**Původní sdělení** | Original research article

# The Effect of Cigarette Exposure on Intima-Media Thickness (IMT) and VCAM-1 Expression in the Aorta of Mus musculus Mice

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ARTICLE INFO

*Article history:*
Submitted: 26. 12. 2024
Accepted: 21. 1. 2025
Available online: 12. 6. 2025

*Klíčová slova:*
Ateroskleróza
Kouření cigaret
Tkáň aorty
Tloušťka intimy-medie
VCAM-1

SOUHRN

**Kontext:** Kouření je nadále jednou z hlavních příčin endotelové dysfunkce, která spouští proces rozvoje aterosklerózy a přispívá k rozvoji řady kardiovaskulárních onemocnění. Jedním ukazatelem endotelové dysfunkce je zvýšení hodnot vaskulární buněčné adhezní molekuly (vascular cell adhesion molecule-1, VCAM-1), molekuly podporující shlukování monocytů a urychlující rozvoj aterosklerózy. Běžným ukazatelem poškození endotelu je i ztluštění vrstvy intimy-medie (intima-media thickness, IMT), jež lze často pozorovat u kuřáků. Cílem této studie bylo zkoumat vliv kouření cigaret na hodnoty VCAM-1 a IMT.
**Metody**: Byla provedena experimentální studie s kontrolní skupinou hodnocenou až na konci studie. Dvanáct myší domácích (*Mus musculus*) bylo náhodně rozděleno do čtyř skupin: skupiny K (-), kontrolní (nevystavena tabákovému kouři); skupiny P1, s denní expozicí kouři po dobu 14 dní; skupiny P2, vystavené kouři denně po dobu 21 dní, a skupiny P3 s expozicí kouři po dobu 28 dní. Po 28 dnech expozice byly vzorky aorty všech skupin analyzovány z hlediska exprese VCAM-1 a hodnoty IMT.
**Výsledky:** Expozice tabákovému kouři zvětšuje IMT aorty. Měření IMT prokázalo statisticky významné rozdíly mezi „třítýdenní", „dvoutýdenní" skupinou a kontrolní skupinou ($p < 0,05$). Marker endotelové dysfunkce VCAM-1 ve vzorcích aorty nicméně statisticky významné změny nevykazoval ($p > 0,05$).
**Závěr:** Po třítýdenní expozici cigaretovému kouři se sice statisticky významně zvýšila hodnota IMT, délka expozice však nebyla dostatečně dlouhá na to, aby došlo ke statisticky významnému zvýšení hodnot VCAM-1 v tkáni myší aorty.



*Keywords:*
Aortic tissue
Atherosclerosis
Cigarette smoking
Intima media thickness
VCAM-1

ABSTRACT

**Background:** Smoking remains one of the leading causes of endothelial dysfunction, which triggers the atherosclerotic process and contributes to various cardiovascular diseases. One marker of endothelial dysfunction is an increase in VCAM-1, a molecule that promotes monocyte aggregation and accelerates the development of atherosclerosis. Thickening of the intima-media layer (IMT) is also a common indicator of endothelial damage, frequently observed in smokers. The aim of this study is to investigate the effect of cigarette smoking on vascular cell adhesion molecule-1 (VCAM-1) levels and intima-media thickness (IMT).
**Methods**: An experimental study with a post-test only controlled group design was conducted. Twelve mice (*Mus musculus*) were randomly subdivided into four groups: Group K (-), the control group (not exposed to tobacco smoke); Group P1, exposed to smoke daily for 14 days; Group P2, exposed to smoke daily for 21 days; and Group P3, exposed to smoke daily for 28 days. After the 28-day exposure period, samples from all groups were analyzed for VCAM-1 expression and aortic intima-media thickness (IMT).
**Results:** The tobacco smoke exposure leads to an increase in aortic intima-media thickness (IMT). The IMT measurements showed a significant difference between the 3-week group compared to the 2-week group and the baseline ($p < 0.05$). However, the endothelial dysfunction marker, VCAM-1, did not show a significant change in the aortic samples ($p > 0.05$).
**Conclusion:** The IMT measurement increased significantly after 3 weeks of cigarette smoke exposure, but the exposure duration was not sufficient to significantly elevate VCAM-1 levels in the aortic tissue of the mice.

