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Molecular Detection of Virulence Genes (spy1258, scpA, sdaB) and Their Association with Clinical Features of *Streptococcus pyogenes* Isolated from Pharyngitis Patients

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Abstract

Background: *Streptococcus pyogenes* is a significant human pathogen responsible for a wide range of diseases.

Objective: This study aimed to identify S. pyogenes isolates from patients with pharyngitis and to investigate their antibiotic resistance profiles and the presence of key virulence genes (scpA, sdaB, and spy1258) using molecular techniques.

Methods: A total of 150 throat swabs were collected from pharyngitis patients attending Tikrit Teaching Hospital, Salah Al-Din General Hospital, and private clinics between January and July 2023. Samples underwent bacteriological identification and biochemical testing for S. pyogenes, with confirmation using the VITEK 2 system. Antibiotic susceptibility testing was conducted, and multidrug-resistant isolates were further analyzed by PCR to detect the presence of virulence genes

Results: Out of 150 collected samples, 122 isolates were identified as Gram-positive bacteria. Among them, 52 isolates (42.6%) were confirmed as S. pyogenes. Antibiotic susceptibility testing showed variable resistance patterns, with four isolates (7.7%) displaying complete resistance to all tested antibiotics. Molecular analysis revealed the presence of the spy1258 gene in three isolates (5.7%), while scpA and sdaB were each detected in one isolate.

Conclusion: The findings highlight the clinical importance of integrating molecular surveillance of virulence factors and antibiotic resistance in S. pyogenes diagnostics. Routine molecular monitoring can improve early detection, guide effective treatment strategies, and help limit the spread of multidrug-resistant strains within the community.

الكشف الجزيئي عن جينات الضراوة (spy1258، scpA، sdaB) وارتباطها بالخصائص السريرية لبكتيريا المكورات العقدية المقيحة المعزولة من مرضى التهاب البلعوم

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الخلاصة

تعد بكتريا العقدية القيحية (المعروفة العقدية من المجموعة أ) من مسببات الامراض البشرية المهمة, والمسؤولة عن مجموعة واسعة من الأمراض التي تتراوح بين العدوى السطحية الخفيفة الى الحالات الشديدة الغازية. وهي السبب البكتيري الأكثر شيوعاً لالتهاب البلعوم وخاصة لدى الأطفال الذين تتراوح اعمارهم بين5-15 عاماً. واذا ترك التهاب البلعوم الناتج العقدية القيحية دون علاج فقد يؤدي الى مضاعفات خطيرة مثل الحمى الروماتيزمية, وخراجات حول اللوزتين, ومتلازمة الصدمة السمية العقدية, مما يؤكد ضرورة التشخيص السريع والدقيق. هدفت هذه الدراسة الى تحديد عزلات العقدية القيحية من مرضى التهاب البلعوم والتحقق من أنماط مقاومتها للمضادات الحيوية, والكشف باستخدام التقنيات الجزيئية. تم جمع 150 مسحة (scpA, sdaB, و(spy1258 عن وجود جينات الضراوة الرئيسة بلعومية من مرضى التهاب البلعوم الذين يراجعون مستشفى تكريت التعليمي, ومستشفى صلاح الدين العام, والعيادات الخاصة, بين يناير ويوليو2023. خضعت العينات للتشخيص البكتيري والاختبارات الكيميائية الحيوية للكشف عن بكتريا العقدية القيحية, وتم تأكيد التشخيص باستخدام جهاز الفايتك. كما أجري اختبار الحساسية للمضادات الحيوية, للكشف (PCR) وتم تحليل العزلات المقاومة للأدوية المتعددة بشكل اكبر بواسطة تقنية تفاعل البوليميراز المتسلسل عن وجود جينات الضراوة. من بين 150 عينة تم جمعها, تم تحديد 122 عزلة على انها بكتريا موجبة لصبغة جرام, من بينها, تم تأكيد 52عزلة (بنسبة 43.6%) هي بكتريا العقدية القيحية. اظهر اختبار حساسية المضادات الحيوية أنماط مقاومة متفاوتة, حيث اظهرت اربع عزلات (7.7%) مقاومة كاملة لجميع المضادات الحيوية المختبرة. اما sdaBمن في ثلاث عزلات (5.7%), بينما تم الكشف عن كلspy1258 التحليل الجزيئي فقد اظهر وجود جين في عزلة واحدة. تبرز هذه النتائج الأهمية السريرية لدمج المراقبة الجزيئية لعوامل الضراوة ومقاومة scp Aو المضادات الحيوية في تشخيص المكورات القيحية. أذ يمكن من الرقابة الجزيئية الروتينية أن تحسن الكشف المبكر, وتوجه استراتيجيات علاجية فعالة, وتساعد في الحد من أنتشار السلالات المقاومة للأدوية المتعددة داخل المجتمع.

