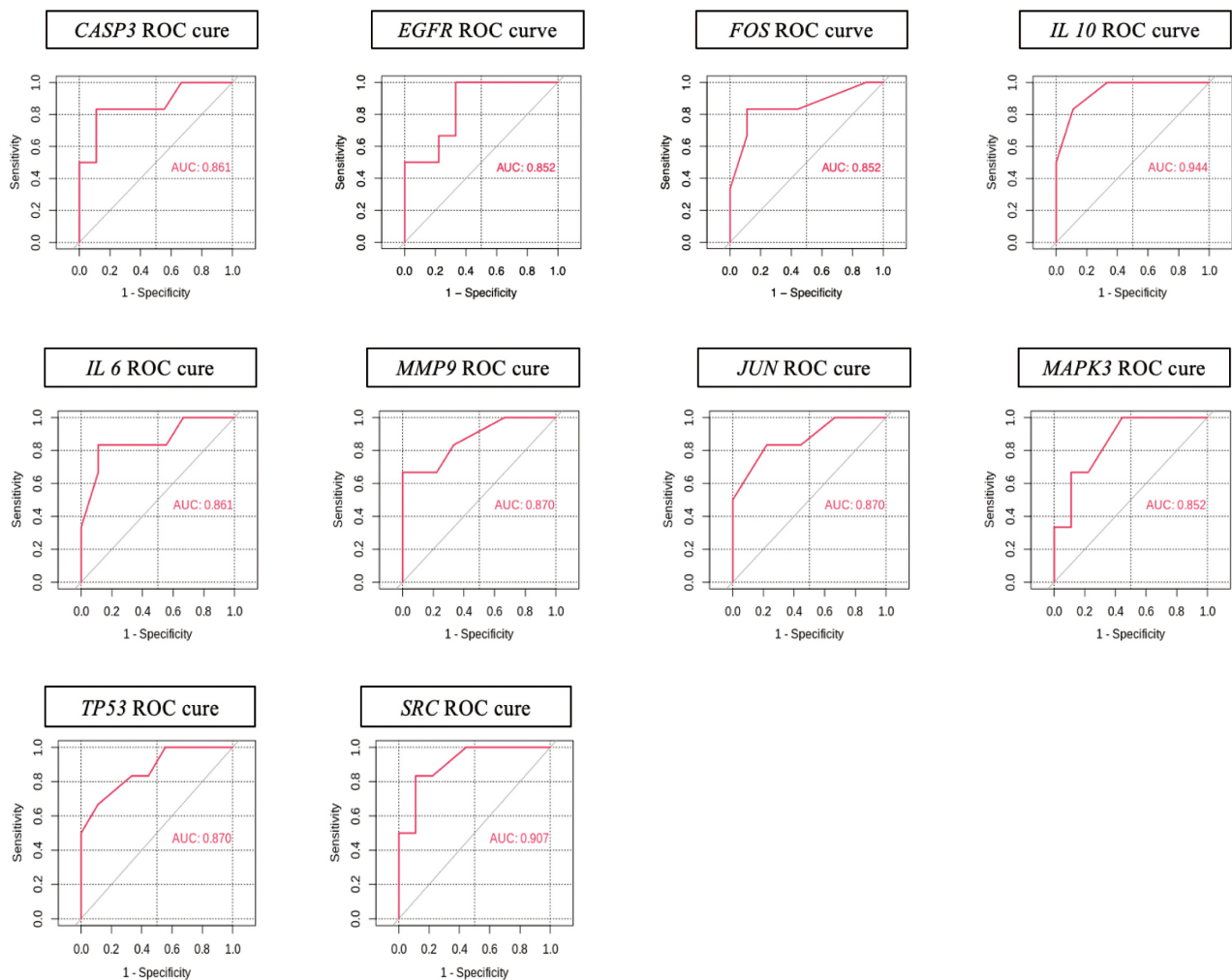
ROC analysis is a common method used to evaluate the effects of diagnostic markers [14]; therefore, it was used to evaluate the function of the hub genes in CAD diagnosis. ROC analysis showed that the AUCs of all 10 hub genes were  $>0.85$ . Therefore, all 10 hub genes could sensitively distinguish the CAD samples from the control samples. Fig. 3 shows the ROC curves of these hub genes.



