

# Nrf2 and Keap1 Gene Polymorphisms and Lower Extremity Atherosclerosis in the Elderly

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**Abstract:** Objective: To investigate the association between Nuclear factor-erythroid 2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) gene polymorphisms and lower extremity atherosclerotic disease (LEAD) in elderly individuals, and to develop a risk prediction model for LEAD. Methods: A total of 198 elderly inpatients diagnosed with lower extremity atherosclerosis by vascular ultrasound at the Affiliated Hospital of North Sichuan Medical College from November 2023 to November 2024 were enrolled as the case group. Sixty-six healthy individuals aged over 60 years without LEAD were randomly selected as controls. Polymorphisms at three gene loci (Nrf2 rs6721961, Nrf2 rs35652124, and Keap1 rs1048290) were analyzed using the TaqMan probe method. Various clinical indicators were included, and multiple logistic regression analyses were performed to construct prediction models. Results 1. Impact of gene polymorphisms on LEAD: Univariate analysis showed significant differences in genotype distributions of Nrf2 rs6721961 and Keap1 rs1048290 between cases and controls ( $P < 0.05$ ), while no significant difference was observed for Nrf2 rs35652124 ( $P > 0.05$ ). Logistic regression analysis indicated that carrying the TT genotype of Nrf2 rs6721961, along with a history of smoking and elevated LDL concentration, were significant risk factors for LEAD. 2. Evaluation of prediction model effectiveness: Among various constructed models, Model 3, which included the Nrf2 rs6721961 genotype, hypertension, smoking history, and LDL levels, showed better agreement between predicted and actual risk compared to models utilizing only genetic or clinical indicators. Model 3 effectively identified high-risk patients. Conclusion: 1. A significant association exists between polymorphisms at Nrf2 rs6721961 and Keap1 rs1048290 and the occurrence of lower extremity atherosclerosis in elderly individuals; however, Nrf2 rs35652124 is not significantly associated. 2. Clinical sequencing of the Nrf2 gene combined with relevant clinical indicators and predictive modeling can aid in early clinical intervention for LEAD in elderly populations.

**Keywords:** Lower extremity atherosclerotic disease; Nrf2; Keap1; Gene polymorphism

## Introduction

Lower extremity atherosclerotic disease (LEAD) is characterized by arte-

rial stenosis or occlusion caused by lipid deposition and plaque formation in the arterial intima. This condition may result in severe clinical manifestations such as intermittent claudication, rest pain, ulceration, and gangrene, significantly impairing patients' quality of life<sup>[1]</sup>. Among atherosclerotic vascular diseases, LEAD has the third-highest incidence, following coronary artery disease and stroke. It represents a major cause of chronic limb ischemia, particularly among elderly populations<sup>[2]</sup>. Epidemiological studies indicate that the prevalence of LEAD rises with age, reaching approximately 15%–20% in individuals older than 60 years<sup>[3]</sup>. LEAD frequently involves the popliteal artery and arteries distal to the knee joint, such as the anterior tibial artery, posterior tibial artery, and peroneal artery, with the anterior tibial artery most commonly affected (64.5%). The disease predominantly manifests as stenosis and occlusion of arteries below the knee joint<sup>[4]</sup>. Although smoking, hypertension, type 2 diabetes, and dyslipidemia are recognized risk factors, the underlying pathogenesis in many high-risk individuals remains unclear<sup>[5]</sup>.

Currently, inflammatory responses, oxidative stress, and lipid infiltration are identified as the primary mechanisms of atherosclerosis. Oxidative stress is defined as an imbalance between the generation and clearance of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Under various internal and external stimuli, the production of ROS and RNS may surpass the body's clearance capacity, triggering pathological responses. Major sources of ROS in the vascular wall include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, mitochondrial electron transport chain, and uncoupled endothelial nitric oxide synthase (eNOS). Studies have demonstrated that oxidative stress promotes critical molecular events involved in atherogenesis, such as oxidative modification of lipoproteins and phospholipids, endothelial cell activation, and macrophage infiltration and activation<sup>[6]</sup>.

Nuclear factor-erythroid 2-related factor 2 (Nrf2), encoded by the nuclear factor erythroid 2-like 2 gene (located at 2q31), belongs to the basic leucine zipper (bZIP) transcription factor family, specifically the Cap'n'Collar (CNC) subfamily, and is widely distributed across various tissues and organs. Nrf2 contains seven conserved Nrf2-ECH homology (Neh) domains, each performing distinct functions and collectively regulating its transcriptional activity. Among these domains, Neh1 and Neh2 are particularly critical. Neh1 primarily forms heterodimers with small musculoaponeurotic fibrosarcoma (sMAF) proteins and other bZIP proteins, enabling recognition of antioxidant response elements (AREs) and regulation the transcription of multiple antioxidant and cellular defense genes. The Nrf2 gene itself contains ARE sites, allowing Nrf2 to activate its own transcription through a positive feedback mechanism, thus amplifying the antioxidant stress response. Neh2 specifically interacts with the Kelch domain<sup>[7]</sup>. Kelch-like ECH-associated protein 1 (Keap1), encoded by the human Keap1 gene (located at 19p13.2), serves as a critical regulator of Nrf2 activity. In the Keap1-CUL3-RBX1 E3 ubiquitin ligase complex, Keap1 acts as the substrate-binding

component, RBX1 binds the ubiquitin-conjugating enzyme, and CUL3 provides scaffolding for the Keap1-RBX1 interaction. Under steady-state conditions, this complex binds to Nrf2, precisely marking it for ubiquitination and subsequent proteasomal degradation, thereby suppressing Nrf2 activity<sup>[8]</sup>. During oxidative stress, conformational changes in Keap1 result in the release of Nrf2, enabling it to escape ubiquitination and translocate into the nucleus. Subsequently, Nrf2 forms a heterodimer with sMAF proteins to regulate genes involved in antioxidant responses. Through the activation of protective target genes, including heme oxygenase-1 (HO-1) and NADPH quinone oxidoreductase 1 (NQO1), Nrf2 enhances cellular capacity to eliminate oxidative products and maintain redox homeostasis<sup>[9-10]</sup>.

