https://africanjournalofbiomedicalresearch.com/index.php/AJBR
Afr. J. Biomed. Res. Vol. 27 (September 2024); 516-521

Research Article

Biomarker Profiling of Nitrosative Stress and Inflammation in Early Detection and Prognosis of Coronary Artery Disease

Reshma Gopan M[1*], Deepika Chandrasekaran[2], Dinesh Roy[3], N Muninathan[4], S Mahila[5], Radhika PR[6]

[1]Research Scholar, Department of Physiology, Meenakshi Academy of Higher Education and Research, Chennai*
[2]Assistant Professor, Department of Physiology, Meenakshi Medical College Hospital and Research Institute, Kanchipuram
[3]CEO and Senior Cytogeneticist, Genetika, Centre for Advanced Genetic Studies, Thiruvananthapuram
[4]Scientist, Central Research Laboratory, Meenakshi Medical College Hospital and Research Institute, Kanchipuram
[5]Professor, Department of Physiology, Panimalar Medical College Hospital and Research Institute, Chennai
[6]Senior Lecturer, Department of Physiology, PMS College of Dental Science & Research, Thiruvananthapuram

Abstract

Background: Coronary artery disease (CAD) is the leading cause of morbidity and death worldwide. For effective treatment and improved outcomes, early detection and accurate prediction are crucial.

This study looks into the possibility of the nitrosative stress marker 3-nitrotyrosine (3-NT) and the pro-inflammatory cytokine interleukin-6 (IL-6) as biomarkers for the early diagnosis and prognosis of CAD.

Materials & Methods: The Institutional Ethics Committee of Genetika granted ethical approval for the conduct of this case-control study. Seventy subjects confirmed with CAD were selected from the Hridayalaya & Lords Hospitals of Thiruvananthapuram, between August 2023 and August 2024. By employing ELISA methods, the levels of the inflammatory marker (Interleukin-6) and the Nitrosative stress marker (3-Nitrotyrosine) were evaluated. 3-Nitrotyrosine & IL-6 were found to be elevated.

Statistical Analysis: The distribution of the CAD cases under the subcategories was compared using the Chi-squared test. The sensitivity of 3-nitrotyrosine and IL-6 was evaluated by plotting the receiver operating characteristic curve (ROC).

Results: When CAD patients were compared to controls, their levels of 3-NT and IL-6 were significantly higher ($p < 0.05$).

Conclusions: According to the study, IL-6 and 3-NT are viable biomarkers for the early diagnosis and prognosis of CAD.

Key Words: Coronary Artery Disease, Nitrosative Stress, 3-Nitrotyrosine, Inflammation, Interleukin-6

***Author for correspondence: Email:** reshmagpn@gmail.com*

Received; 08/07/2024 Accepted: 10/08/2024

DOI: https://doi.org/10.53555/AJBR.v27i3.2250

Introduction

A complicated multifactorial ailment, coronary artery disease (CAD) is caused by a combination of environmental and hereditary factors. Nitrosative stress, a primary underlying cause of CAD, is characterized by the excessive formation of reactive nitrogen species (RNS), which can cause cellular damage and lead to the establishment and progression of atherosclerosis. This is one of the key components implicated in the pathogenesis of CAD.

Reactive nitrogen species (RNS) are formed when there is an abnormal increase in the level of nitric oxide (NO) produced by the inducible nitric oxide synthase (iNOS) and/or by the

uncoupled endothelial nitric oxide synthase (eNOS).[1] iNOS-derived RNS can react with superoxide anion radicals to form peroxynitrite, a highly reactive oxidant that can cause lipid peroxidation, protein nitration, and DNA damage.

Since nitrotyrosine and interleukin 6 are important markers of nitrosative stress and inflammation, respectively, they are utilized as biomarkers in this article. Reactive nitrogen species, such as peroxynitrite, nitrate proteins to generate nitrotyrosine, which causes tissue damage and cellular malfunction. Low-density lipoprotein (LDL) isolated from human atheroma and human atherosclerotic lesions have been reported to contain higher concentrations of nitrotyrosine. [2] A key component of the inflammatory response is the pro-inflammatory cytokine interleukin 6 (IL-6). Monitoring IL-6 levels helps assess the extent of inflammation and its role in the progression of CAD.