الكلمات المفتاحية: التهاب البلعوم، VITEK 2، scpA، sdaB، MDR، spy1258، PCR، GAS.

Introduction

Sore throat is the third most frequent cause of pediatric outpatient visits, accounting for approximately 7.3 million consultations per year. Bacterial infections are responsible for about 30-40% of pediatric pharyngitis episodes [(1)]. A sore throat (also known as pharyngitis) is referred to as inflammation of the mucous membranes and submucosal tissues of the oropharynx, typically caused by viral or bacterial infection. There are two forms of sore throat: acute and chronic [(2)]. An acute sore throat is more common and resolves in ten days. When treating it, the primary objective is usually to address the symptoms. Whereas, in a chronic sore throat, symptoms persist for a longer time and do not improve with standard acute treatment [(3)].

The common bacterial causes of this illness are Group A Streptococci (GAS), *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Chlamydia pneumoniae*, *Pseudomonas aeruginosa*, and Diphtheria [(4)]. *Streptococcus pyogenes*, also known as Group A Streptococcus (GAS) is considered a serious human pathogen that can cause a wide range of localized and systemic diseases, including pharyngitis, rheumatic heart disease (RHD), acute rheumatic fever (ARF), scarlet fever, necrotizing fasciitis, impetigo, and streptococcal toxic shock syndrome (STSS) [(5)]. It thrives on human skin and mucosal surfaces and also secretes a wide range of virulence factors that play a critical role in infecting and damaging the host [(6)].

These virulence factors include enzymes and toxins; among the key enzymes are streptokinase, hyaluronidase, and streptodornase, which facilitate the dissemination of infection throughout host

tissues. The Streptodornase B (also known as DNase B) is a secreted nuclease considered a possible virulence factor in *Streptococcus pyogenes*, encoded by the sdaB gene[7]. These bacteria also secrete the C5a peptidase encoded by the scpA gene, which is a virulence factor that helps the bacteria evade the host's immune response by cleaving human serum chemotaxis at the leukocyte binding site[8]. In addition, GAS produces many toxins, such as streptolysin S and streptolysin O, which are responsible for β-hemolysis observed on a blood agar plate, as well as erythrogenic toxin, which has been linked to the pathogenesis of scarlet fever [9]. Accurate identification of S. pyogenes is important to prevent its spread. The spy1258 gene, a transcriptional regulator belonging to the TetR/AcrR family, is specific to *Streptococcus pyogenes* and serves as a reliable molecular marker for its detection, and this gene offers a valuable tool for the rapid diagnosis of GAS-associated tonsillitis[10].

Thus, pathogenic bacteria employ various strategies to alter the expression of virulence factors to adapt effectively to the changing conditions within the host. This modification occurs in response to different growth conditions and stresses encountered in several niches and phases of infection [11].