Recent studies have identified multiple single nucleotide polymorphism (SNP) sites within Nrf2 and Keap1 associated with various diseases. Current research mainly explores the associations between gene polymorphisms in oxidative stress-related pathways and conditions such as cancer, endocrine disorders, and cardiovascular diseases<sup>[11-12]</sup>. However, no research has yet examined the relationship between Nrf2 gene polymorphisms and LEAD. Therefore, the relationship between Nrf2 and Keap1 gene polymorphisms and the development of LEAD warrants further investigation. This study selected Nrf2 rs6721961, rs35652124, and Keap1 rs1048290 loci to examine their association with LEAD in elderly individuals and aimed to construct a prediction model to guide early diagnosis and intervention strategies.

## 1. Materials and Methods

### 1.1 General Information

A total of 198 hospitalized patients at the Affiliated Hospital of North Sichuan Medical University, diagnosed with LEAD through vascular ultrasound examination from November 2023 to November 2024, were enrolled as the disease group. The group comprised 103 males and 95 females, with a median age of 76 (interquartile range [IQR]: 70–81) years. Additionally, 66 healthy individuals, including 41 males and 25 females with a median age of 75.5 (IQR: 69–81) years, undergoing physical examination at the hospital's health examination center during the same period, were selected as the control group. Lower extremity atherosclerosis was excluded in the control group using vascular ultrasound.

Inclusion Criteria: 1. Color Doppler ultrasound of blood vessels in the disease group indicated atherosclerotic plaques or stenosis and occlusion of lower extremity arteries, meeting diagnostic criteria for LEAD as defined by the Transatlantic Inter-Society Consensus (TASC) II guidelines<sup>[13]</sup>; The age and gender of the control group were matched with those of the disease group, and lower extremity atherosclerosis was excluded by ultrasound; 2. Age  $\geq 60$  years; 3. Signed informed consent.

Exclusion Criteria: 1. Concurrent malignant tumors, autoimmune diseases,

infectious diseases, mental/cognitive dysfunction, or chronic infectious diseases; 2. Acute cardiovascular and cerebrovascular events or acute exacerbations of chronic diseases; 3. Use of glucocorticoids, immunosuppressants, or other drugs affecting oxidative stress status within the past 3 months; 4. Presence of lower extremity deep vein thrombosis, varicose veins, arteriovenous malformations, or history of lower extremity surgery.

## 1.2 Methods

Peripheral venous blood (5–8 ml) was collected in the early morning. Biochemical indicators measured on the day of collection were included in the study data. Blood samples were anticoagulated with EDTA, and genomic DNA was extracted using an Ezup Column Extraction Kit. Gene polymorphisms were analyzed using the TaqMan probe method. Primers were designed based on sequences retrieved from the GeneBank database, using Primer Express Software v2.0 (ABI), and synthesized by Shanghai GeneRay Biotechnology Co., Ltd. (Tables 1 and 2)

**Table 1.** Primer Names and Sequences

| Primer name  | Sequence (5'to3')      |
|--------------|------------------------|
| rs6721961-F  | CGTGCCACTGCCAACTG      |
| rs6721961-R  | AGGGCGTTCAGGGTGACT     |
| rs35652124-F | AGCTCGTGTTCGCAGTCA     |
| rs35652124-R | TGAGCTTAGGAGAATGGAGACA |
| rs1048290-F  | GAATTAAGGCGGTTTGT      |
| rs1048290-R  | ATGCTGACACGAAGGAT      |

Note: F=forward primer; R=reverse primer.

**Table 2.** Probe Names and Sequences

| Primer name     | Sequence (5'to3')    | 5'-modification | 3'-modification |
|-----------------|----------------------|-----------------|-----------------|
| rs6721961FAM-T  | CTGCCGGaGCTGTC       | 5'-FAM          | 3'-MGB          |
| rs6721961VIC-G  | CTGCCGGcGCTGTC       | 5'-VIC          | 3'-MGB          |
| rs35652124FAM-T | TGAACGCCcCCTCTGAA    | 5'-FAM          | 3'-MGB          |
| rs35652124VIC-C | TGAACGCCcCCTCTGAA    | 5'-VIC          | 3'-MGB          |
| rs1048290FAM-G  | CGGCATAAAGgAGACGATTG | 5'-FAM          | 3'-MGB          |
| rs1048290VIC-C  | CGGCATAAAGcAGACGATTG | 5'-VIC          | 3'-MGB          |

The PCR reaction system (10 µl) consisted of 3.05 µl ddH<sub>2</sub>O, 5 µl 2× Probe PCR MIX, 0.05 µl ROX, 0.3 µl each of forward and reverse primers, 0.15 µl each probe, and 1 µl DNA template. Reaction conditions were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 59°C for 10 s, extension at 72°C for 40 s, and final extension at 72°C for 10 min. Reactions were performed on an ABI ViiA 7 Real-Time PCR system, and results were automatically analyzed, typed, and recorded by the instrument's software (Figures 1-3.)

