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Evaluation of Anti-Aging Klotho and Apolipoprotein E4 Levels in Coronary Artery Disease Patients and Their Relation with Some Biomarkers of Alzheimer's Disease: Case-Control Study

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Abstract

Background: Coronary artery disease (CAD) is a common heart disease that ranks as the leading cause of death and morbidity worldwide.

Objectives: This study is designed to evaluate the levels of Anti-Aging Klotho and Apolipoprotein E4(APOE4) in the serum of CAD patients and their correlation with established Alzheimer's disease biomarkers, Amyloid-β42 (Aβ42) and Phosphorylated-tau protein (P-tau).

Method: In a case-control study, included two groups, the first group was 60 adult males in the age group 40-80 years, who were clinically diagnosed with CAD by a cardiologist, and the second group was 30 adult males who looked healthy. These assessments were conducted through the measurement of Klotho, Aβ42, P-tau protein, and APOE4.

Results: Serum Klotho levels did not differ between CAD and the control group. In contrast, the mean level of APOE4 in CAD patients increased significantly compared to healthy individuals. Klotho was significantly and positively related to the Aβ42, P-tau protein, and APOE4. Binary logistic regression showed that P-tau protein and APOE4 were negatively associated with CAD (as a risk factor) and performed better in predicting CAD compared with the control group.

Conclusion: The positive relationship of Klotho with Aβ42 and P-tau implies that higher Klotho levels might be connected to the regulation or response to neurodegenerative changes and aging-related processes. Higher Klotho levels reduce the odds of having CAD, identifying it as a protective factor, while P-tau and APOE4 are risk factors.

تقييم مستويات كلوثو المضاد للشيخوخة وأبوليبوبروتين E4 لدى مرضى الشريان التاجي وعلاقتها مع بعض المؤشرات الحيوية لمرض الزهايمر

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الخلاصة

مرض الشريان التاجي هو أحد أمراض القلب الشائعة ويُعد السبب الأول للوفيات والاعتلالات على مستوى العالم. هدفت هذه الدراسة إلى تقييم مستويات بروتين كلوثو المضاد للشيخوخة وأبوليبوبروتينE4 في مصل دم مرضى الشريان التاجي، وعلاقتها مع المؤشرات الحيوية المعروفة لمرض الزهايمر امالويد بيتا و بروتين تاو المفسفر.

تم تنفيذ هذه التقييمات من خلال قياس كل من كلوثو، Aβ42، بروتين تاو المفسفر، و APOE4 باستخدام دراسة حالة-ضابطة. أظهرت نتائج الدراسة أن مستويات كلوثو في المصل لم تختلف بشكل ملحوظ بين مرضى الشريان التاجي والمجموعة .في المقابل، ارتفع متوسط مستوى APOE4 لدى مرضى الشريان التاجي بشكل كبير مقارنة بالأشخاص الأصحاء . كانت مستويات كلوثو مرتبطة بشكل إيجابي وكبير مع Aβ42 ، ومع بروتين تاو المفسفر، ومعAPOE4 . أظهرت نتائج تحليل الانحدار اللوجستي الثنائي أن بروتين تاو المفسفر و APOE4 يرتبطان سلبًا بمرض الشريان التاجي (كعوامل خطر)، وكان أداؤهما أفضل في التنبؤ بوجود المرض مقارنة بالمجموعة الضابطة. تشير العلاقة الإيجابية بين كلوثو و Aβ42وتاو المفسفر إلى أن المستويات الأعلى من كلوثو قد تكون مرتبطة بتنظيم أو استجابة للتغيرات التنكسية العصبية والعمليات المرتبطة بالشيخوخة. تقلل المستويات المرتفعة من كلوثو من احتمالية الإصابة بمرض الشريان التاجي، مما يجعله عامل حماية، في حين يُعتبر كل من تاو المفسفر و APOE4 من عوامل الخطر.