Considering how crucial nitrosative stress is to the pathophysiology of CAD, comprehending the fundamental mechanisms governing these processes may help create new therapeutic approaches for the disease's prevention and therapy. For instance, targeting the iNOS-derived RNS pathway or developing antioxidants that can scavenge RNS may represent promising therapeutic approaches to mitigate Nitrosative stress-induced damage in CAD.

Aim

To investigate the molecular mechanisms by which nitrosative stress contribute to the pathogenesis and progression of CAD, with the goal of identifying potential biomarkers and therapeutic targets for improving CAD diagnosis, prevention, and treatment.

Materials and Methods

Study Population

- **Subjects**: The study included 140 participants, comprising 70 patients with clinical diagnosis of CAD aged between 18-50 years and 70 age- and sex-matched healthy controls with no risk factors of CAD. Prior to being included in the study, all participants gave their informed consent. Demographic, clinical and lifestyle characteristics were recorded using a proforma.
- **Inclusion and Exclusion Criteria**:
  - Inclusion Criteria- Patients diagnosed with CAD based on clinical, angiographic, or diagnostic criteria. Controls were free of any cardiovascular disease.
  - Exclusion Criteria- Participants with a history of autoimmune diseases, chronic inflammatory conditions, malignancies, or other severe systemic illnesses were excluded.

Ethical Approval

The study was approved by the Institutional Ethics Committee of Genetika (Ref. No. 14/2023/IECG).

Sample Collection

The samples were referred from Hridayalaya, Institute for Preventive Cardiology to Genetika, Centre for Advanced Genetic Studies, Thiruvananthapuram.

- **Blood Sample Collection:** Venous blood samples (5 mL) were collected from all participants after overnight fasting. Samples were drawn into EDTA tubes for plasma isolation and serum-separator tubes for serum collection.
- **Plasma and Serum Preparation:** Blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C. Plasma and serum were aliquoted and stored at -20°C until further analysis.

Biomarker Assays

In this study, 3-nitrotyrosine and Interleukin-6 test was carried out in each subject. IL-6 test was performed for quantitating the extent of inflammation and 3-nitrotyrosine test was conducted to evaluate nitrosative stress.

- **Nitrosative Stress Marker (3-Nitrotyrosine Level):** Plasma levels of 3-nitrotyrosine was quantified using ELISA [Origin kit, Cat: OPK7866]. The assay was performed following the manufacturer's protocol. The microplate reader was used to measure the absorbance at 450 nm. 3-NT levels were expressed as ng/mL of plasma.
- **Inflammatory Marker (Interleukin-6):** Plasma levels of IL-6 were quantified using high-sensitivity ELISA kits [ABclonal kit, Cat: RK00004]. All samples and standards were run in duplicate, and the average values were used for analysis. Results were expressed in pg/mL of plasma.

Biochemical Parameters

- **Fasting Blood Glucose (FBS)**- FBS was measured using the glucose oxidase method on a semi-automated analyser.
- **Lipid Profile**- Total cholesterol, HDL, LDL, and triglycerides were measured using enzymatic methods with a semi-automated analyser.

Statistical Analysis

- Data were analyzed using jamovi 2.5.3. Continuous variables were presented as mean ± standard deviation. Group comparisons were performed using either the independent t-test or the Mann-Whitney U test for non-normally distributed data. Categorical variables were compared using the chi-square test.
- **Correlation Analysis**: Pearson correlation coefficient was calculated to assess the relationships between Nitrosative stress and inflammatory marker with clinical parameters.
- **Significance Level**: A p-value of < 0.05 was considered statistically significant.