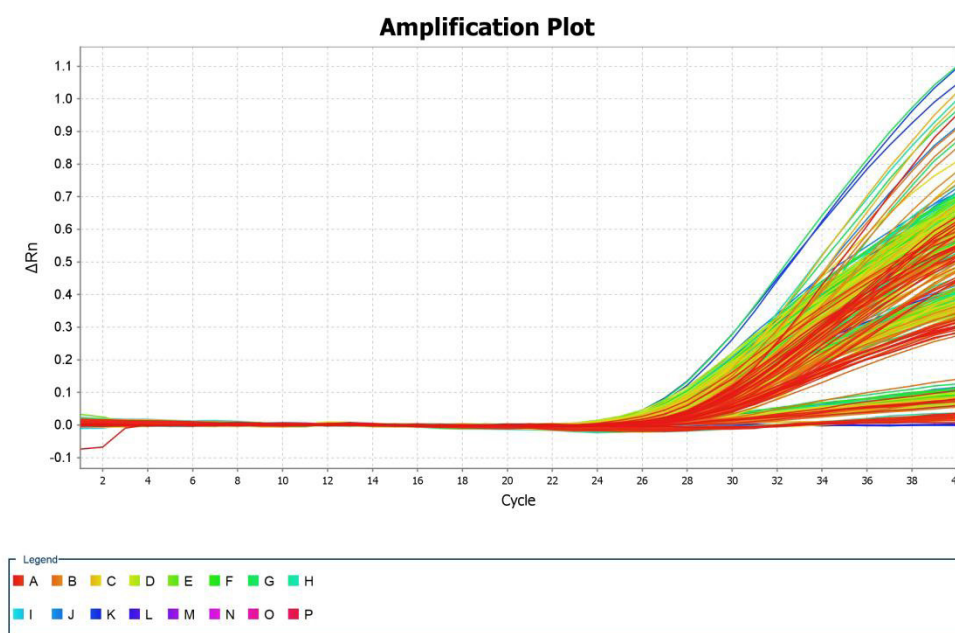


**Figure 1.** Melting Curve of Nrf2 rs6721961

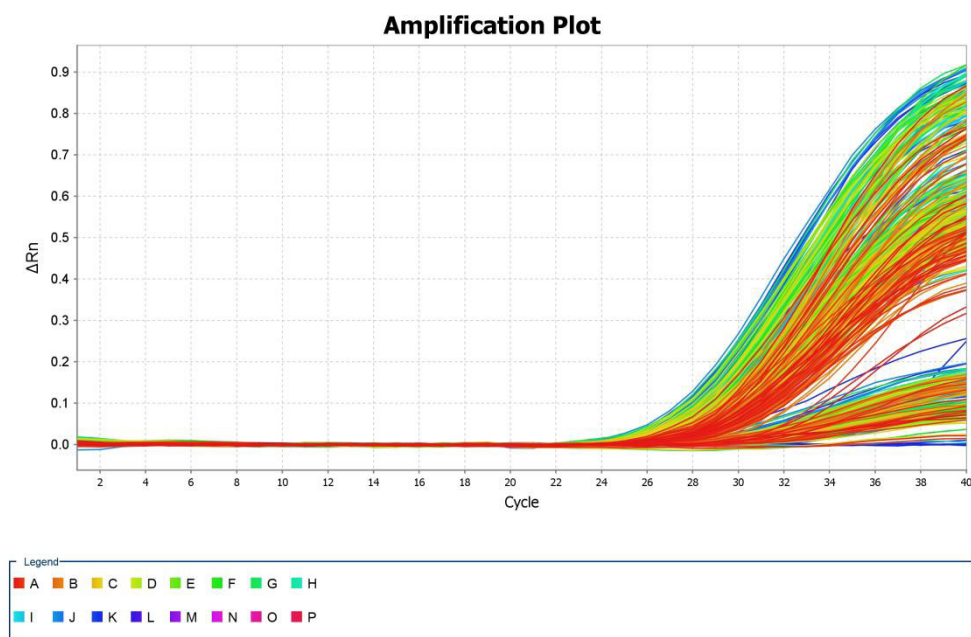


**Figure 2.** Melting Curve of Nrf2 rs35652124

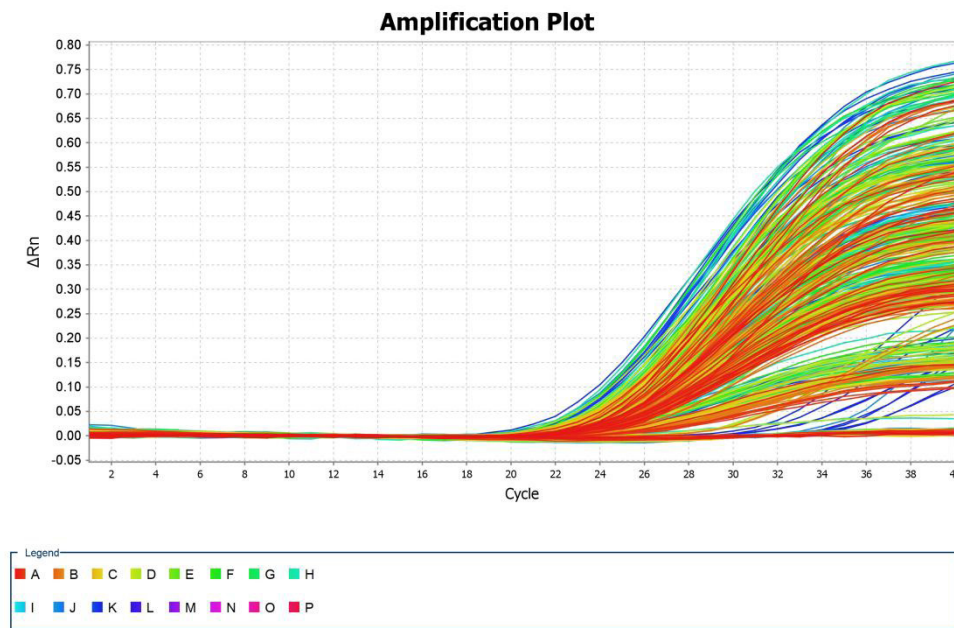


**Figure 3.** Melting Curve of Keap1 rs1048290

### 1.3 Statistical Methods

Statistical analyses were performed using SPSS 25.0 and R 4.0.3 software. Categorical data were expressed as frequencies with percentages,  $n$  (%), and compared using the chi-square test ( $\chi^2$ ). Continuous variables with normal distribution were presented as mean  $\pm$  standard deviation (SD) and compared between groups using independent-samples t-tests. Data that did not follow a normal distribution were expressed as median (IQR) and analyzed using the Mann–Whitney U test (z statistic).