الكلمات المفتاحية: كلوثو، أبوليبوبروتين E4، Aβ42، بروتين تاو المفسفر، مرض الشريان التاجي، مرض الزهايمر.

Introduction

Coronary artery disease is A common cardiac disease that can be defined by the buildup of atherosclerotic plaque in the arterial lumen. Impaired blood flow decreases the myocardium's ability to receive oxygen[(1)]. Atherosclerotic cardiovascular diseases are the number one cause of mortality and morbidity globally [(2)]. Cardiovascular disease ranks first as a cause of disease-related death in Iraq, concerns have also been raised regarding the rising prevalence of disease among the young population in Iraq. Since 1991, the prolonged conflict in Iraq has continued to have a negative impact on the population's health and nutritional status. Iraq's exposure to westernization has also resulted in a rise in the consumption of nutrient-poor and fast food products. The Iraqi population is more susceptible to cardiovascular diseases, such as obesity, diabetes, and hypertension, as a result of these dietary and lifestyle modifications [(3)]. The total number of cases has increased significantly over the last 30 years, from 271 million in 1990 to 523 million in 2019 [(4)]. Among the various forms of cardiovascular disease, CAD is the main cause of mortality and morbidity worldwide [(5)]. Atherosclerosis has been known as the basis of CAD for decades. These processes are highly regulated by several specialized protein and lipid mediators [(6)]. The buildup of lipids and other organic molecules leads to a proliferation of specific cell types within the artery wall that progressively encroaching on the vessel lumen and obstructing the blood flow in large and medium-sized arteries [(7)].

Alzheimer's disease (AD), being the number one in terms of dementia burden, is an insidious age-related neurodegenerative disease characterized by premature neuronal death and is presently considered a global public health threat [(8)]. In 2024, 6.9 million Americans who are 65 years of age or older are predicted to be living with AD [(9)]. Due to the large number of people who suffer from Alzheimer's and other dementias (more than 55 million), research centers throughout the

world are focusing on developing strategies to prevent, delay, improve, and cure these diseases and the devastating consequences they have for individuals, families, communities, and medical systems [10]. An essential goal in current AD research is to develop fluid biomarkers that can identify these pathologies, since these markers could be used to monitor patients' disease progression and assess the effectiveness of drug treatment [11]. Amyloid-β42 (Aβ42) and Phosphorylated-tau protein (P-tau) accumulation are followed by damage to and destruction of neurons (called neurodegeneration) and other brain cells; these are key features of AD [12]. Modifiable atherosclerotic risk factors, such as hypertension, are responsible for one-third of AD-related dementias because they encourage the buildup of amyloid, a misfolded protein, in AD patients' brains. It is difficult to research the relationship between AD and CAD because there are no clear diagnostic criteria. Furthermore, inflammation coexists with the pathophysiology of AD and CAD and is implicated as a major factor in the development of the diseases as well as a consequence [13]. At the molecular level, disequilibrium in Aβ clearance and degradation is linked to endothelial dysfunction, inflammation, and oxidation—all of which are indications of atherosclerosis [14].

The anti-aging protein Klotho has been related to protective effects against cardiovascular disease. Increasing experimental evidence supports that α-Klotho expression is closely related to longevity and cardiovascular benefits, but investigations in humans are still limited [15]. Klotho has been linked to several age-related illnesses, including cardiovascular disease, cancer, neurological diseases, and chronic kidney disease [16,17]. Research has also revealed a decline in *klotho* expression levels in the brains of people with early-stage AD and aging brains. An accurate understanding of the role played by the Klotho protein in the development of AD is still lacking [18]. The toxicity of Aβ and neurofibrillary tangles is believed to be prevented by Klotho, which also gives neurons resistance to oxidative and endoplasmic reticulum stress[19]. Studies suggest Klotho modulates vascular function, inflammation, and amyloidogenesis, processes implicated in both conditions [20]. Klotho-deficient animals demonstrated disruption of endothelial integrity, while Klotho-deficient cells displayed increased Ca^{2+} influx and hyperactivity of Ca^{2+}-dependent proteases. These findings may contribute to vascular hyperpermeability and widespread vascular calcification. Cellular necrosis, lipid accumulation, and inflammation are all part of this process [16].