Results

Table 1: Comparison of Sociodemographic and Clinical Characteristics between Control and Case Groups

		Control		Case		χ^2	df	p
		N	%	N	%			
Educational level	>10th	43	61.4	31	44.3			
	Up to 10th	27	38.6	39	55.7	4.128	1	0.042
Occupational	Non sedentary	42	60	39	55.7			

Type	Sedentary	28	40	31	44.3	0.264	1	0.608
Socioeconomic status	APL	64	91.4	63	90			
	BPL	6	8.6	7	10	0.085	1	0.771
Diabetes	No	70	100	46	65.7			
	Yes	0	0	24	34.3	28.97	1	<0.001
Hypertension	No	69	98.6	45	64.3			
	Yes	1	1.4	25	35.7	27.21	1	<0.001
Dyslipidaemia	No	69	98.6	51	72.9			
	Yes	1	1.4	19	27.1	18.9	1	<0.001
Alcohol consumption	No	69	98.6	55	78.6			
	Yes	1	1.4	15	21.4	13.83	1	<0.001

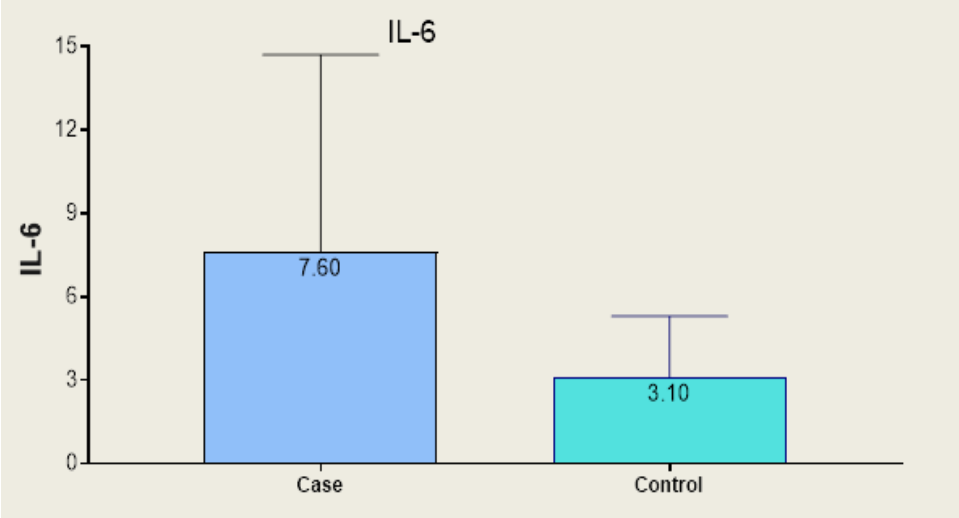
A higher percentage of participants in the control group had an education level above 10th standard (61.4%) compared to the case group (44.3%) (p=0.042).The analysis of occupational type and socioeconomic status revealed no significant difference between both the groups. None of the control group participants had diabetes, whereas 34.3% of the case group participants were diabetic (p<0.001). Hypertension was also significantly more prevalent in the case group (35.7%) compared to only 1.4% in the control group. The presence of dyslipidaemia was significantly higher in the case group (27.1%) compared to the control group (1.4%). A significantly higher proportion of participants in the case group reported alcohol consumption (21.4%) compared to the control group (1.4%).

Table 2: Comparison of Anthropometric, Biochemical, and Inflammatory Markers between Control and Case Groups

	Control (n=70)		Case (n=70)		T test	
	Mean	SD	Mean	SD	t	p
Anthropometric Measurements						
Abdominal Circumference (cm)	86.3	14.2	96.7	11.6	-4.741	<0.001
Height (cm)	160.5	9.2	158.0	11.4	1.449	0.150
Weight (kg)	59.7	8.3	68.5	12.5	-4.916	<0.001
BMI	23.1	2.2	27.5	4.6	-7.218	<0.001
Biochemical Parameters						
Fasting Blood Sugar	88.3	15.6	126.4	44.1	-6.813	<0.001
Serum Triglyceride	108.8	20.5	152.5	36.5	-8.728	<0.001
Serum Total Cholesterol	163.4	19.3	227.0	51.9	-9.614	<0.001
Serum HDL Cholesterol	50.9	6.0	40.1	5.7	10.924	<0.001
Serum LDL Cholesterol	90.7	19.8	156.4	47.2	-10.738	<0.001
Inflammatory and Nitrosative Stress Markers						
IL-6	3.1	2.2	7.6	7.1	-5.146	<0.001
3-nitrotyrosine	18.9	6.7	38.8	24.0	-6.705	<0.001