Although S.pyogenes causes a considerable amount of sickness, there is no licensed vaccine to protect against this infection. Therefore, worldwide, it is estimated that S. pyogenes causes more than 500,000 deaths each year. Those numbers include thousands of people who initially complained of strep throat, then rapidly declined and expired[12]. Therefore, to prevent serious infections and reduce related mortality, research must continue in order to discover effective vaccinations or other therapeutic options [13].

Materials and Methods:

Source of sample

The research was conducted in several hospitals in Tikrit City, including Salah Al-Din General Hospital, Tikrit Teaching Hospital, and private clinics between January and July 2023. A total of 150 swabs were collected from children diagnosed with a sore throat. Many of whom had a documented history of tonsillar hyperplasia and/or recurrent tonsillitis following repeated failures of antimicrobial treatment. Additionally, relevant demographic and clinical information, including age, sex, and other pertinent details, was recorded for each patient.

Isolation and identification of S. pyogenes

The identification of bacterial colonies was initially based on morphological characteristics and growth patterns , including a distinct colony appearance and the presence of β-hemolysis on blood agar. Further confirmation of *Streptococcus pyogenes* was achieved through biochemical testing and bacitracin disc susceptibility. Final confirmation of Group A Streptococcus (GAS) was performed using the VITEK 2 Compact system.

Antibacterial susceptibility test

Confirmed S. pyogenes isolates were tested for antimicrobial susceptibility using the standard disc diffusion method on Mueller–Hinton agar enriched with 5% blood. The inoculated media were incubated at 37 °C for 24 hours under an atmosphere enriched with 5–10% CO_2. The appropriate disc with known concentration was used, such as (10 µg) Penicillin G, (30 µg) Ceftriaxone, (30 µg) Amoxicillin, (30 µg) Oxacillin, (30 µg) Vancomycin, (15 µg) Azithromycin, (2 µg) Clindamycin, (10 µg) Gentamicin, (5 µg) Levofloxacin, and (30 µg) Chloramphenicol. The interpretation of antimicrobial resistance patterns followed the protocols recommended by the Clinical and Laboratory Standards Institute [14].

DNA Extraction

Genomic DNA extraction was performed from confirmed S. pyogenes isolates cultured in Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 hours. DNA was extracted using a commercial kit (Tinzyme, China) following the manufacturer's instructions. The quality and purity of the extracted DNA were assessed using a

NanoDrop spectrophotometer by measuring the absorbance ratio at 260/280 nm, and further confirmed by electrophoresis on a 1% agarose gel.

Genotypic identification of spy1258, scpA, and sdaB genes

Polymerase chain reaction (PCR) was employed to detect the spy1258 (407 bp), scpA (622 bp), and sdaB (440 bp) genes, and was performed in a total reaction volume of 20 µL. Each PCR mixture consisted of 3 µL of DNA template, 10 µL of Master Mix, 1 µL of each reverse and forward primer, and 5 µL of nuclease-free water. PCR-amplified products were resolved by electrophoresis on a 2% agarose gel at 5 V/cm for 90 minutes. Following separation, the gel was stained with ethidium bromide by immersion with gentle agitation for 45 to 60 minutes, then visualized and documented utilizing a gel documentation system. The specific primer sequences used for gene amplification are presented in Table 1, while the optimized thermal cycling conditions for detecting spy1258, scpA, and sdaB genes are shown in Table 2.

Table 1: Sequence of oligonucleotide primers for spy1258, scpA, and sdaB genes (*F: Forward primer, R: Reverse primer).

Primer name	Primer sequence		Size of Product (bp)
SPY1258	F	AAAGACCGCCTTAACCACCT	407 bp
	R	TGCCAAGGTAAACTTCTAAAGCA	
scpA	F	GCTCGGTTACCTCACTTGTCC	622 bp
	R	CAATAGCAGCAAACAAGTCACC	
sdaB	F	TATAGCGCATGCCGCCTTTT	440 bp
	R	TGATGGCGCAAGCAAGTACC	

Table 2a: The optimum condition of detection SPY1258 gene.