Apolipoprotein E4 (APOE4) is a glycoprotein that is mostly produced by the liver but is also produced by the brain and a variety of other peripheral tissues.APOE4 is an important constituent of the high-density lipoprotein (HDL-like particles) in the central nervous system, despite its significantly lower concentration than plasma HDL. The three main alleles of AD, ε2, ε3, and ε4, have worldwide frequencies of 8%, 78%, and 14%, respectively, making it the strongest genetic risk factor for AD. In the presence of particular genetic variations, such as in carriers of the APOε4 polymorphism, the association is extremely strong [7,11]. This study is designed to evaluate the levels of Anti-Aging Klotho and APOE4 in the serum of CAD patients and their correlation with established Alzheimer's disease biomarkers, Aβ42 and P-tau protein.

Materials and Methods:

Study Design

A case-control design was selected because it is efficient for investigating the association between CAD and biomarker levels. This design allows for a clear comparison between patients with confirmed CAD and matched controls, making it suitable for exploring potential diagnostic or risk-related biomarkers. This study was done in Tikrit City/Iraq, for patients who underwent a

Coronary Care Unit at Tikrit Teaching Hospital from the period between 1st June to 15th November 2024. This study included two groups, the first group was 60 adult males in the age group 40-80 years, who were clinically diagnosed with CAD by a cardiologist, clinical assessment revealed elevated myocardial enzymes, ischemic alterations in the electrocardiogram (ECG), chest discomfort, and a more than 50% decrease in coronary artery diameter in at least one of the major arteries, as demonstrated by coronary angiography. Exclusion Criteria: percutaneous coronary intervention or coronary artery bypass grafting, percutaneous or surgical revascularization within the previous 3 months, Individuals with a history of neurological disorders, Patients with renal and liver diseases, Patients who had cerebrovascular disease and peripheral artery disease, Patients with tumors, respiratory diseases, congenital heart disease, and autoimmune or inflammatory diseases, Women were excluded from this study. The second group 30 adult males in the age group 40-80 years, who looked healthy with no prior history, nor family history for CAD, and a normal ECG as a control group in this study.

Data and Sample Collection

All subjects underwent a comprehensive medical health examination and filled out questionnaires on health and lifestyle at the time of enrollment. The collected data included: age, Body mass index (BMI), educational level, and CAD risk factors (Diabetes mellitus, hypertension, smoking status, family history, and physical activity). Blood samples were collected from participants at 9:00 am. Five milliliters of blood were drawn from the antecubital veins of the patients and controls, placed in gel tubes devoid of any anticoagulant, allowed to clot for ten to fifteen minutes at room temperature, and then centrifuged for ten minutes at 3000 rpm. Four clear, dry Eppendorf tubes were pipetted with the clear serum for each sample and stored at -80°C until used for determination of Klotho, Aβ42, P-tau protein, and APOE4. Samples from the control subjects were collected and processed in the same way. All biomarkers were measured by the enzyme-linked immunosorbent assay (ELISA) using the commercially available human kit by Sunlong, BioTech/China.

Ethical approval

The research protocol received formal permission No. 107/253 from the scientific committee of Tikrit University's faculty of medicine on April 15, 2024. This clearance was granted for the collection of patient samples and had earlier approved the methodology at Tikrit Teaching Hospital in Tikrit City.