**Fig. 1. Identification of DEIOGs.** (A) Volcanic map of genes expression between CAD and control groups. The 10 most upregulated and downregulated genes were marked. (B) Heatmap of these 20 DEGs between the CAD and control groups. (C) Venn diagram: a total of 66 DEIOGs were identified. (D) Bubble chart of GO annotation. (E) Bubble chart of KEGG enrichment analysis: most of the DEIOGs were related to MAPK signalling pathway, lipid metabolism, and atherosclerosis formation. CAD, coronary artery disease; DEGs, differentially expressed genes; DEIOGs, differentially expressed immune and oxidative stress-related genes; MAPK, mitogen-activated protein kinase; GO, Gene Ontology.



**Fig. 2. Identification of hub DEIOGs.** (A) The PPI network constructed by the proteins encoded by DEIOGs. (B) Ten proteins were identified according to node degree of the PPI network, and the genes encoding these proteins were regarded as hub genes. (C) Violin plot of hub genes: the expression of these 10 hub genes between CAD and control group were significantly different. (D) Circos map of hub genes. From outside to inside: the first circle is the name of different hub genes, the second circle is the expression level of hub genes in the control group, the third circle is the expression level of hub genes in the CAD group, and the innermost circle represents the connection between various hub genes. (E) The regulation network of hub genes. \* $p < 0.05$ ; \*\* $p < 0.01$ ; PPI, protein-protein interaction; TFs, transcription factors; miRNAs, microRNAs.



**Fig. 3. ROC curve of the different hub genes.** The AUC of these hub genes were all above 0.70. ROC, receiver operating characteristic; AUC, area under curve.

### Clinical Correlation Analysis and Validation of Hub Genes

To explore the relationship between clinical factors (age and sex) and the expression of hub genes, the samples in GSE23561 were divided into two groups according to age ( $\leq 51$  and  $> 51$ ) and sex (female and male) (see Table 2). The results demonstrated that in the group divided by sex, there were no significant differences except in IL-10 expression, which was significantly higher in males than in females ( $p = 0.0455$ , Fig. 4A). In the group divided by age, there were no significant differences in hub gene expression between the ages ( $p > 0.05$ , Fig. 4B). Therefore, we can preliminarily rule out the influence of these confounding factors on the results. However, owing to the limited sample size, this conclusion needs to be verified by future large-scale clinical studies. In addition, the diagnostic potential of hub genes was validated using GSE166780. The ROC curve showed AUCs for *Fos*, *Il6*, *Jun*, *Mapk3*, and *Mmp9*  $> 0.70$ . That means that all these five hub genes could sensitively distinguish CAD samples from control samples. Combining the results of the training and valida-

tion sets indicated that *Fos*, *Il6*, *Jun*, *Mapk3*, and *Mmp9* are potential CAD diagnostic biomarkers. The expression data and ROC curves of hub genes in GSE166780 are shown in **Supplementary Table 3** and Fig. 5.