The mean of waist circumference (case- 96.7 ± 11.6 cm; control-19.3 mg/dL) and serum LDL cholesterol levels (case- 156.4 ± 47.2 86.3 ± 14.2 cm), weight (case-68.5 ± 12.5 kg; control-59.7 ± 8.3mg/dL, control- 90.7 ± 19.8 mg/dL) of the case group kg), BMI (case- 27.5 ± 4.6; control 23.1 ± 2.2) of the case groupwere significantly higher when compared to the control group. In were significantly higher when compared to the control group. Incontrast to the control group (50.9 ± 6.0 mg/dL), the case group's addition to this there was no significant difference between the twomean serum HDL cholesterol was significantly lower (40.1 ± 5.7 groups for height: case group (158 ±11.4cm) versus control groupmg/dL). The mean IL-6 levels were significantly higher in the case (160.5 ±9.2cm). The mean of fasting blood sugar level (case- 126.4group (7.6 ± 7.1 pg/mL) compared to the control group (3.1 ± 2.2 ± 44.1 mg/dL; control- 88.3 ± 15.6 mg/dL), serum triglyceridepg/mL). The case group's mean 3-nitrotyrosine levels were 38.8 ± levels (case- 152.5 ± 36.5 mg/dL; control- 108.8 ± 20.5 mg/dL),24.0 nmol/L, which was substantially higher than the control serum total cholesterol (case- 227.0 ± 51.9 mg/dL, control- 163.4 ±group's 18.9 ± 6.7 nmol/L.

Graph 1: Comparison of Mean IL-6 Levels between Case and Control Groups

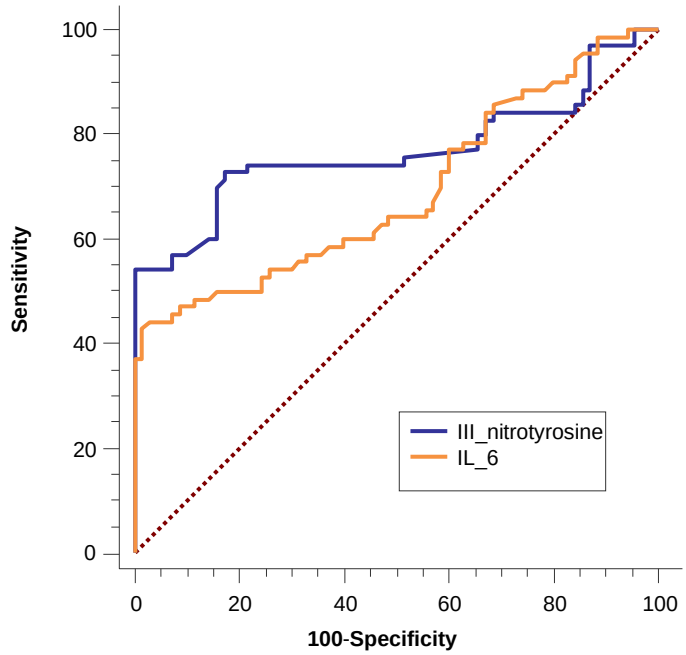


The mean IL-6 levels were significantly higher in the case group (7.60 ± SD) compared to the control group (3.10 ± SD). The case group showed more than double the IL-6 levels compared to the control group, suggesting a higher inflammatory response among the patients.

Table 3: Area Under the Curve Analysis for 3-Nitrotyrosine & Interleukin-6

	AUC	SE	95% CI
3- Nitrotyrosine	0.769	0.0431	0.690 to 0.836
Interleukin-6	0.691	0.0453	0.607 to 0.766

Graph 2: ROC curve of various parameters to predict CAD



The AUC for 3-nitrotyrosine was 0.769 (SE = 0.0431) & IL-6 was 0.691 (SE = 0.0453), demonstrating good diagnostic accuracy in distinguishing between the case and control groups. These results indicate that among the biomarkers studied, 3-nitrotyrosine exhibited the highest diagnostic accuracy for distinguishing cases from controls.