Statistical Analysis

The data analysis for this work was generated using the Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA). Additionally, GraphPad Prism version 8.0 was used for graphical representation. The distribution of the data was checked using the Shapiro-Wilk test as a numerical means of assessing normality. The comparisons between groups were performed by Student's t-test, and variables were presented by mean ± standard deviation for normal data. All hypothesis test results with two-sided p-values less than 0.05 were taken to be statistically significant. Through the use of receiver operating characteristic (ROC) analysis, a suitable threshold with high specificity and sensitivity for the study cases was determined**.**

Results

Relation of Age Groups and BMI between CAD Patients and Control Groups

The age range of participants was 40-80 years, and the mean age of this study was significantly higher in patients compared to the control group (58.25 ± 9.19 vs. 54.07 ± 9.45, p-value 0.047). Also, there was no significant difference in BMI between CAD patients and the control group (28.33±4.39 vs.

28.95±4.11 kg/m^2, p-value 0.520) as clarified in Table 1.

Table 1: Mean levels of age and BMI among patients with coronary artery disease and healthy controls

Variable	Patient Mean±SD	Control Mean±SD	P value
Age(year)	58.25±9.19	54.07±9.45	0.047 **[S]**
BMI (kg/m^2)	28.33±4.39	28.95±4.11	0.520 [NS]

Serum Levels of Klotho, Aβ42, P-tau protein, and APOE4 in CAD Patients and Control

As depicted in Table 2, the mean levels of klotho of patients and control groups showed no significant difference (1311.93±654.12 vs. 1563.14±668.42 (pg/ml), p-value 0.217), The Klotho level was decreased in the CAD group, but differences did not reach statistical significance. Aβ42 shows non-significant differences between coronary artery disease patients and controls (1514.86±400.02 vs.1487.49±611.02 (pg/ml), p-value 0.483). In contrast, the mean levels of P-tau protein in CAD patients were increased significantly compared to healthy individuals (20.65±5.86 vs.15.13±4.59 (ng/L), p-value <0.001). Also, this study found that there was a significant difference in APOE4 level between the case and control group, which is increased in CAD patients compared to the control group (97.36±24.97 vs. 72.31±20.69 (ng/ml), p-value <0.001).

Table 2: Mean levels of biomarkers among patients with coronary artery disease and healthy controls

Biomarker	**Patient Mean ± SD**	**Control Mean ± SD**	**P value**
Klotho (pg/ml)	1311.93±654.12	1563.14±668.42	0.217 [NS]
Aβ42 (pg/ml)	1514.86±400.02	1487.49±611.02	0.483 [NS]
P-tau protein (ng/L)	20.65±5.86	15.13±4.59	**<0.001[S]**
APOE4 (ng/ml)	97.36±24.97	72.31±20.69	**<0.001[S]**
T-test was *: significant at p ≤ 0.05 SD: standard deviation; S: significant; NS: Non-significant.			

Correlation of Study Biomarkers

The response relationship among parameters was examined using Pearson's correlation test analysis in CAD patients. The most important correlations were between serum Klotho levels, which were significantly and positively related to the Aβ42 (r =0.5, p <0.001), P-tau protein (r =0.3, p = 0.029), and APOE4 (r =0.7, p <0.001), as shown in Figure 1. Also, Aβ42 shows a significant positive correlation with P-tau protein (r =0.5, p <0.001), and APOE4 (r =0.6, p <0.001). About P-tau protein shows a significant positive correlation with APOE4 (r =0.5, p <0.001).