**Table 2. Age and sex of samples in GSE23561.**

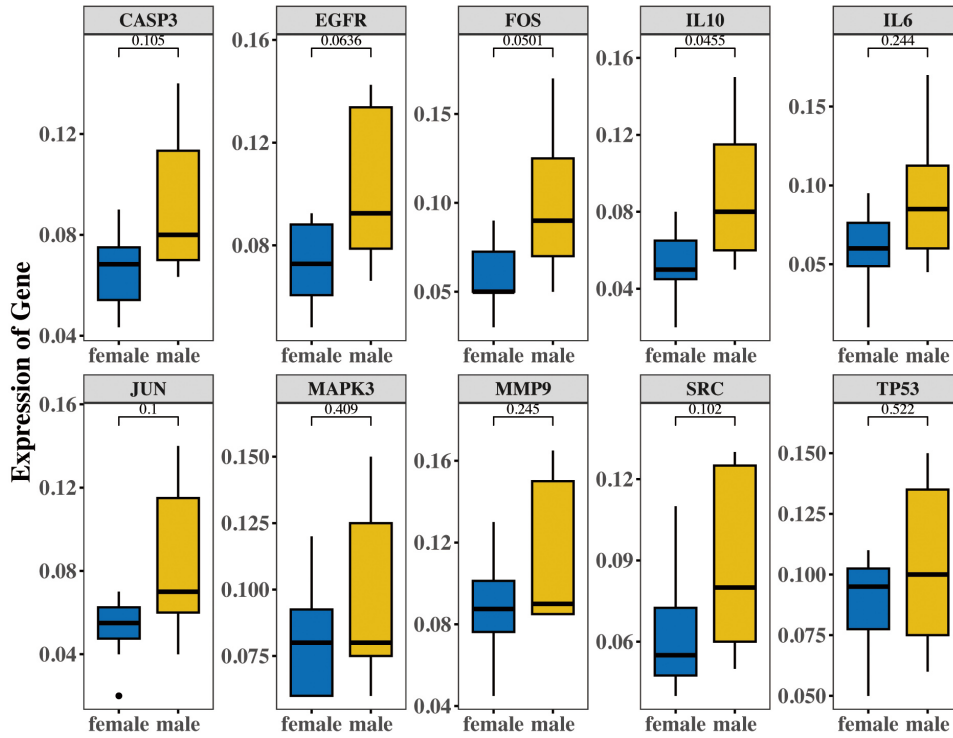
Group	Patients	Sex	Age (y)
CAD	6	Female: 1 (16.7%); Male: 5 (83.3%)	56.00 $\pm$ 11.04
Control	9	Female: 7 (77.8%); Male: 2 (22.2%)	46.44 $\pm$ 9.81
<i>p</i> value	-	0.041	0.102

CAD, coronary artery disease.