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**DOI:** 10.33678/cor.2025.015

**Please cite this article as:** Ardiana M, Andrianto, Hermawan HO, et al. The Effect of Cigarette Exposure on Intima-Media Thickness (IMT) and VCAM-1 Expression in the Aorta of Mus musculus Mice. Cor Vasa 2025;67:376–380.

## Introduction

Smoking is recognised as a major cause of cardiovascular disease (CVD), accounting for one in every four CVD-related deaths worldwide.[1,2] Smoking is the most significant modifiable risk factor, proven to greatly contribute to premature death, both related and unrelated to CVD.[3] Smoking just 1–4 cigarettes per day has also been shown to increase the risk of death from ischemic heart disease by 2.74 times compared to never having smoked.[4] A recent meta-analysis revealed that smoking increases the risk of atrial fibrillation and coronary artery disease by 1.23 (95% CI 1.10–1.36) and 1.33 (95% CI 1.27–1.40), respectively.[5]

From a pathogenic perspective, cardiovascular diseases (CVDs) are recognised as some of the most complex human conditions, arising from the interplay between genetics, environment, and lifestyle.[6] Smoking is one of the most significant modifiable risk factors, and among lifestyle risks, it remains the most complex and least understood due to the 9,000 different chemicals in cigarettes (ranging in size from atoms to particulate matter), which contribute to the development of complex CVDs that are difficult to fully comprehend.[7]

One of the well-known harmful substances that contribute to atherosclerosis is nicotine. Nicotine is absorbed in the alveoli of the lungs and rapidly enters the bloodstream, reaching the brain within just 10–12 seconds. This triggers the release of dopamine, which is the primary reason for nicotine addiction, encouraging continued smoking.[8]

A critical review of the current scientific literature leads to the hypothesis that nicotine mediates the effects of cigarette smoke on the cardiovascular system by enhancing MAPK signalling, inflammation, and oxidative stress via NADPH oxidase 1 (Nox1), which induces vascular smooth muscle cell (VSMC) ageing.[9] In fact, atherosclerosis is driven by two key pathological mechanisms: inflammation and cell death.[10]

Previous studies on mice exposed to cigarette smoke have shown that nicotine exposure leads to atherosclerotic lesions, especially in those on a high-fat diet (HFD).[11] The combination of platelet aggregation, lipid profile modification, endothelial dysfunction, direct actions on cellular elements involved in plaque formation, and vascular smooth muscle cell (VSMC) proliferation accelerates atherosclerosis progression due to nicotine exposure.[8]

Several biomarkers have been investigated in recent years to evaluate their association with various cardiovascular diseases, as they may assist in achieving an optimal diagnosis or predicting prognosis. One important biomarker of endothelial dysfunction caused by smoking is Vascular Cell Adherens Molecule-1 (VCAM-1). VCAM-1 is a key pro-inflammatory and pro-atherogenic protein, serving as a critical indicator of endothelial dysfunction and atherosclerosis. Among other cell adhesion molecules, VCAM-1 plays a crucial role in neointima proliferation following arterial damage induced by nicotine, a major focus of research into atherosclerotic cardiovascular diseases. In the nicotine-induced arterial injury model, VCAM-1 expression is notably elevated, contributing to the proliferation and migration of neointimal smooth muscle cells.[12]

VCAM-1 is also known to have increased expression in various cardiovascular conditions, including hypertension, atherosclerosis, ischemic disease, stroke, heart failure, and atrial fibrillation. Recent studies suggest that VCAM-1 could be a potential biomarker for atrial fibrillation, post-operative atrial fibrillation, and cardiovascular mortality in patients with coronary artery disease undergoing coronary artery bypass surgery.

In this study, the researchers aim to examine whether there is a change in aortic IMT size and an increase in VCAM-1 expression in *Mus musculus* subjects exposed to cigarette smoke.

## Material and method

### *Ethics approval*

This study adhered to the ARRIVE guidelines for animal experimentation protocols. The animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee of Universitas Airlangga (UNAIR), Surabaya, Indonesia (Animal Approval No: 270/EC/KEPK/FKUA/2023), with Meity Ardiana as the principal investigator. The study was carefully conducted to prevent harm or discomfort to the experimental animals. At the conclusion of the study, anesthesia was administered to minimize pain during euthanasia.