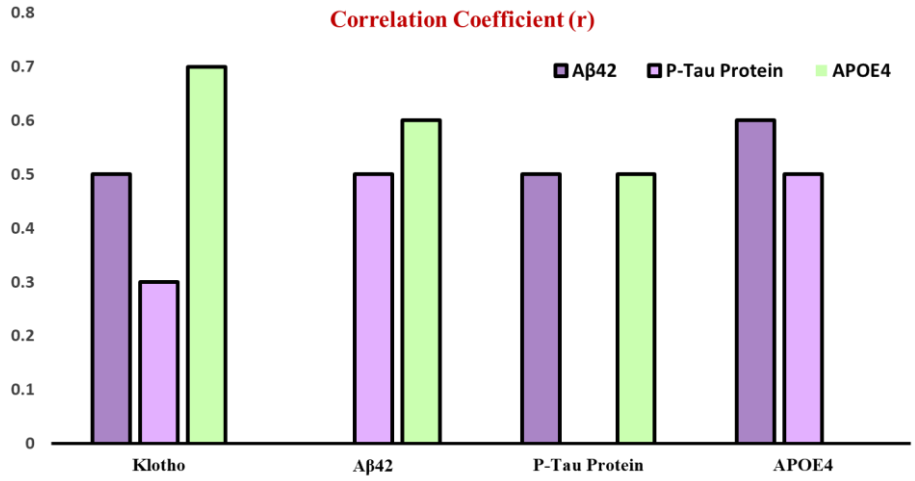

Figure 1: The correlation coefficient (r) between biomarkers among patient groups of the coronary artery disease group compared to the control group.

Prediction of Incident CAD by Study Biomarkers

The results were analyzed using forward logistic regression once binary logistic regression was completed. It was found that the biomarker Klotho was positively associated with CAD (a protective factor) and had a highly statistically significant difference (OR (95% CI): 0.997 (0.995-0.999), p-value 0.003). Aβ42 were non-significant, while P-tau protein and APOE4 were negatively associated with CAD (a risk factor) and had a highly statistically significant difference (OR (95% CI): 1.161(1.015-1.327), p-value 0.030) and (OR (95% CI):1.141(1.055-1.234), p-value < 0.001) respectively, as shown in Table 3.

Table 3: Binary logistic regression represented by Odds Ratio and 95% CIs among patients with coronary artery disease and healthy controls

Variable	OR (Lower-Upper)	P value
Klotho	**0.997 (0.995-0.999)**	**0.003 [S]**
Aβ42	**0.998 (0.996-1.002)**	**0.409 [NS]**
P-tau protein	**1.161 (1.015-1.327)**	**0.030 [S]**
APOE4	**1.141 (1.055-1.234)**	**<0.001 [S]**
p<0.05 considered significantly different- [S]= Significant, [NS]= Non significant, OR= odd ratio		

ROC curve and AUC analysis for Klotho, Aβ42, P-tau protein, and APOE4 patients and healthy subjects

Receiver operating curve (ROC) results and area under the curve (AUC) study for the Klotho, Aβ42, P-tau protein, and APOE4 as analytical parameters were done. P-tau protein and APOE4 showed a good performance in predicting CAD compared with a control group; data are shown in Table 4. Klotho levels: (sensitivity = 91.2%, specificity 38.5%) AUC = 50.4. Aβ42 levels: (sensitivity = 23.7%, specificity 92.6%) at a level = 51.3, for P-tau protein levels: (sensitivity = 76.7 %, specificity 85.2%) at a level = 80.1. Finally, APOE4 levels: (sensitivity = 73.3 %, specificity 72%) at a level = 78.8, the p-values of the AUC were <0.05 and highly statistically significant. The AUC's p-values were statistically significant and less than 0.05. Youden's J statistics of the parameters in

(Figure 2) support these results for Klotho, Aβ42, P-tau protein, and APOE4.

Table 4: AUC and proposed markers obtained by the ROC Curves for prediction in coronary artery disease compared to the control group.