Table 4: Logistic Regression Analysis of Factors Associated with Case Status

	B	S.E.	Wald	df	p	OR	95% C.I.for OR	
							Lower	Upper
Hypertension	3.171	1.359	5.443	1	0.02	23.84	1.66	342.15
IL-6	0.285	0.123	5.352	1	0.021	1.33	1.04	1.69
3-nitrotyrosine	0.127	0.041	9.454	1	0.002	1.14	1.05	1.23
Constant	-11.434	2.287	24.995	1	0	0		

A binary logistic regression analysis was conducted to evaluate the impact of hypertension, Interleukin-6 (IL-6), and 3-nitrotyrosine on the likelihood of being in the case group. People with hypertension were approximately 24 times more likely to be in the case group compared to those without hypertension, controlling for other variables in the model. The probability of being in the case group rose by 33% and 14% for each unit rise in the IL-6 and 3-nitrotyrosine levels.

Discussion

The present investigation has successfully discovered 3-nitrotyrosine (3-NT) and interleukin-6 (IL-6) as biomarkers for the prediction of Coronary Artery Disease (CAD).

3-NT was higher in CAD patients than in the control group. The significance of nitrosative stress in the pathogenesis of CAD is shown by this rise. With an AUC of 0.769, 3-NT also demonstrated strong discriminatory power, indicating that it holds considerable potential as a CAD predictive biomarker. Studies showing the importance of nitrosative stress in coronary artery disorders are compatible with the raised 3-nitrotyrosine levels, which indicate enhanced nitrosative stress in CAD patients.

IL-6, was also found to be significantly elevated in CAD patients. This elevation indicates a heightened inflammatory response in these individuals. The correlation between IL-6 levels and CAD suggests that inflammation plays a critical role in the development and progression of the disease IL-6 had moderate discriminating ability despite a somewhat lower AUC of 0.691, making it a valuable biomarker in the context of CAD.

The study revealed a positive correlation between the levels of 3-NT and IL-6 and the severity of CAD. Patients with elevated levels of these biomarkers may experience a lower risk of adverse cardiovascular events if they receive early care. Individuals with advanced stages of the disease were more likely to have higher amounts of these biomarkers. The accuracy of CAD diagnosis and prognosis could be increased by incorporating this combination of indicators into standard clinical practice.

In studies conducted by Savio C, Murray EC, et al., in 2011 and 2021, it was found that endothelial dysfunction may raise peripheral resistance via a number of different pathways. The development and problems of hypertension are linked to these pathways, which result in increased constriction and vascular remodeling (including structural, mechanical, and functional modifications) of resistant arteries. [3-5] This is in accordance with our present study, which showed that hypertension was substantially more common in the case group (35.7%) than in the control group (1.4%).

Diabetes mellitus and endothelial dysfunction are linked by a substantial body of research. Patients with Type 1[6-7] and Type 2 diabetes mellitus [6-9] have less endothelium-dependent vasodilation in their peripheral and coronary arteries, according to several cross-sectional researches. [12]

Recently, many studies have shown an association of the lipid profile with endothelial function. [13-16] Similarly in this study, the presence of dyslipidaemia was significantly higher in the case group (27.1%) compared to the control group (1.4%).

Conclusion

Globally, cardiovascular illnesses are now the leading cause of mortality. Consequently, understanding its pathophysiological underpinnings is essential for developing preventative and therapeutic measures. This study investigated NSS's role in the pathophysiology of CAD, which will be helpful when anti-NSS treatments are proposed in the future. The findings suggest that early detection of nitrosative stress, indicated by elevated nitrotyrosine levels, and the assessment of inflammation through interleukin-6 (IL-6) levels, could play a critical role in the early diagnosis and improved prognosis of CAD. One possible approach to mitigate nitrosative stress-induced damage in CAD is to target the RNS pathway originating from iNOS or develop antioxidants that have the ability to scavenge RNS.

Acknowledgement: We are grateful to Team Genetika for their kind assistance and unwavering support during this study project.

Conflicts of interest: The author(s) have no conflict of interest to declare.