(2) Variables identified as significant in the initial analysis were included in a logistic regression model. Regression coefficients ( $\beta$ ), standard errors (SE), Z-values ( $Z = \beta/SE$ ), P-values, odds ratios (OR), and 95% confidence intervals (95% CI) were calculated. Various predictive models were constructed and compared to determine the optimal model. model performance was evaluated using decision curve analysis (DCA) and calibration curves (CAL).

## 2 Result

### 2.1 General Information of the Control and Case Groups

In total, 198 participants were enrolled in the case group and 66 participants in the control group. All subjects were of Han ethnicity. There were no statistically significant differences between the groups regarding age ( $Z = 0.525$ ,  $P = 0.600$ ) or gender ( $\chi^2 = 2.037$ ,  $P = 0.154$ ) ( $P > 0.05$ ) (**Table 3**).

**Table 3.** General Data of the Study Subjects

| Variable      | Control Group (n=66) | Case Group (n=198) | $\chi^2/z$     | P     |
|---------------|----------------------|--------------------|----------------|-------|
| Gender, n (%) |                      |                    | $\chi^2=2.037$ | 0.154 |
| Female        | 25(37.9%)            | 95(48%)            |                |       |
| Male          | 41(62.1%)            | 103(52%)           |                |       |
| Age ( years)  | 75.5(69,81)          | 76(70,81)          | $z=0.525$      | 0.600 |

## 2.2 Comparison of Clinical Data between Control and Case Groups

Comparisons of clinical characteristics and laboratory parameters indicated that histories of hypertension and smoking were significantly more prevalent in the case group compared to the control group ( $P < 0.05$ ). Additionally, levels of white blood cell count (WBC), absolute neutrophil count (ANC), low-density lipoprotein (LDL), erythrocyte sedimentation rate (ESR), and fibrinogen (Fib) were significantly higher in the case group than in the control group ( $P < 0.05$ ) (Table 4).

**Table 4.** Clinical and Laboratory Characteristics of Participants

| Variable                              | Control Group(n =66) | Case Group(n =198) | $\chi^2/z$     | P     |
|---------------------------------------|----------------------|--------------------|----------------|-------|
| History of hypertension, n (%)        | 30(45.5%)            | 120(60.6%)         | $\chi^2=4.632$ | 0.031 |
| History of diabetes, n (%)            | 48(72.7%)            | 130(65.7%)         | $\chi^2=1.127$ | 0.288 |
| History of alcohol consumption, n (%) | 10(15.2%)            | 39(19.7%)          | $\chi^2=0.677$ | 0.411 |
| History of smoking, n (%)             | 9(13.6%)             | 65(32.8%)          | $\chi^2=9.038$ | 0.003 |
| Total glycosylated hemoglobin (%)     | 9.735(8.22,13.65)    | 9.71(7.505,12.845) | $z=-0.914$     | 0.361 |
| PCT(ng/mL)                            | 0.078(0.05,0.976)    | 0.074(0.05,0.548)  | $z=0.274$      | 0.784 |
| CRP(mg/L)                             | 8.46(1.4,63.03)      | 6.24(1.31,59.81)   | $z=-0.155$     | 0.877 |
| ESR(mm/h)                             | 12(2,34)             | 29.5(8,65)         | $z=2.711$      | 0.007 |
| WBC( $\times 10^9/L$ )                | 6.45(4.96,7.76)      | 7.385(5.64,9.395)  | $z=2.412$      | 0.016 |
| ANC( $\times 10^9/L$ )                | 3.88(3.12,5.54)      | 5.245(3.695,7.29)  | $z=3.045$      | 0.002 |
| TC(mmol/L)                            | 4.704 $\pm$ 1.214    | 4.852 $\pm$ 1.129  | $t=-0.903$     | 0.367 |
| TG(mmol/L)                            | 1.465(0.93,2.3)      | 1.185(0.875,1.765) | $z=-1.677$     | 0.093 |
| LDL(mmol/L)                           | 2.59(1.91,3.26)      | 2.9(2.355,3.4)     | $z=2.754$      | 0.006 |
| HDL(mmol/L)                           | 1.185(0.94,1.36)     | 1.13(0.895,1.42)   | $z=-0.404$     | 0.686 |
| TT(s)                                 | 17.8(17.1,18.2)      | 17.6(16.85,18.2)   | $z=-1.905$     | 0.057 |
| PT(s)                                 | 12(11.5,13.5)        | 12.5(11.7,13.55)   | $z=1.050$      | 0.294 |
| APTT(s)                               | 32.7(29.9,36.1)      | 32.95(29.55,38.7)  | $z=0.813$      | 0.416 |
| Fib(g/L)                              | 3.365(2.75,4.32)     | 4.025(3.13,5.16)   | $z=3.134$      | 0.002 |

## 2.3 Population Genetic Analysis

The genotype frequencies of Nrf2 rs6721961 (G/T), Nrf2 rs35652124 (C/T), and Keap1 rs1048290 (C/G) were all consistent with Hardy-Weinberg equilibri-

um ( $P > 0.05$ ).

## 2.4 Comparison of Genotype Frequencies between Control and Case Groups

Comparison of genotype frequencies between groups revealed statistically significant differences for Nrf2 rs6721961 and Keap1 rs1048290 ( $P < 0.05$ ), while no significant difference was found for Nrf2 rs35652124 ( $P > 0.05$ ) (Table 5).