Test Variable	AUP	Sensitivity %	Specificity %	Youden index	Cut-off points	CI (95%)
Klotho	50.4%	91.2%	38.5%	0.297	639.1429	0.371-0.637
Aβ42	51.3%	23.7%	92.6%	0.163	1802.9412	0.384-0.643
P-tau	80.1%	76.7%	85.2%	0.619	16.8539	0.699-0.903
APOE4	78.8%	73.3%	72%	0.453	82.2963	0.688-0.888

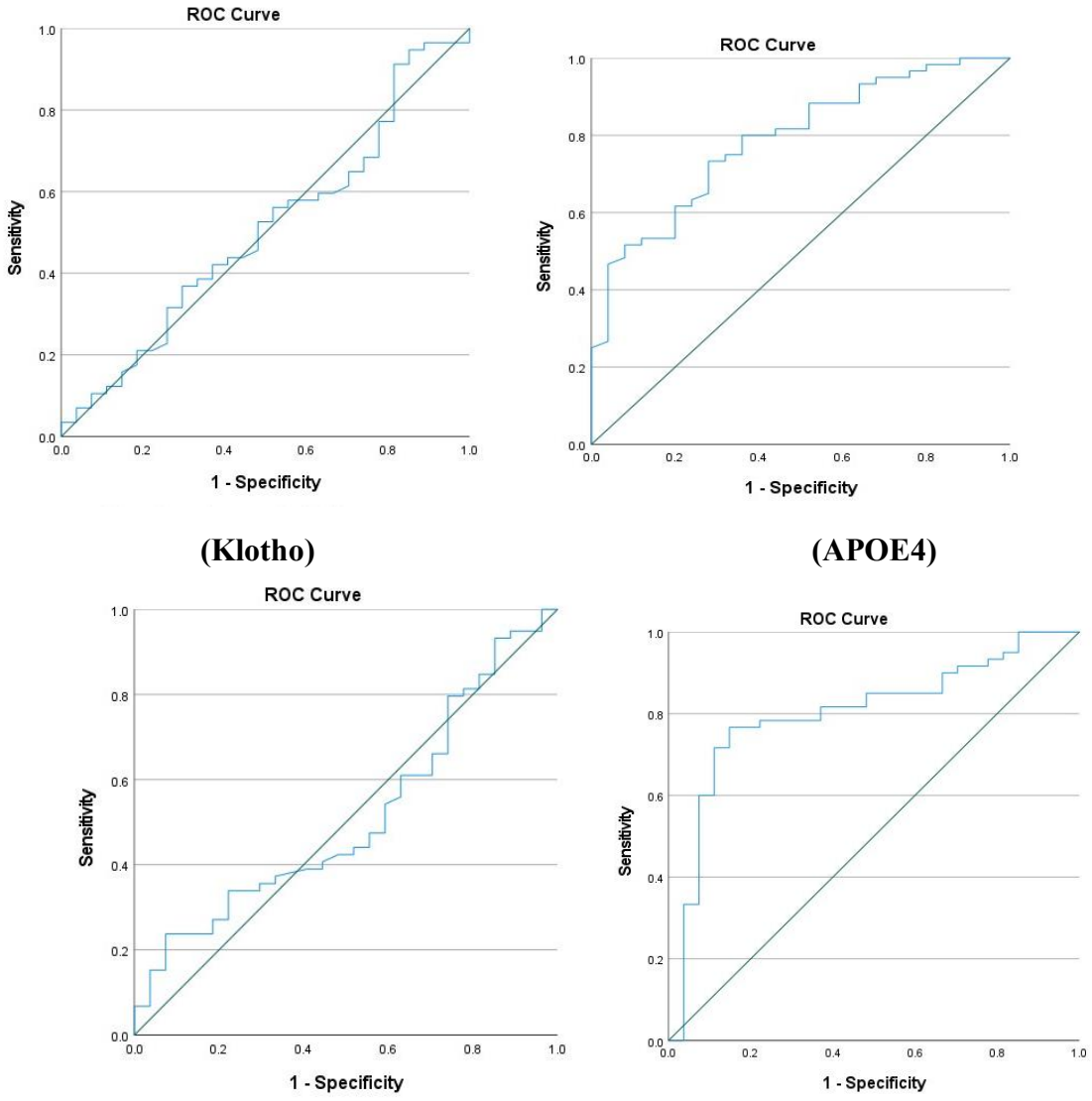

Figure 2: ROC curves for biomarker levels among patients with coronary artery disease to evaluate the best diagnostic criteria for expecting these cases in comparison to the control group.