### *Animals*

This study used 12 male Balb/c mice (Mus musculus), aged eight weeks and weighing between 30 and 50 grams. Before the experiment commenced, the mice were acclimatised to their new environment for seven days. The animals were housed in the pharmacology animal laboratory at the Faculty of Medicine, Universitas Airlangga, in individual polycarbonate cages measuring 480 mm × 265 mm × 210 mm.

Each cage contained three or four mice, with wood shavings covering the cage floor. The animals were kept in microisolator cages under controlled room temperatures ranging between 22 °C and 25 °C, with a 12-hour light/dark cycle. Humidity was maintained at a constant level of 50% to 60%. The mice were fed a standard diet and had access to water ad libitum. Group assignment was carried out using simple random sampling.

At the end of the study, the mice were administered isoflurane and either to minimise the impact on nitric oxide levels. Subsequently, the mice were euthanised by cardiac puncture, performed only after confirming deep anaesthesia, as evidenced by the absence of a pain response. Our method was approved by the Animal Ethics Committee, in accordance with the IACUC policy on laboratory animal use. The study was conducted following international standards as outlined in the National Institute of Health's Guide for the Care and Use of Laboratory Animals and adhered to the ARRIVE guidelines for reporting in vivo experiments.

After euthanasia, the aortic tissues were collected to isolate the intima-media, involving the removal of surrounding adipose tissue, longitudinal cutting along the edges, and peeling off of the adventitia.

| Table 1 – Immunoreactivity Scoring System for IHC[13] | | |
|---|---|---|
| **Percentage of positive cells** | **X intensity of staining** | **= IRS (0–12)** |
| 0 = no positive cells | 0 = no colour reaction | 0–1 = negative |
| 1 = < 10% of positive cells | 1 = mild reaction | 2–3 = mild |
| 2 = 10–50% positive cells | 2 = moderate reaction | 4–8 = moderate |
| 3 = 51–80% positive cells | 3 = intense reaction | 9–12 = strongly positive |
| 4 = > 80% positive cells | | |

| Table 2 – IRS points and classification[13] | |
|---|---|
| **IRS points** | **IRS classification** |
| 0–1 | 0 = negative |
| 2–3 | 1 = positive, weak expression |
| 4–8 | 2 = positive, mild expression |
| 9–12 | 3 = positive, strong expression |

### *VCAM-1 immunohistochemistry*

The VCAM-1 reagent for immunohistochemistry (IHC) was purchased from Santa Cruz Biotechnology (VCAM-1 Antibody [E-10], sc-13160; Dallas, TX, USA). The analysis was performed using the streptavidin-biotin method, with a biotin-conjugated secondary antibody used to link the primary antibody to the streptavidin-peroxidase complex. The labeled streptavidin-biotin (LSAB) method was employed to assess VCAM-1 expression in mouse aortic tissue.

The procedure began with deparaffinisation and fixation of the aortic tissue, followed by rehydration using xylene and various concentrations of ethanol. After rehydration, the tissue was washed with Phosphate Buffer Solution and immersed in 3% $H_2O_2$ solution for 20 minutes. We then added 1% Bovine Serum Albumin and incubated the samples for 30 minutes.

The primary anti-VCAM-1 antibody was applied, incubated, and washed, followed by the addition of the secondary antibody and further incubation. The SA-HRP complex was then added and incubated for 10 minutes. DAB chromogen was applied and incubated, followed by another wash.

Finally, counterstaining with Haematoxylin-Eosin was carried out, and VCAM-1 expression was measured and analysed using a biological microscope at 400× magnification. Semiquantitative analysis was performed using an immunoreactivity scoring system (**Tables 1, 2**).[13]

### *IMT measurement and histology*

The thoracic aorta was prepared by cutting the distal arch from the left ventricle of each mouse. Post-mortem samples of the descending thoracic aorta, obtained through dissection, were then fixed using 10% formaldehyde to preserve tissue integrity. Following fixation, the samples were embedded in paraffin and sectioned into 6 µm slices for histological analysis.

After sectioning, the tissue slices were stained with haematoxylin and eosin to enhance contrast and facilitate the observation of cellular morphology. The intima-media thickness of the aorta was measured using a Leica DMD 108 (Leica Microsystems GmbH, Wetzlar, Germany), which enables precise and consistent measurements.