**Table 5.** Genotype Distribution in Control and Case Groups

|                    | Genotype | Control Group(n,%) | Case Group(n,%) | $\chi^2$       | P     |
|--------------------|----------|--------------------|-----------------|----------------|-------|
| Nrf2<br>rs6721921  | GG       | 40(60.6%)          | 99(50%)         | $\chi^2=7.428$ | 0.024 |
|                    | GT       | 25(37.9%)          | 76(38.4%)       |                |       |
|                    | TT       | 1(1.5%)            | 23(11.6%)       |                |       |
| Nrf2<br>rs35652124 | CC       | 25(37.9%)          | 50(25.3%)       | $\chi^2=3.792$ | 0.155 |
|                    | CT       | 25(37.9%)          | 87(43.9%)       |                |       |
|                    | TT       | 16(24.2%)          | 61(30.8%)       |                |       |
| Keap1<br>rs1048290 | CC       | 17(25.8%)          | 63(31.8%)       | $\chi^2=6.211$ | 0.043 |
|                    | CG       | 39(59.1%)          | 83(41.9%)       |                |       |
|                    | GG       | 10(15.2%)          | 52(26.3%)       |                |       |

## 2.5 Comparison of Allele Frequencies between Control and Case Groups

Allele frequencies differed significantly at Nrf2 rs6721961, with G allele frequencies of 79.55% (105/132) in controls and 69.19% (274/396) in cases, and T allele frequencies of 20.45% (27/132) and 30.81% (122/396), respectively (OR=0.021, 95% CI: 0.022–0.025,  $P=0.022$ ). No significant differences were observed at Nrf2 rs35652124 and Keap1 rs1048290 ( $P > 0.05$ ) (Table 6).

**Table 6.** Allele Frequency Comparison between Control and Case Groups

|                 | Control Group(n,%) | Case Group(n,%) | OR    | 95%CI        | P     |
|-----------------|--------------------|-----------------|-------|--------------|-------|
| Nrf2 rs6721921  |                    |                 |       |              |       |
| G               | 105(79.55%)        | 274(69.19%)     | 1.000 |              |       |
| T               | 27(20.45%)         | 122(30.81%)     | 0.021 | 0.025 ~0.022 | 0.022 |
| Nrf2 rs35652124 |                    |                 |       |              |       |
| C               | 75(56.82%)         | 187(47.22%)     | 1.000 |              |       |
| T               | 57(43.18%)         | 209(52.78%)     | 1.469 | 0.988 ~2.192 | 0.056 |
| Keap1 rs1048290 |                    |                 |       |              |       |
| C               | 73(55.3%)          | 209(52.78%)     | 1.000 |              |       |
| G               | 59(44.7%)          | 187(47.22%)     | 1.106 | 0.745 ~1.648 | 0.614 |

## 2.6 Logistic Regression Models

Three predictive logistic regression models were established using different

inclusion criteria. Model 1 (genotype data alone) showed that individuals carrying the TT genotype at Nrf2 rs6721961 had an 8.658-fold higher risk for LEAD (OR=8.658, 95% CI: 1.715–157.95, P=0.038). Model 2 (clinical data alone) identified smoking history (OR=3.652, 95% CI: 1.695–8.687, P=0.002), hypertension, and elevated LDL as independent risk factors. Model 3, integrating significant variables from both Models 1 and 2, demonstrated that the TT genotype at Nrf2 rs6721961 (OR=8.359, 95% CI: 1.608–153.95, P=0.043), smoking history (OR=3.451, 95% CI: 1.622–8.108, P=0.002), and elevated LDL (OR=1.835, 95% CI: 1.284–2.699, P=0.001) significantly increased LEAD risk (Tables 7–9).

**Table 7.** Logistic Regression Model 1 (Genotypes of rs6721961 and rs1048290)

|                    | $\beta$ coef | SE    | Z      | P     | OR    | 95%CI          |
|--------------------|--------------|-------|--------|-------|-------|----------------|
| Nrf2 rs6721961 GT  | 0.202        | 0.301 | 0.671  | 0.502 | 1.223 | 0.682 ~ 2.224  |
| Nrf2 rs6721961 TT  | 2.159        | 1.041 | 2.073  | 0.038 | 8.658 | 1.715 ~ 157.95 |
| Keap1 rs1048290 CG | -0.515       | 0.340 | -1.517 | 0.129 | 0.597 | 0.301 ~ 1.149  |
| Keap1 rs1048290 GG | 0.334        | 0.445 | 0.750  | 0.453 | 1.397 | 0.591 ~ 3.441  |

**Table 8.** Logistic Regression Model 2 (Clinical Factors)

|                          | $\beta$ coef | SE    | Z     | P     | OR    | 95%CI         |
|--------------------------|--------------|-------|-------|-------|-------|---------------|
| Hypertension, n (%)      | 0.640        | 0.311 | 2.060 | 0.039 | 1.897 | 1.035 ~ 3.512 |
| History of smoking, n(%) | 1.295        | 0.413 | 3.135 | 0.002 | 3.652 | 1.695 ~ 8.687 |
| ESR(mm/h)                | 0.007        | 0.006 | 1.171 | 0.241 | 1.007 | 0.995 ~ 1.02  |
| WBC( $\times 10^9/L$ )   | -0.190       | 0.178 | 1.068 | 0.286 | 0.827 | 0.579 ~ 1.149 |
| ANC( $\times 10^9/L$ )   | 0.227        | 0.189 | 1.206 | 0.228 | 1.255 | 0.895 ~ 1.839 |
| LDL(mmol/L)              | 0.638        | 0.195 | 3.280 | 0.001 | 1.893 | 1.31 ~ 2.815  |
| Fib(g/L)                 | 0.153        | 0.126 | 1.216 | 0.224 | 1.166 | 0.976 ~ 1.519 |

**Table 9.** Logistic Regression Model 3 (Genotypes and Clinical Factors)

|                          | $\beta$ coef | SE    | Z     | P     | OR    | 95% CI         |
|--------------------------|--------------|-------|-------|-------|-------|----------------|
| Nrf2 rs6721961 GT        | 0.322        | 0.312 | 1.032 | 0.302 | 1.380 | 0.752 ~ 2.568  |
| Nrf2 rs6721961 TT        | 2.123        | 1.051 | 2.021 | 0.043 | 8.359 | 1.608 ~ 153.95 |
| Hypertension, n (%)      | 0.514        | 0.303 | 1.698 | 0.090 | 1.672 | 0.925 ~ 3.044  |
| History of smoking, n(%) | 1.239        | 0.407 | 3.046 | 0.002 | 3.451 | 1.622 ~ 8.108  |
| LDL(mmol/L)              | 0.607        | 0.189 | 3.216 | 0.001 | 1.835 | 1.284 ~ 2.699  |