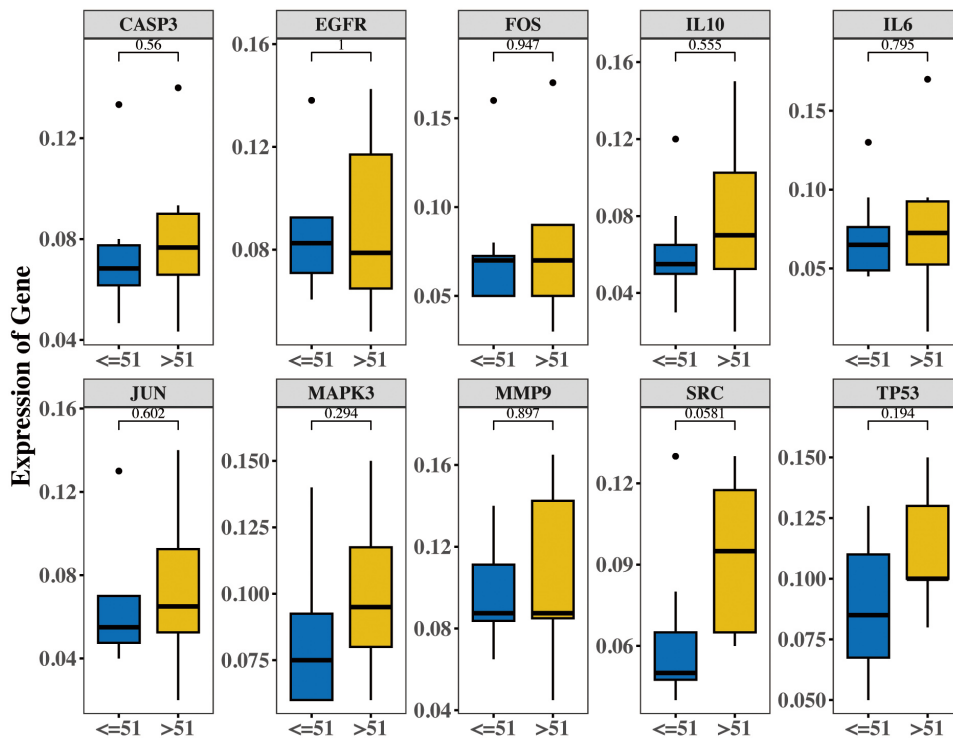
### Discussion

Abnormal coronary artery inflammation leads to vascular endothelial cell injury, inflammatory cell infiltration, and atheromatous plaque formation [4]. Oxidative

**A**

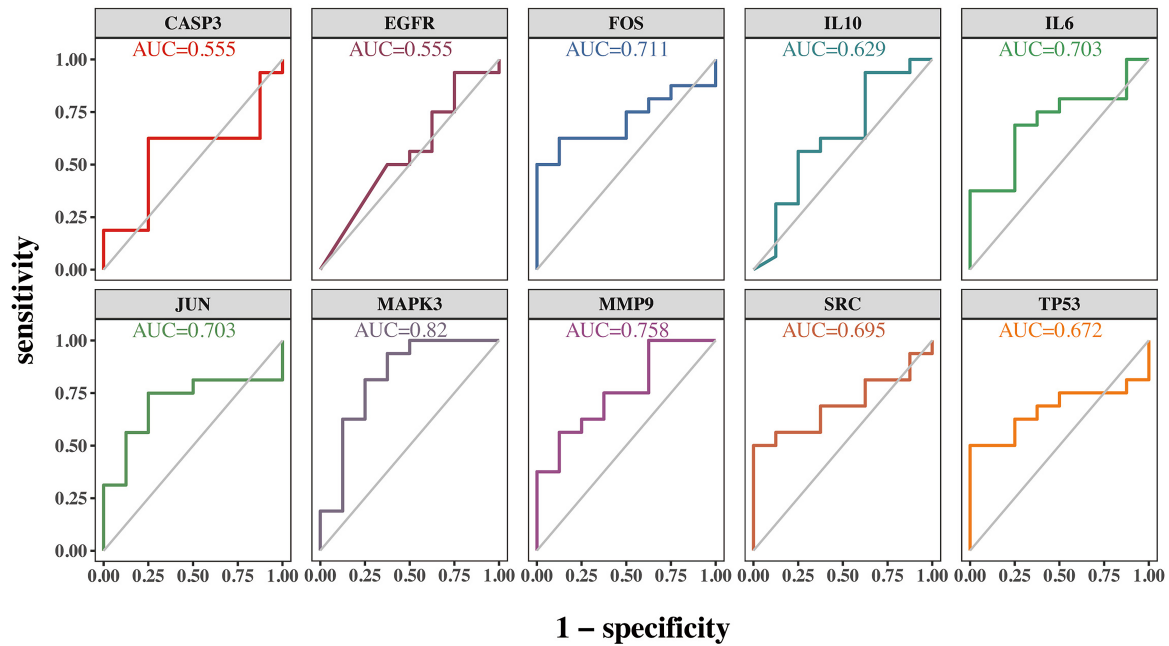


**B**



**Fig. 4. Expression of hub genes in different groups.** (A) For groups divided by sex, there were no significant differences except for *Il10* expression, which was significantly higher in males than in females ( $p = 0.0455$ ). (B) For groups divided by age ( $\leq 51$  and  $> 51$ ), there were no significant differences in hub gene expression between different groups ( $p > 0.05$ ).