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	94°C	2 min	1
2-	Denaturation -2	94°C	30s	30
3-	Annealing	58°C	30s	
4-	Extension	72°C	30s	
5-	Final extension	72°C	2 min	1

Table 2b: The optimum condition of detection scpA gene.

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	94°C	2 min	1
2-	Denaturation -2	94°C	30s	30
3-	Annealing	59°C	30s	

4-	**Extension**	**72°C**	**30s**	
5-	**Final extension**	**72°C**	**2 min**	**1**

Table 2c: The optimum condition of detection sdaB gene.

No.	Phase	Tm (°C)	Time	No. of cycle
1-	**Initial Denaturation**	**94°C**	**2 min**	**1**
2-	**Denaturation -2**	**94°C**	**30s**	**30**
3-	**Annealing**	**61°C**	**30s**	
4-	**Extension**	**72°C**	**30s**	
5-	**Final extension**	**72°C**	**2 min**	**1**

Results

The results of this research showed that out of 150 throat swab samples collected from children with pharyngitis, 122 isolates (81.3%) were identified as Gram-positive bacteria, while the remaining 28 isolates (18.6%) were Gram-negative (Table 3). Among the Gram-positive isolates, *Streptococcus pyogenes* (GAS) was detected in 52 samples (42.6%) based on clinical evaluation and bacterial culture, with confirmation achieved through the VITEK 2 system.

Table (3): Number and percentage of isolated bacterial isolates.

Type of Microorganism	Number of isolate	%
Gram-positive bacteria	122	81.3%
Gram-negative bacteria	28	18.6%
Total	150	100 %

As seen in Figure 1, the highest rate of GAS pharyngitis was in 5–7 year olds, followed by the 11-13year age group, then the 8- 10 year age group, and the lowest rate (9.61%) was found in the age group 14-17.

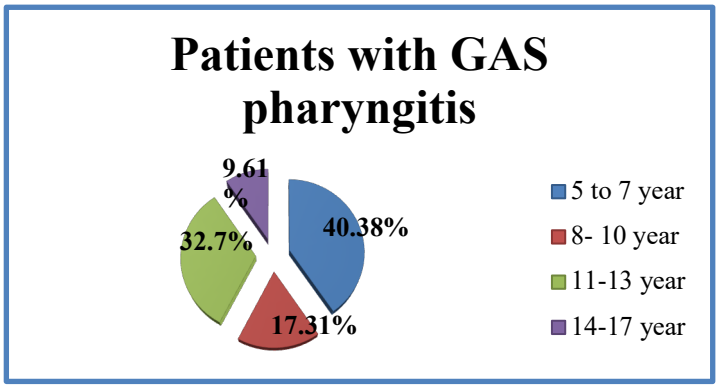

Figure 1: Distribution of patients with GAS pharyngitis according to age.

Figure 2 illustrates the antibiotic susceptibility profiles of *Streptococcus pyogenes* isolates. The strains demonstrated diverse resistance patterns across the tested antimicrobial agents. Remarkably, 7.7% of the isolates (n = 4) exhibited complete resistance to all antibiotics typically prescribed for pharyngitis. Conversely, most of the isolates were susceptible to Clindamycin, Penicillin G, Gentamicin, and Levofloxacin. Resistance rates to other drugs were recorded as follows: Oxacillin (100%), Vancomycin (98%), Amoxicillin (88%), Ceftriaxone (67%), and (46%) for each Chloramphenicol and Azithromycin.