## 2.7 Comparison of Model Performance

Receiver operating characteristic (ROC) curves were constructed to compare the predictive performances of the three models. The area under the curve (AUC) values were 0.636 (Model 1), 0.749 (Model 2), and 0.726 (Model 3). Although all models demonstrated predictive capability ( $AUC > 0.5$ ), Model 1 performed significantly less effectively compared to Models 2 and 3, which showed similar predictive accuracy. However, Model 3, combining genetic and clinical indicators, demonstrated superior applicability in clinical practice and was therefore selected for subsequent analyses (**Figure 4**).

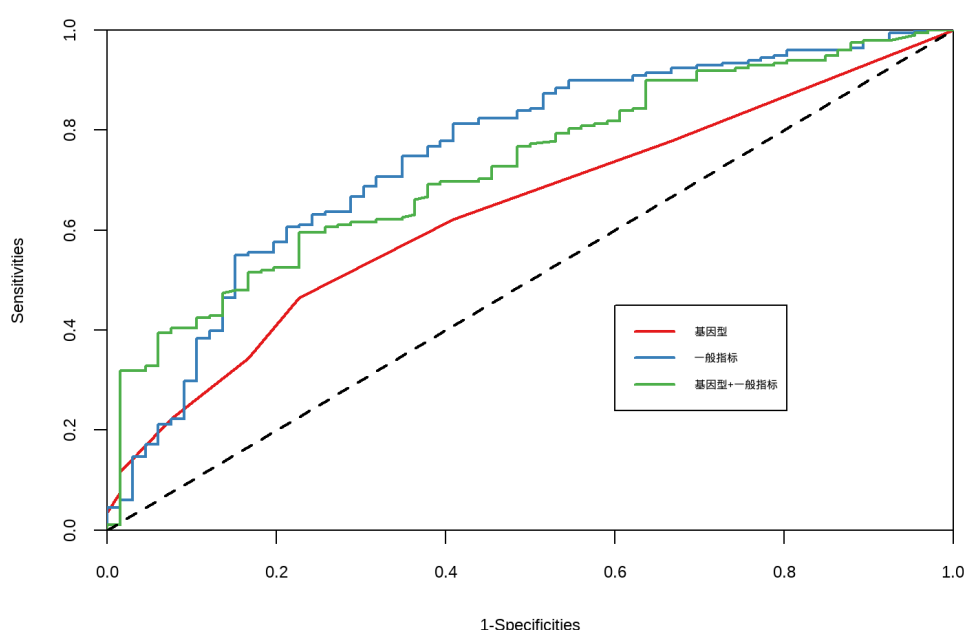


**Figure 4.** ROC Curves of the Three Predictive Models

Note: The x-axis represents specificity (Spe), and the y-axis represents sensitivity (Sen). ROC curves were generated by plotting sensitivity versus specificity across various threshold values. The red curve denotes Model 1, the blue curve denotes Model 2, and the green curve denotes Model 3.

## 2.8 DCA

Decision curves were plotted using the risk threshold (Pt) as the x-axis and Net Benefit as the y-axis, along with reference lines representing the scenarios of “universal treatment” and “no treatment.” Results showed that the model provided beneficial clinical utility across a broad range of threshold probabilities, particularly maintaining a high net benefit at thresholds exceeding 0.6. This highlights the model’s advantage in identifying high-risk patients, thereby offering valuable clinical decision-making insights (**Figure 5**).