## GSE166780



**Fig. 5. ROC curve of the different hub genes according to validation set.** The AUCs of *Fos*, *Il6*, *Jun*, *Mapk3*, and *Mmp9* were  $>0.70$ . ROC, receiver operating characteristic; AUC, area under curve.

stress refers to the imbalance between oxidation and anti-oxidation, which leads to coronary endothelial cell injury and atheromatous plaque formation [5]. Although correlations among inflammation, oxidative stress, and CAD have been reported, most studies investigated such correlations separately. However, oxidative stress has been reported to increase in various oxidative products, which in turn, activates inflammatory cells and leads to abnormal inflammation [5]. Therefore, we speculated that inflammation and oxidative stress have a synergistic effect on the progression of CAD. This study was designed to explore the synergistic effects of inflammation and oxidative stress and identify hub genes involved in their regulation. A total of 66 DEIOGs were identified, most of which were involved in MAPK signalling pathway, lipid and atherosclerosis, fluid shear stress, and atherosclerosis. This result is consistent with a previous study that abnormal oxidative stress and MAPK signalling pathway activation can aggravate myocardial ischaemic injury and accelerate CAD progression [15]. To further explore the synergistic effect of inflammation and oxidative stress on CAD progression, a PPI network was constructed according to the DEIOGs, and 10 hub genes were identified.

The main functions of these 10 identified hub genes were as follows: (1) *Casp3*: CASP3 is a cysteine-aspartic acid protease that plays an important role in cell apoptosis. Abnormal CASP3 expression leads to ischaemia [16]. (2) *Egfr*: EGFR is a receptor for members of the epidermal growth factor family. Abnormal *EGFR* expression is closely correlated with inflammation [17]. (3) *Fos*: FOS

have been implicated in cell proliferation and differentiation regulators. Abnormal FOS expression has also been associated with cell apoptosis [18]. (4) *Il10*: IL-10 has a profound anti-inflammatory function. Therefore, IL-10 may have a protective effect on CAD by inhibiting the abnormal inflammation [19]. (5) *Il6*: IL-6 is primarily produced at sites of inflammation, where it is secreted into the serum and induces a transcriptional inflammation [20]. (6) *Jun*: JUN plays an important role in cell death induced by active T cells [21,22]. (7) *Mapk3*: MAPK3 functions as an essential component of the MAPK signal transduction pathway. Abnormal MAPK signal transduction pathway activation aggravates myocardial ischaemic injury and accelerates CAD progression [23]. (8) *Mmp9*: MMP9s are members of the matrix metalloproteinase family, which is involved in the breakdown of the extracellular matrix [24]. (9) *Src*: Overexpression of SRC can activate the reactive oxygen species system, induce oxidative stress and inflammation through oxidative products, and further aggravate CAD [25,26]. (10) *Tp53*: TP53 overexpression can aggravate the apoptosis of coronary endothelial cells and impair cardiac function in patients with CAD [27,28]. Subsequently, the regulatory mechanisms of these hub genes were analysed, and a TF-gene-miRNA regulatory network targeting these hub genes was constructed. Through the analysis of this network, we found that *Tp53* was the most targeted gene by both TFs and miRNAs. Finally, all of these findings indicated that inflammation and oxidative stress have a synergistic effect on CAD progression.



## Clinical Implications

Currently, the main options for CAD treatment are pharmacological therapy, catheter intervention, and surgery, all of which require early diagnosis to effectively manage CAD [29,30]. Diagnosing CAD mainly depends on coronary computed tomography angiography, cardiac magnetic resonance imaging, and coronary angiography. However, such examinations are inconvenient and cannot be applied at a large scale. Recently, the feasibility of using blood biomarkers for the large-scale screening of high-risk patients to diagnose CAD has been reported [31,32]. However, most of the reported CAD biomarkers are limited to a single CAD pathological process, such as inflammation, diabetes, and oxidative stress. Furthermore, their sensitivity and specificity for CAD diagnosis are limited [33]. Studies on the application of biomarkers simultaneously involved in different pathological process are lacking. In the present study, hub genes simultaneously involved in inflammation and oxidative stress were identified, and their role in CAD diagnosis was explored. The results showed that *Fos*, *Il6*, *JUN*, *Mapk3*, and *Mmp9* could sensitively distinguish CAD. These genes could be potential biomarkers for CAD diagnosis.

