# STUDY PROTOCOL

**Official Title:** Association of OR6A2 Expression on Monocytes with Inflammation and Major Adverse Cardiovascular Events in Myocardial Ischemia-Reperfusion Injury

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# Study Protocol

## 1. Rationale

Early reperfusion therapy following acute myocardial infarction (AMI) is crucial for salvaging ischemic myocardium. However, concomitant myocardial ischemia-reperfusion (IR) injury significantly compromises clinical outcomes [1,2]. Studies indicate that the pathological core of myocardial IR injury lies in a self-amplifying interplay between oxidative stress and inflammation: mitochondrial fission-fusion imbalance triggers explosive release of reactive oxygen species (ROS), while aberrant activation of myeloid immune cells (e.g., monocytes/macrophages) synergistically propagates cardiomyocyte oxidative damage and pro-inflammatory milieu [3,4]. The hyperinflammatory response elicited by myocardial IR injury is strongly associated with left ventricular remodeling and adverse cardiovascular outcomes [5–9]. Recent clinical trials validate anti-inflammatory interventions: the CANTOS trial demonstrated that IL-1β neutralization reduces major adverse cardiovascular events (MACEs) by 15–17%, and low-dose colchicine achieves 23% MACE risk reduction via NLRP3 inflammasome inhibition [10–12].

During early IR injury, monocytes infiltrating the infarct zone primarily derive from the spleen and bone marrow [13,14]. These infiltrating monocytes differentiate into monocyte-derived macrophages, which act as pivotal mediators of myocardial injury progression through secretion of IL-1α/β and other pro-inflammatory cytokines. Consequently, targeted modulation of inflammatory polarization and oxidative stress in infiltrating monocytes may represent a novel therapeutic avenue for mitigating myocardial IR injury. Nevertheless, current anti-inflammatory strategies face substantial challenges, notably limited target specificity and safety concerns.

Emerging research reveals expanding roles of olfactory receptors (ORs) in non-olfactory pathologies. OR6A2—a specific receptor for octanal—activates G protein-coupled signaling to promote ROS generation and cytokine release in macrophages, thereby driving atherosclerotic plaque progression [15]. However, the precise molecular mechanisms linking octanal-OR6A2 signaling to oxidative stress activation, along with its clinical implications in myocardial IR injury, remain elusive.

Existing evidence identifies mitochondrial dynamics dysregulation as central to ROS burst [16–

19]. Our preliminary work demonstrates that octanal stimulation triggers mitochondrial homeostasis dysregulation in monocytes via OR6A2 activation, manifesting as aberrant fission, membrane depolarization, and pathological accumulation of mitochondrial ROS (mtROS) [20–22]. Excessive mtROS and mitochondrial fragments cooperatively act as "Signal 2" to activate the NLRP3 inflammasome, initiating IL-1α/β secretion. These findings implicate OR6A2 as a critical molecular nexus connecting immunometabolic dysregulation to myocardial IR injury, though clinical validation and translational potential warrant urgent exploration.

This clinical cohort study addresses three pivotal questions: (1) Whether plasma octanal levels and monocyte OR6A2 expression demonstrate specific alterations in myocardial IR injury patients; (2) Whether these biomarkers correlate with oxidative stress, inflammatory phenotypes, and MACEs risk; (3) Whether OR6A2 expression patterns across monocyte subsets (classical $CD14^{+}CD16^{-}$, intermediate $CD14^{+}CD16^{+}$, non-classical $CD14^{-}CD16^{+}$) hold prognostic value. Integrating multi-omics profiling with long-term follow-up data, this research will systematically characterize clinicopathological features of octanal-OR6A2 signaling in myocardial IR injury, advancing novel biomarker discovery and precision-targeted therapeutic strategies.

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## 2. Research Content

This project investigates the "Association of OR6A2 Expression on Monocytes with Inflammation and Major Adverse Cardiovascular Events in Myocardial Ischemia-Reperfusion Injury."

### 2.1. Study Objectives

Primary Aim: Elucidate the clinical relevance of octanal-OR6A2 signaling in myocardial IR injury and its prognostic value for MACEs.