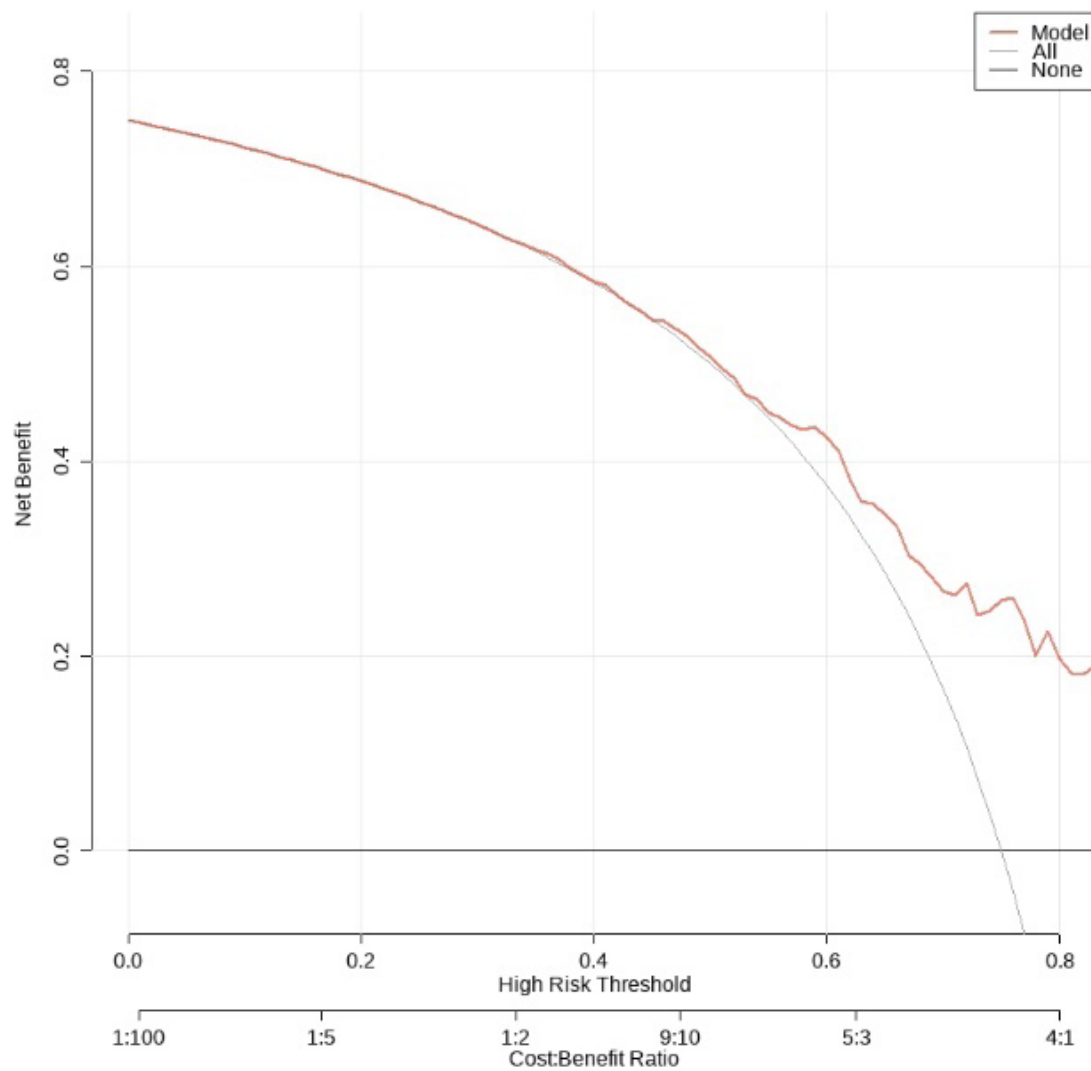


**Figure 5.** Decision Curve of Model 3

## 2.9 CAL

CALs were generated with predicted probabilities as the x-axis and observed probabilities as the y-axis to evaluate consistency between the model's predicted and actual event probabilities. When the predicted probability exceeded 0.4, the slope of the apparent CAL (red curve) approached 1, closely aligning with the ideal curve. This indicates high consistency between the predicted and observed probabilities. The bias-corrected CAL (green solid line) also closely aligned with the ideal curve, demonstrating good agreement between the predicted and actual risks of LEAD (**Figure 6**).

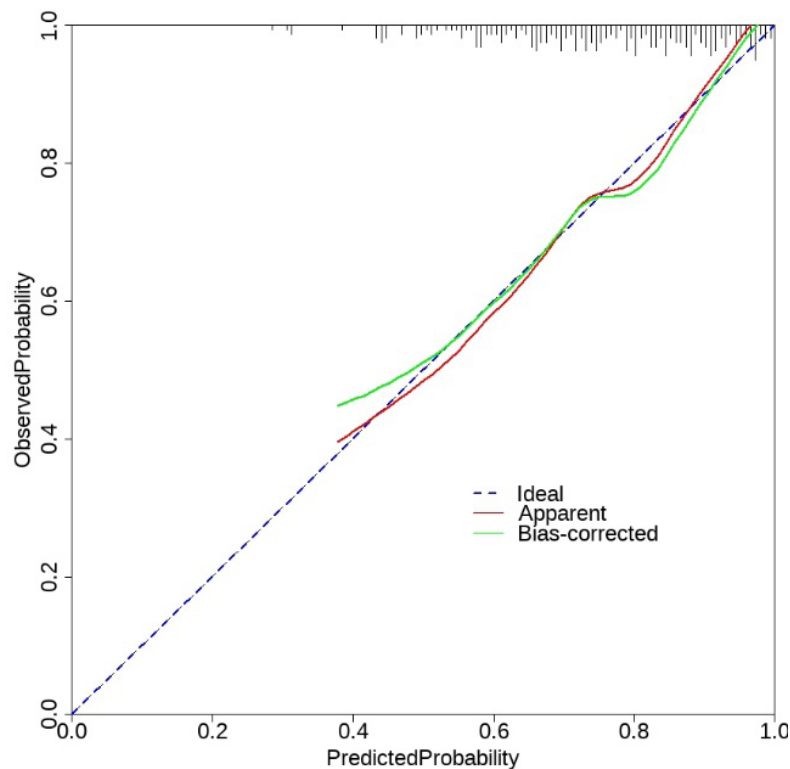


Figure 6. CAL of Model 3

### 3 Discussion

#### 3.1 Association between Nrf2 Gene Polymorphism and LEAD

Nrf2, a critical transcription factor, plays an essential role in cellular physiological processes by regulating antioxidant stress responses. Under oxidative stress conditions, Nrf2 binds specifically to AREs, activating the expression of key antioxidant genes, such as heme oxygenase 1 (HO-1) and NADPH quinone oxidoreductase 1 (NQO1), thereby protecting cells against oxidative damage<sup>[14-15]</sup>. Additionally, Nrf2 is an important transcriptional regulator of ferritin heavy chain (FTH), upregulating its expression to inhibit autophagic degradation of ferritin, reducing excessive iron accumulation, and mitigating ferroptosis induced by lipid peroxidation under oxidative stress conditions<sup>[16]</sup>. These mechanisms collectively influence endothelial cell damage and contribute to atherosclerosis. Therefore, it is plausible that Nrf2 gene polymorphisms may compromise its function and impair antioxidant capacity.