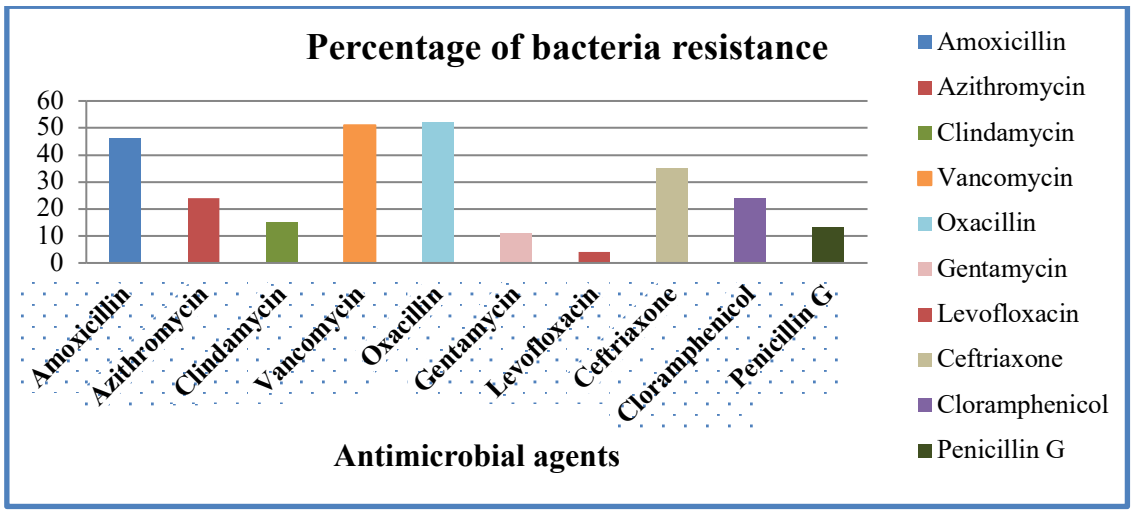

Figure 2 : Antimicrobial susceptibility of *S. pyogenes.*

Detection of spy1258, scpA, and sdaB genes by Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from four (7.7%) *Streptococcus pyogenes* isolates that exhibited resistance to all antibiotics tested in this study. The presence of DNA was confirmed by the appearance of distinct bands following agarose gel electrophoresis. The resulting PCR products were analyzed by comparing the observed bands on agarose gel electrophoresis to a 100 bp DNA ladder to verify the expected fragment sizes. The analysis revealed that three of the multidrug-resistant (MDR) isolates carried the spy1258 gene (representing 5.7%), while the scpA and sdaB genes were each detected in one isolate. Figure 3,4.

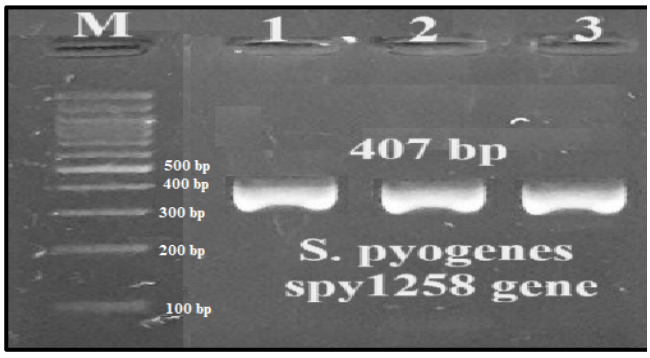

Figure 3: Agarose gel electrophoresis showing PCR product of the spy1258 gene.

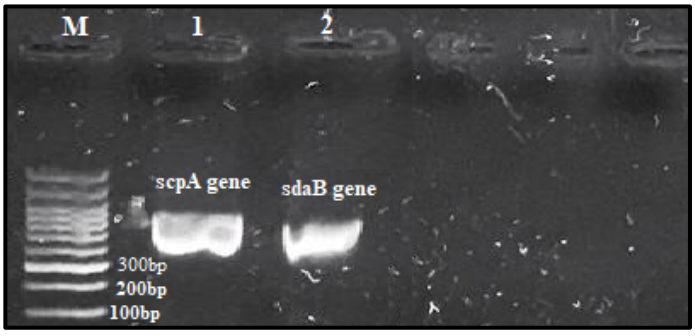

Figure 4: Electrophoretic analysis of PCR amplification products of the scpA and sdaB gene**.**

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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