Specific Objectives:

(1) Characterize pathobiomarker profiles in IR injury:

- Plasma octanal dynamics (LC/MS/MS)
- OR6A2 expression kinetics on monocyte subsets:
    - Classical ($CD14^{+}CD16^{-}$)
    - Intermediate ($CD14^{+}CD16^{+}$)
    - Non-classical ($CD14^{-}CD16^{+}$)
- Correlations with oxidative stress (mtROS, MDA, $H_2O_2$) and inflammation (IL-1α/β, CRP)

(2) Define prognostic utility:

- Association of baseline $OR6A2^{+}$ monocyte subsets with 44-month MACEs
- Predictive value of plasma octanal levels for 44-month MACEs

(3) Validate translational potential:

- Develop risk-stratification models integrating OR6A2 biomarkers

**2.2 Research Design**

(1) Study Site

Department of Cardiology, Zhongda Hospital, Southeast University

(2) Study Population

- 200 AMI patients (STEMI/NSTEMI) with confirmed myocardial IR injury
- Inclusion: Meeting myocardial IR diagnostic criteria (multimodal assessment)
- Exclusion: active systemic infections, advanced heart failure (NYHA III-IV), acute cerebrovascular events, myocarditis, cardiomyopathy, refractory ventricular tachycardia, malignancies diagnosed within the preceding 5 years, severe renal insufficiency (estimated glomerular filtration rate [eGFR] <30 mL/min/1.73m$^2$ or dialysis dependence), and Child-Pugh class C hepatic dysfunction.

(3) Diagnostic criteria for myocardial ischemia-reperfusion injury

Myocardial ischemia-reperfusion injury was diagnosed based on clinical evidence meeting both temporal and multimodal criteria. The diagnostic window encompassed clinical manifestations or objective findings within 24 hours following percutaneous coronary intervention (PCI), extendable to 48 hours if symptoms persisted.

- Diagnostic Requirements (≥2 criteria):
    - Symptomatic criteria: Sudden recurrent chest pain post-procedure (excluding in-stent thrombosis or re-occlusion);
    - Electrocardiographic evidence: Delayed ST-segment resolution or recurrent ST elevation (>1 mm in at least two contiguous leads); New-onset ventricular arrhythmias (e.g., frequent premature ventricular complexes, ventricular tachycardia, ventricular flutter, ventricular fibrillation accelerated idioventricular rhythm); New-onset bradyarrhythmias (e.g., sinus bradycardia, atrioventricular block, intraventricular conduction delays).
    - Imaging abnormalities: New myocardial edema (manifested by high-signal intensity on T2-weighted magnetic resonance imaging [MRI]); Lack of improvement in regional wall motion abnormalities; Evidence of microvascular obstruction (e.g., perfusion defect on myocardial contrast echocardiography or late gadolinium enhancement on MRI).

- Biomarker kinetics: Abnormal elevation of cardiac troponin (cTnI/cTnT) or CK-MB within 6 hours post-procedure, excluding procedural re-infarction.

- Coronary Flow Validation:
  - Confirmation of successful coronary flow restoration (TIMI flow grade ≥2) to exclude ischemia from technical failure or residual stenosis;
  - Post-procedural confirmation of stent patency through coronary angiography or computed tomography angiography (CTA) is required to exclude acute thrombosis or re-occlusion.
- Exclusion Criteria:
  - Non-ischemic myocardial injury etiologies (e.g., Takotsubo cardiomyopathy, myocarditis, toxic/metabolic insults) were systematically excluded.

(4) Sample Size

- Myocardial IR cohort: n=200 (longitudinal follow-up)
- Power analysis: 80% power ($\alpha$=0.05) for HR≥2.5 between OR6A2 expression strata

(5) Recruitment & Group Assignment

- Consecutive enrollment of eligible patients (September 1st, 2019-August 30th, 2027)
- Non-interventional cohort design (observational)

(6) Ethical Considerations

- Approved by Zhongda Hospital IEC (2020ZDSYLL051-P01)
- Written informed consent obtained pre-enrollment

**2.3 Methodology**

(1) Biosample Collection & Analysis Timeline

| Timepoint | Assessments |
|---|---|
| T0 (Baseline) | Plasma octanal (LC/MS/MS)<br>Flow cytometry: Monocyte subsets + OR6A2 MFI<br>Serum: IL-1$\alpha$/$\beta$, MDA, $H_2O_2$, CRP |
| T1 (24h post-PCI) | Repeat flow cytometry<br>Caspase-9/mPTP activity assays |
| T2 (Month 3,6,12...44) | MACEs surveillance |