Each sample was measured in micrometres (µm) at six different locations along the vessel wall, providing a comprehensive view of tissue thickness. The arithmetic mean of these six measurements was calculated and presented in the results section to offer detailed data on the morphological characteristics of the aorta.

## Results

### *Smoking causes increased aortic intima-media thickness due to the progression of atherosclerosis*

This study evaluated the thickness of the aortic intima--media layer, measured using the MIT scale. The statistical analysis employed was a one-way ANOVA test, conducted after confirming the normality and homogeneity of the data. The results showed a significant difference between the treatment groups (**Fig. 1**).

Based on the results of the post hoc LSD test, the study demonstrated a significant difference between the control group and the group exposed to cigarette smoke for 3 weeks, as well as between the 2-week and 3-week exposure groups. These findings confirm that cigarette smoke exposure at certain doses and durations can lead to endothelial dysfunction and thickening of the aortic intima-media layer, which is clinically significant in the development of atherosclerosis.

### *Lack of significant increase in VCAM-1 expression in mice exposed to cigarette smoke for 1 month*

This study examined VCAM-1 expression using IHC staining, evaluated by an anatomical pathology expert according to the Immunoreactivity Scoring System (IRS) (Kaemmerer, 2012). Initial assessment results indicated that the data were not normally distributed or homogeneous. The statistical analysis employed was the Kruskal-Wallis test, with the results presented in **Figure 2**.

Based on the Kruskal-Wallis statistical analysis, no significant differences were found between the groups. This suggests that the level of cigarette smoke exposure may have been insufficient to cause an increase in the exposure groups. The brown colour observed on the elastic fibers and blood cells was identified as a false positive and was not included in the analysis. Adjustments were made before evaluation to avoid false positives in the samples. Not all samples could be observed across 10 fields of view due to the small or overlapping aortas; however, a re-

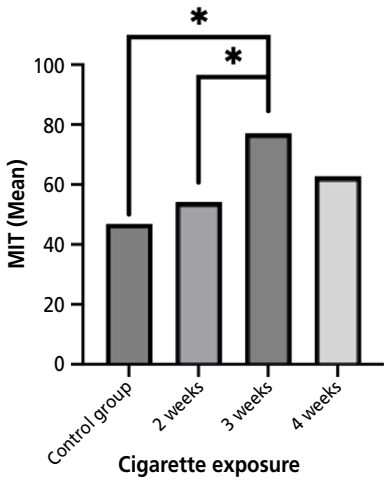


**Fig. 1 – Measurement of aortic IMT in mice exposed to cigarette smoke (Sig <0.05).**

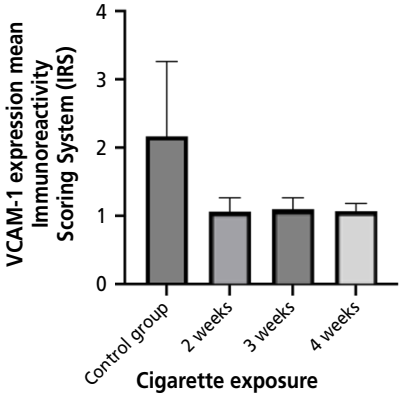


**Fig. 2 – Assessment of VCAM-1 expression in the aorta of mice exposed to cigarette smoke.**

duced number of fields still adequately represented the entire aortic wall.

## Discussion

### *The effect of smoking on the IMT aortic*

The thickening of carotid intima-media (IMT) is recognised as a proxy indicator of early-stage atherosclerosis and serves as a predictor of cardiovascular disease (CVD) development, independent of conventional CVD risk factors.[14] Prior studies have highlighted the significant role of inflammation in the initial thickening of IMT resulting from exposure to cigarette smoke. Smoking's impact on IMT is partially mediated by leukocytes, high-sensitivity C-reactive protein, and fibrinogen, all of which are early biomarkers of atherosclerosis.[15]

Based on the results of the study above, it shows that there is a significant difference between giving smoked cigarettes to mice on increasing aortic IMT with a *p*-value <0.05, especially between the control group and the group exposed to cigarette smoke for 3 weeks. This finding is consistent with another research,[16] that there is a significant relationship to the aortic IMT exposed to cigarette smoke in experimental animals. Another research[17] also showed that there was an increase in the average carotid artery IMT in smokers than in non-smokers.