Discussion

Almost one in three deaths worldwide are believed to be caused by heart and circulation diseases. Nowadays, in developing countries, including Iraq, there are alarming signals of higher rates of CAD at a young age [3]. This work investigated serum levels of anti-aging Klotho and APOE4 in CAD patients; only male patients were included because a previous study showed that the incidence of acute myocardial infarction in male patients was significantly higher than that in female patients in the Iraqi population [21]. Furthermore, a Saudi Arabian study on gender differences in CAD showed that the mean age at which men are more likely to present with CAD is 55 years, compared to 59 years for women. Males were also more likely to smoke. Smoking is a significant risk factor, especially for men, but there is no gender difference in the severity of CAD [22]. Take into account the neurotoxic effects associated with gender in addition to getting clearer results. Evidence suggests that the male brain is more susceptible to numerous toxic exposures than the female brain. This difference includes the male brain's increased neuroinflammatory response, the female brain's decreased susceptibility to oxidative stress, and the neuroprotective effects of progesterone and estrogen, particularly in reducing oxidative stress and inflammation [23].

This study focused on the calculation of the mean differences in biomarkers Klotho, Aβ42, P-tau, and APOE4 among patients with CAD compared to the control group. The main findings of the present study show that patients with CAD have lower concentrations of Klotho. The difference between the two groups was nonsignificant, which means that any difference found was not large enough to be considered statistically significant, and this may be due to the sample size, which may have influenced the results. Additionally, important aspects in the present study include the fact that none of the individuals had renal insufficiency, as chronic kidney disease has been described as a state of Klotho deficiency, and that the subjects were older (58.25±9.19 years in patients vs. 54.07±9.45 years in controls), as lower Klotho has been observed in older age.

Reduced serum Klotho concentration has been associated with increased oxidative stress and apoptotic factors in atherosclerosis. Additionally linked to an increase in cellular age, this decline stimulates atherosclerosis as an age-related condition [24]. The findings of this study could be explained by the fact that a number of medications raise the levels of Klotho in the blood, and some of them may act in the brain via crossing the blood-brain barrier. In medicine, statins and renin-angiotensin-aldosterone system (RAAS) inhibitors are frequently used drug combinations, and this type of medication may have antiaging benefits [19]. Since blood pressure, cholesterol, renal function, cardiovascular disease, and other variables are all significantly impacted by these medications [25,26], it will be difficult to determine Klotho's exact contribution. [19]. The most well-established Klotho-enhancing clinical medications are RAAS inhibitors, particularly valsartan and losartan, which block the angiotensin II receptor. For instance, in diabetic patients, losartan raised Klotho levels by 23% [27]. The most commonly prescribed antidiabetic drug, metformin, raised the levels of Klotho in the blood, kidneys, and urine. It caused mTOR levels to decline, and Klotho suppression reestablished this effect. Further research is needed to fully understand the mechanisms and clinical implications of these drugs [19]. Our observations contrast with the previous study, where the authors observed a significant association between lower concentrations of circulating Klotho and an increased risk of developing long-term atherosclerotic cardiovascular disease (CAD and stroke) [28].

According to clinical research, patients with CAD who have higher Aβ have an increased likelihood of mortality from cardiovascular

disease, in agreement with our results, a study shows that the Plasma Aβ42 levels did not significantly differ between CAD and control groups [29]. Furthermore, plasma Aβ42 levels were significantly higher in *APOE ε*4 allele carriers. Also, as in the brain, two forms of Aβ (Aβ42 and Aβ40) are present in the heart, and their expression is increased in AD. Aβ42 shows the highest tendency to aggregate due to its β-sheet conformation, and it is the main constituent of senile plaques, in association with the hyperphosphorylated tau protein. The heterogeneity may result from variations in patient attributes, such as treatment, or from modifications in Aβ, which occur relatively late in the course of the disease. The pathogenesis and clinical relevance of these biomarkers for cardiac function remain unclear [30].