(2) Core Techniques

| Method | Target | Instrument/Kit |
|---|---|---|
| Flow Cytometry | OR6A2 surface expression (CD14/CD16 subsets) | Attune™ NxT (Thermo) |
| LC/MS/MS | Plasma octanal | d16-octanal isotope standard |
| Western Blot | Mitochondrial OR6A2 | Invitrogen PA5-102216 |
| Multiplex ELISA | IL-1α/β, oxidative stress markers | Cusabio/Beyotime kits |

(3) Primary endpoint

- MACEs (composite of AMI, cardiac death, stroke, hospitalization for unstable angina, or unplanned coronary revascularization)

(4) Statistical Analysis

Data Presentation & Normality Assessment:

- Continuous variables are reported as mean ± standard deviation (SD) for normally distributed data or median with interquartile range (IQR) for non-normally distributed data. Normality was evaluated using the Shapiro-Wilk test.
- Categorical variables are presented as frequencies and percentages.

Hypothesis Testing:

- Normally Distributed Data: Parametric tests were used: Student's t-test (two groups) or one-way ANOVA with Tukey's post hoc test (≥3 groups).
- Non-Normally Distributed Data: Non-parametric tests were applied: Wilcoxon rank-sum test (two independent groups) or Kruskal-Wallis H test with Dunn's post hoc correction (≥3 groups).
- Categorical Variables: Analyzed using the $\chi^2$ test or Fisher's exact test (sparse data).
- Correlations: Assessed using Spearman correlation analysis.
- Time-to-Event Data: Survival curves generated via Kaplan-Meier estimation.

Primary Exposure Analysis (OR6A2 & Octanal):

- OR6A2 median fluorescence intensity (MFI) was analyzed both as a continuous variable (per 1000-unit increment) and categorically (dichotomized at the median).
- Plasma octanal concentration was similarly evaluated as a continuous metric and

categorically (median-dichotomized).

- Association with MACEs: Quantified using multivariable Cox proportional hazards regression, yielding adjusted hazard ratios (HRadj) and 95% confidence intervals (CIs). Three sequential adjustment models were employed:
    - Model 1: Age, gender.
    - Model 2: Model 1 + body weight, smoking status, hypertension, diabetes mellitus.
    - Model 3: Model 2 + lipid-lowering therapy (with/without PCSK9 inhibitors), eGFR, white blood cell count, C-reactive protein (CRP), Creatine Kinase-Myocardial Band (CK-MB), onset-to-reperfusion time, echocardiographic parameters (ejection fraction, interventricular septum thickness, left ventricular internal diameter, left ventricular posterior wall thickness), discharge medications (aspirin, P2Y12 inhibitors, statins, β-blockers, ACEI/ARB/ARNI, aldosterone receptor antagonists).
- Non-Linear Association (Octanal): The relationship between continuous plasma octanal levels and MACEs was further examined using multivariable Cox regression with restricted cubic spline functions, fully adjusted for Model 3 covariates, allowing flexible assessment of potential non-linearity.

Follow-up & Software:

- Complete 44-month follow-up data were obtained for all participants with no loss to follow-up.
- Analyses were performed using SPSS (v25.0), GraphPad Prism (v8.0), and R software (v4.2.3).

Significance:

- Statistical significance was defined as a two-sided p-value $< 0.05$.
- Significance levels denoted as: *P $< 0.05$, **P $< 0.01$, ***P $< 0.001$, ****P $< 0.0001$.

### 2.4 Feasibility & Innovation

Strengths:

- Novelty: First clinical validation of olfactory receptor (OR6A2) in IR injury prognosis
- Methodological rigor:
    - Dual biomarkers (octanal + cell-type-specific OR6A2)

    - Advanced statistics (spline-adjusted dose-response models)
- Resources:
    - Hospital's accredited flow cytometry core (ISO-15189)
    - Established biorepository with 90% follow-up rate

### 2.5 Expected Outcomes

(1) Clinical validation of OR6A2 as a prognostic biomarker for IR injury

(2) Risk stratification algorithm integrating monocyte subset profiling

(3) ≥3 high-impact publications (*European Heart Journal/Circulation*)

(4) Patent application for OR6A2-targeted diagnostic kits