There are three main advantages to our study: (1) The identified hub genes are involved in both inflammation and oxidative stress, indicating better diagnostic sensitivity than biomarkers involved in a single pathological process. (2) The hub genes could be identified in blood samples through RT-qPCR assay. Compared with common diagnostic methods, this approach is more cost-effective and suitable for large-scale screening. (3) Based on the detection of these hub genes, CAD could be diagnosed in its early stages, which would provide support for early intervention and improve prognosis. However, challenges remain in applying hub genes as diagnostic markers in real-world clinical settings. For example, although CAD diagnosis based on hub gene detection is feasible, whether the sensitivity of this approval is better than that of traditional diagnostic methods remains to be confirmed. Furthermore, since many diseases and injuries, such as ischemia–reperfusion injury, are closely related to inflammation and immune-oxidative stress, the specificity of hub genes for CAD diagnosis needs to be further clarified. Finally, the hub genes can be used not only for CAD diagnosis but also as treatment targets. Thus, our findings provide insights into novel pharmacological therapies for CAD.

## Limitations and Future Direction

This was a retrospective bioinformatics study based on limited samples. Therefore, our conclusions still need to be validated in future studies. Future research should focus on the correlations among inflammation, oxidative stress, and CAD. Further prospective and large-scale clinical studies (collecting patient peripheral blood and testing

using RT-qPCR) are needed to verify the sensitivity of hub genes for CAD diagnosis. Basic studies are needed to explore the mechanisms underlying how these hub genes simultaneously regulate inflammation and oxidative stress. For example, ApoE<sup>-/-</sup> mice fed a high-fat diet could be used as a model of atherosclerosis. The expression of hub genes could be regulated by a specially designed lentivirus injection. Then, blood samples should be collected to detect changes in inflammation and oxidative stress. Moreover, the RNA and protein of mice aorta could be extracted, and RT-qPCR and western blotting could be used to confirm the correlation among hub genes, TF, and miRNA. Finally, hub gene detection for CAD diagnosis should be compared with common CAD diagnostic methods (e.g., coronary CTA, cardiac MRI, and coronary angiography) to explore the cost-effectiveness and specificity.

## Conclusions

Immune and oxidative stress play an important role in CAD development. Bioinformatics analysis technology was used to identify 10 hub DEIOGs that might play a crucial role in CAD progression, and five special hub genes (*Fos*, *Il6*, *Jun*, *Mspk3*, and *Mmp9*) could be regarded as potential biomarkers for CAD diagnosis. However, further studies are required to verify the functions of these hub genes.

## Abbreviations

AUC, area under the curve; CAD, coronary artery disease; DEIOGs, differentially expressed immune and oxidative stress-related genes; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNAs, microRNAs; PPI, protein–protein interaction; ROC, receiver operating characteristic; STRING, Search Tool for the Retrieval of Interacting Genes; TFs, transcription factors.

## Availability of Data and Materials

All data generated or analysed during this study are included in this published article.

## Author Contributions

YS, YZ, and SH conceptualized and designed the study. SH provided administrative support. YG and DZ collected and assembled all data. YZ, YS, and SH analysed and interpreted the data. YS wrote the manuscript. All authors provided their final approval of the manuscript. All

authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

## Ethics Approval and Consent to Participate

This study was approved by the Clinic Review Board of the Guangdong Medical University (ethics approval number: GDMCRB-2023-0123). The need for patient informed consent was waived by the Clinic Review Board of the Guangdong Medical University because of the retrospective design of the study. The protocol of this study was performed in accordance with the Declaration of Helsinki.

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Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.59958/hsf.5799>.

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