In contrast, the mean levels of p-tau protein in CAD patients increased significantly compared to healthy individuals. To our knowledge, there is limited direct research specifically on P-tau protein in CAD patients. One of the main pathological characteristics of AD is tau pathology; however, no research has shown a causal link between tau and CAD to demonstrate the pathological association between AD and CAD [31]. In patients with chronic heart failure, a prominent increase in serum P-tau over time was observed, which was predicted by prevalent myocardial dysfunction. When combined, these findings provide insight on the peripheral role of tau protein and raise concerns about the therapeutic approach of reducing tau protein [32]. Notably, P-tau levels in the insoluble fraction determined by each ELISA differ depending on the epitopes of antibodies, it can result in prejudice toward tau isoforms that are processed differently.

The present study found that there was a significant difference in APOE4 levels between the case and control group, which was higher in CAD patients compared to the control group. APOE gene functional inactivation causes athero-susceptibility in mice, and even when kept on a regular low-fat chow diet, APOE−/−mice exhibit substantial lipid depositions in the arterial wall [33]. Aging and APOE4 are the two most significant risk factors for late-onset Alzheimer's disease (LOAD). According to in vitro research, APOE4-treated APOE−/− neurons had higher levels of phosphorylated tau proteins than neurons treated with APOE3 and APOE2. These findings imply that tau phosphorylation may be isoform-specifically facilitated by APOE4. However, the molecular basis of the findings has not been investigated in this study [34]. This finding aligns with the present study results, in which P-tau protein is also significantly increased in the patient group, which means it is increased due to raised APOE4 levels in the same group.

APOE4 binds to triglyceride-rich lipoproteins (such as VLDL-C and chylomicron remnants) with greater affinity than it binds to LDL-C. Comparing APOE4 carriers to those who carry APOE 2 and 3, this characteristic causes a decrease in LDL-C clearance, which raises LDL-C levels [35]. Because APOE plays a crucial role in receptor-mediated endocytosis, where it binds to triglyceride-rich lipoproteins and acts as a ligand for the LDL receptor and LDL receptor-related protein, the mechanisms generating elevated APOE levels are probably attributable to this. According to a review, the elevated level of LDL-C in carriers of the E4 allele is due to under expression of LDL receptors because of accelerated absorption (by the liver) of VLDL-C enriched in APOE4[36].

Although the present study has some strengths, which allowed us to establish the association between biomarkers and CAD and measure the odds ratio. However, our study has some limitations. First, it was difficult to interpret the causality of these relationships. Second, there is a possibility of unknown confounding variables that could affect biomarker levels and were not considered in the study, such as treatment used for CAD patients, systemic inflammatory disorders (chronic systemic inflammation can promote neuroinflammation, oxidative stress, and

blood-brain barrier dysfunction, which may transiently impair clearance) or recent acute cardiovascular events that may cause transient increases in P-tau. Third, the study involved a relatively small sample size, so the findings may not be generalizable to the broader community. Further studies are needed to confirm the reliability of our findings, using a larger number of datasets and participants.

Conclusion

This study did not find enough evidence to conclude that there is a meaningful or statistically significant difference in Klotho and Aβ42 levels between the groups they were comparing. In contrast, P-tau and APOE4 may reflect an association with CAD, in which serum levels were significantly increased in the patient group. The positive relationship of Klotho with Aβ42 and P-tau implies that higher Klotho levels might be connected to the regulation or response to neurodegenerative changes and aging-related processes. The association with APOE4 indicates Klotho's involvement in lipid metabolism and cardiovascular risk factors. Higher Klotho levels reduce the odds of having CAD, identifying it as a protective factor, while P-tau and APOE4 are risk factors.

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