One of the most harmful components of cigarettes known to cause various cardiovascular diseases is nicotine. Nicotine is recognised for causing systemic hemodynamic disturbances, reducing coronary blood flow, and inducing myocardial tissue remodelling. It is also known as a pro-atherogenic agent with several effects, such as: 1. triggering endothelial dysfunction, 2. increasing inflammatory response, 3. modifying lipid profile, 4. inducing catecholamines, which are responsible for raising blood pressure and heart rate, 5. enhancing platelet aggregability, 6. triggering smooth muscle cell proliferation and migration into the intima, and 7. promoting vascular smooth muscle cell (VSMC) proliferation and migration to the lesion cap.[8] These pathogenic mechanisms contribute to permanent vascular damage and the progression of atherosclerosis.

Based on the number of cigarettes smoked per day,[18] showed that the lowest average IMT value was in the study population, who consumed 1–5 cigarettes per day, and the highest average IMT value was in the study population, who consumed 11–15 cigarettes per day. This shows that IMT increases with the number of cigarettes smoked per day, especially in chronic smokers. He showed significant changes in the structure and function of the carotid artery IMT in respondents who had smoked for 5–10 years.[18]

However, another research[19] obtained different results where there were no changes in the tunica intima observed from the mouse experiment. Another experimental study[20] found that exposure to cigarette smoke for eight weeks only resulted in disorganization of vascular smooth muscle cells in the tunica media. Vacuolization is one of the complications of the cytotoxic process in cells and an early marker of preclinical atherosclerosis. Vacuolization is a sign of oxidative stress in blood vessels due to exposure to cigarette smoke. Vacuolization makes vascular smooth muscle cells have different shapes and sizes, resulting in cells becoming irregular and causing atherosclerosis.[21]

### *VCAM-1 expression in mice exposed to cigarette smoke*

Based on the results of the study above, it showed that there was no significant difference in VCAM-1 expression between control and treatment. This result is appropriate with the study of Ardiana et al. (2023)[16] that there is no significant difference in VCAM-1 expression between the group exposed to cigarette smoke and the control group. VCAM-1 is expressed in vascular endothelial cells, and VCAM-1 expression can increase leukocyte adhesion to endothelial cells. VCAM-1 accelerates the migration of adherent leukocytes along the endothelial surface, and promotes the proliferation of vascular smooth muscle cells. So, VCAM-1 may play an important role as a pro-atherogenic molecule.[22]

It is believed that smoking can cause increased oxidative stress due to direct damage by reactive oxygen species (ROS) and inflammatory responses. The production of oxidative stress and ROS due to cigarette smoke is

expected to increase VCAM-1 expression and decrease e-NOS levels.[23] According to previous studies,[24,25] there was an increase in oxidative stress, ROS, and VCAM-1 expression in endothelial cell cultures after exposure to cigarette smoke.

However, increased VCAM-1 expression is a multifactorial process, smoking cannot increase VCAM-1 independently without other risk factors such as dyslipidemia.[26] have proven this hypothesis by examining VCAM-1 expression in aortic tissue of dyslipidemia patients. The results showed that VCAM-1 expression was positively correlated with triglycerides, total cholesterol, and LDL levels while VCAM-1 and HDL had a negative correlation.[26] VCAM-1 expression in endothelial cells requires a trigger which is high lipid levels, especially LDL. Increased oxidized LDL in the endothelium will be phagocytized by macrophages which require the role of VCAM-1.[27] In our study, other factors contributing to the development of atherosclerosis such as dyslipidemia were not included. Our study did not use experimental animals with high-fat diets and serial lipid profile measurements. Therefore, our results showed that there was no significant difference of VCAM-1 expression between the group exposed to cigarette smoke and the control group.

## Conclusion

IMT increased significantly after 3 weeks of cigarette smoke exposure, but the exposure duration was not sufficient to significantly elevate VCAM-1 levels in the aortic tissue of the mice.

### Conflict of intereset

The authors declare no conflict of interest.

### Acknowledgment

The authors would like to express their sincere gratitude to the advisors for the valuable guidance and support throughout this research journey.

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