The Nrf2 rs6721961 polymorphism, located in the gene promoter region, reportedly interferes with transcription factor binding efficiency, thereby reducing the expression of essential antioxidant genes such as HO-1 and NQO1, and exacerbating vascular oxidative injury<sup>[17-18]</sup>. Previous studies have investigated the associations between genetic polymorphisms at this locus and various oxidative stress-related diseases. Research has demonstrated a significant correlation

between the TT genotype of Nrf2 rs6721961 and coronary artery disease (CAD), suggesting a substantial role of this genotype in atherosclerotic development<sup>[19]</sup>. Oxidative stress is critical in diabetic retinal microvascular complications, and diabetic patients with higher antioxidant exposure show significantly reduced retinopathy risk and severity<sup>[20]</sup>. Li et al. reported that the Nrf2 rs6721961 polymorphism was strongly associated with diabetic retinopathy (DR) susceptibility, with patients carrying the homozygous mutant (AA genotype) more prone to proliferative lesions, indicating the A allele as a risk factor for DR in T2DM patients. During radiotherapy for non-small cell lung cancer (NSCLC), ionizing radiation induces abundant ROS, leading to DNA fragmentation, endothelial damage, and inflammatory cytokine accumulation<sup>[21]</sup>. Studies have shown that NSCLC patients with the Nrf2 rs6721961 AA genotype exhibit higher susceptibility to radiation pneumonitis. In some previous studies, the rs6721961 alleles were labeled as A/C, whereas in our study, the alleles were labeled as T/G. Both are located at position -617 of Nrf2, with the labeling discrepancy due to differences in forward and reverse DNA strand referencing during probe design. We hypothesized that the TT genotype and T allele might impair the body's antioxidant stress response. Consistent with this hypothesis, our findings revealed significantly higher frequencies of the TT genotype (homozygous mutant) and T allele of Nrf2 rs6721961 in the LEAD group compared to controls. This strongly suggests that a homozygous mutation at this locus likely weakens Nrf2 antioxidant function (e.g., reducing Nrf2 binding affinity to ARE), disrupts oxidative stress homeostasis, and promotes atherosclerosis.

Multiple studies have examined associations between Keap1 gene polymorphisms and diseases closely linked to oxidative stress. A study conducted in China found that the C/G genotype at Keap1 rs1048290 may serve as an independent prognostic marker for endometrial cancer<sup>[22]</sup>. Another study showed that the G allele of Keap1 rs1048290 may enhance Nrf2-mediated antioxidant responses by affecting Keap1 function, thereby reducing the risk of drug-resistant epilepsy<sup>[23]</sup>. Research from West China Hospital, Sichuan University, observed that the CC genotype at this locus correlated with increased colorectal cancer risk<sup>[24]</sup>. Additionally, genotyping at this SNP locus was significantly associated with carotid atherosclerosis. Although the frequency of the CC genotype was notably higher in the case group in our study, logistic regression analysis did not demonstrate statistical significance. We attribute this to either an insufficient sample size or relatively weak functional effects associated with this SNP polymorphism. Therefore, further investigations with larger sample sizes are necessary to validate these findings.

This study revealed a significant difference in the prevalence of hypertension between the LEAD and control groups, suggesting hypertension as a critical risk factor for LEAD. Hypertension can induce mechanical damage and dysfunction of the vascular wall, including cellular migration, inflammation, calcification, proliferation, and apoptosis, consequently accelerating the development

of atherosclerosis and vascular stenosis, which are closely related to the pathophysiology of LEAD. Smoking was also identified as a significant risk factor for LEAD. Tobacco components (e.g., nicotine and carbon monoxide) directly injure vascular endothelial cells, trigger inflammatory responses, induce endothelial dysfunction, remodel smooth muscle cell morphology and function, and promote thrombosis, collectively contributing to atherosclerosis<sup>[25]</sup>. Furthermore, regarding lipid metabolism, LDL is critical in the progression of atherosclerosis. LDL particles entering the arterial intima undergo oxidative modification, forming oxidized LDL (ox-LDL), which macrophages phagocytose to become foam cells. The subsequent accumulation of foam cells within the arterial wall leads to atherosclerotic plaque formation<sup>[26]</sup>. This study confirmed LDL as an independent risk factor for LEAD.

Numerous studies have reported significant neutrophil accumulation at atherosclerotic lesions, indicating their essential role in the initiation and progression of atherosclerosis<sup>[27]</sup>. Myeloperoxidase (MPO), an oxidase released by neutrophils under oxidative stress and inflammatory stimuli, is extensively found in atherosclerotic plaques. MPO produces various cytotoxic substances, mediates irreversible modifications of vascular wall proteins and circulating lipoproteins, and thereby promotes atherosclerosis<sup>[28]</sup>. Tissue ischemia and hypoxia in LEAD further activate inflammatory cells, resulting in local neutrophil and inflammatory cell aggregation and releasing additional inflammatory mediators. This cycle exacerbates disease progression. Collectively, these findings underscore the critical role of inflammation in the pathogenesis of LEAD.

As a common manifestation of atherosclerosis, early diagnosis and intervention for LEAD are crucial for improving patient outcomes. However, patients with LEAD frequently present non-specific early symptoms, often leading to delayed diagnosis and missed optimal intervention opportunities. The predictive model developed in this study integrates multiple risk factors to effectively identify high-risk individuals, providing a foundation for timely interventions. By incorporating genotyping data, the model demonstrates greater robustness compared to traditional clinical indicators and laboratory parameters alone. ROC curve analysis showed that this model possesses high sensitivity and specificity, allowing accurate discrimination between LEAD patients and non-patients. CALs further validated the accuracy of the model's predicted probabilities. DCA highlighted the model's distinct advantage in identifying high-risk patients, assisting clinicians in personalized interventions (e.g., smoking cessation, risk factor management, and pharmacological prevention), supporting clinical decision-making, and optimizing healthcare resource allocation.

In conclusion, the study findings regarding Nrf2 gene polymorphisms and LEAD susceptibility are as follows: the frequencies of the TT genotype and T allele at the Nrf2 rs6721961 locus, as well as the GG genotype at the Keap1 rs1048290 locus, were significantly higher in the LEAD group compared to controls. No significant difference was observed in genotype frequency at the Nrf2

rs35652124 locus. Logistic regression analysis further identified the TT genotype at Nrf2 rs6721961, smoking history, and elevated LDL concentration as independent risk factors for LEAD.

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