Funding: The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

Pérez-Torres, I.; Manzano-Pech, L.; Rubio-Ruíz, M.E.; Soto, M.E.; Guarner-Lans, V. Nitrosative Stress and Its Association with Cardiometabolic Disorders. *Molecules* **2020**, *25*, 2555. https://doi.org/10.3390/molecules25112555

Shishehbor MH, Aviles RJ, Brennan ML, Fu X, Goormastic M, Pearce GL, Gokce N, Keaney JF Jr, Penn MS, Sprecher DL, Vita JA, Hazen SL. Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. JAMA. 2003 Apr 2;289(13):1675-80. doi: 10.1001/jama.289.13.1675. PMID: 12672736.

Savoia C, D'Agostino M, Lauri F, Volpe M. Angiotensin type 2 receptor in hypertensive cardiovascular disease. Curr Opin Nephrol Hypertens. 2011 Mar;20(2):125-32. doi: 10.1097/MNH.0b013e3283437fcd. PMID: 21245762.

Murray EC, Nosalski R, MacRitchie N, Tomaszewski M, Maffia P, Harrison DG, Guzik TJ. Therapeutic targeting of

inflammation in hypertension: from novel mechanisms to translational perspective. Cardiovasc Res. 2021 Nov 22;117(13):2589-2609. doi: 10.1093/cvr/cvab330. PMID: 34698811; PMCID: PMC9825256.

Gallo Giovanna , Volpe Massimo , Savoia Carmine. Endothelial Dysfunction in Hypertension: Current Concepts and Clinical Implications. Frontiers in Medicine. 2022(8).https://www.frontiersin.org/journals/medicine/articles/10.3389/fmed.2021.798958. DOI=10.3389/fmed.2021.798958. ISSN=2296-858X

Johnstone MT, Creager SJ, Scales KM, Cusco JA, Lee BK, Creager MA. Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation.* 1993;88:2510–2516

Nicolls MR, Haskins K, Flores SC. Oxidant stress, immune dysregulation, and vascular function in type I diabetes. *Antioxid Redox Signal.* 2007;9:879–889.

McVeigh GE, Brennan GM, Johnston GD, et al. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*. 1992;35:771–776.

Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest.* 1996;97:22–28. [PMC free article] [PubMed] [Google Scholar]

Williams SB, Cusco JA, Roddy MA, Johnstone MT, Creager MA. Impaired nitric oxide-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Am Coll Cardiol.* 1996;27:567–574. [PubMed] [Google Scholar]

Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD. Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *J Clin Invest.* 1996;97:2601–2610.

Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. Rev Endocr Metab Disord. 2010 Mar;11(1):61-74. doi: 10.1007/s11154-010-9134-4. PMID: 20186491; PMCID: PMC2882637.

Yeboah J., Folsom A.R., Burke G.L., Johnson C., Polak J.F., Post W., Lima J.A., Crouse J.R., Herrington D.M. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: The multi-ethnic study of atherosclerosis. *Circulation.* 2009;120:502–509. doi: 10.1161/CIRCULATIONAHA.109.864801

Maruhashi T., Soga J., Idei N., Fujimura N., Mikami S., Iwamoto Y., Kajikawa M., Matsumoto T., Hidaka T., Kihara Y., et al. Relationship between flow-mediated vasodilation and cardiovascular risk factors in a large community-based study. *Heart.* 2013;99:1837–1842. doi: 10.1136/heartjnl-2013-304739.

Matsui S., Kajikawa M., Matsumoto T., Iwamoto Y., Iwamoto A., Oda N., Kishimoto S., Hidaka T., Kihara Y., Chayama K., et al. Optimal target level of low-density lipoprotein cholesterol for vascular function in statin naïve individuals. *Sci. Rep.* 2017;7:8422. doi: 10.1038/s41598-017-09043-1.

Higashi Y. Endothelial Function in Dyslipidemia: Roles of LDL-Cholesterol, HDL-Cholesterol and Triglycerides. Cells. 2023 May 1;12(9):1293. doi: 10.3390/cells12091293. PMID: 37174693; PMCID: PMC10177132.