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Original article

Surface-enhanced Raman Spectroscopy of Blood Serum for Clustering Patients with Clinically Evident Atherosclerosis

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Abstract: *Abstract* — A current focus in modern cardiology is the identification of novel circulating metabolomic and proteomic parameters that may serve as integral biomarkers of atherosclerosis and its phenotypes, including ischemic heart disease (IHD) and its combinations with chronic heart failure (CHF). Conventional analytical techniques for assessing blood biochemical parameters, such as chromatography and mass spectrometry, are labor-intensive, complex, and costly. Surface-enhanced Raman spectroscopy (SERS) emerges as a promising technique that generates a spectral *portrait* of disease, enabling the identification of unfavorable features predictive of atherosclerosis development and progression through mathematical modeling.
This study aimed to evaluate the utility of serum SERS in classifying patients according to distinct phenotypes of peripheral atherosclerosis and IHD.
Materials —The multifocal atherosclerosis (MFA) group included 69 patients of both sexes, while the IHD complicated by CHF group comprised 61 age- and sex-matched patients. The control group consisted of 75 patients without clinical signs of atherosclerosis.
Results —Mathematical modeling using projection to latent structures discriminant analysis (PLS-DA) of serum SERS data demonstrated high accuracy (0.93–1.00) in distinguishing patients with clinical manifestations of atherosclerosis from those without, based on spectral frequencies of 670–680, 718, 1004, 1073, 1146, and 1439 cm^{-1}. The SERS method enables the detection of a metabolic blood profile associated with clinically manifest CHF syndrome (NYHA class II–III) complicating IHD at frequencies of 672, 728, 1077, 1123, 1214, 1284, and 1402 cm^{-1}. Given the relative simplicity and high discriminative power of the SERS method, it holds promise for conducting studies aimed at detecting subclinical stages of diseases associated with atherosclerosis, refining IHD risk stratification, and optimizing therapy for affected patients.
Summary — Significant differences detected by SERS enable reliable classification of patient group membership among the three studied groups.

Keywords: Surface-enhanced Raman spectroscopy (SERS), multifocal atherosclerosis, chronic heart failure, mathematical modeling.

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Introduction

Atherosclerosis-related diseases – including ischemic heart disease, cerebrovascular disease, and peripheral artery disease – pose significant medical and societal challenges in modern healthcare. Fundamentally, atherosclerosis is a chronic condition marked by an extended subclinical phase during which arterial damage to vital organs often remains clinically silent. The initial arterial event is invariably clinically significant and frequently leads to disability or death. Detecting early, preclinical indicators of atherosclerosis and assessing disease progression in symptomatic patients remain critical objectives in contemporary cardiology [1].

Currently, validated cardiovascular disease (CVD) risk stratification systems demonstrate insufficient accuracy in predicting individual-level events. Personalized risk assessment should incorporate a broader array of parameters beyond traditional factors such as age, sex, smoking status, blood pressure, and cholesterol levels. Consequently, a major focus in modern cardiology is the identification of novel genetic, metabolic, and instrumental biomarkers [2]. However, conventional analytical techniques for assessing circulating biochemical parameters, including chromatography and mass spectrometry, remain labor-intensive, complex, and costly.

Raman spectroscopy (RS) fundamentally involves recording the scattering spectrum generated when a sample – such as dried blood serum – is irradiated with a laser. Unlike conventional scattering, Raman scattering arises from molecular vibrations at frequencies different from the incident radiation. Different molecular compounds vibrate at characteristic frequencies, providing a basis for their identification. However, extracting this useful signal is challenging, as it is thousands of times weaker than the incident radiation. Immobilizing biological molecules on substrates composed of silver nanoparticles exploits the plasmon resonance phenomenon to amplify the Raman scattering signal, thereby enhancing the method's sensitivity by several orders of magnitude.

Surface-enhanced Raman scattering (SERS) is a technique that generates a metabolic portrait of disease, highlighting unfavorable features predictive of atherosclerosis development and progression, identifiable through mathematical modeling. This study represents an initial step toward this broader objective, aiming to evaluate the potential of blood serum Raman spectroscopy to identify patients with distinct phenotypes of peripheral atherosclerosis and ischemic heart disease.

Material and Methods

Clinical Characteristics of the Study Population

A total of 205 participants were included in the study. In alignment with the objectives of this cross-sectional study, the design (refer to *Figure* 1) consisted of two primary groups:

Multifocal Atherosclerosis (MFA) Group: This group comprised patients from the Department of Cardiovascular Surgery at the V. D. Seredavin Samara Regional Clinical Hospital, including both sexes, aged 33 to 76 years, who exhibited no clinical signs of chronic heart failure (CHF). A combination of ischemic heart disease (IHD) with clinically significant stenosis of the brachiocephalic trunk (BCT) of 50% or more was identified in 15 (22%) of the patients. Additionally, 13 (19%) of the participants had peripheral artery atherosclerosis accompanied by manifestations of chronic arterial insufficiency (CAI). Furthermore, in 35 (51%) of the cases, BCT stenosis was associated with CAI, while in 6 (8.7%) of the cases, it was linked to renal artery stenosis.

IHD with CHF Group: The second comparison group included 61 patients from the Cardiology Department of the N. I. Pirogov Municipal Clinical Hospital in Samara, who were participants in the Russian national registry of CHF patients known as 'Priority,' where CHF complicated the course of IHD. Patients in this group were matched for gender and age. The diagnosis of CHF was established based on the 2021 criteria set forth by the European Society of Cardiology (ESC) [3]. All patients in this group had their NT-proBNP levels measured, as international guidelines recommend this marker for confirming CHF syndrome. CHF was diagnosed if NT-proBNP levels were >300 pg/ml for patients in sinus rhythm and >900 pg/ml for those with chronic atrial fibrillation (AF). The study received ethical approval from the Ethics Committee of Samara State Medical University, Ministry of Health of Russia. All patients included in the study provided informed consent to participate.

Inclusion Criteria: A confirmed diagnosis of IHD is required, either clinically confirmed or via instrumental methods such as coronary angiography, a history of myocardial infarction, and verification of angina syndrome through stress tests. Additionally, clinical manifestations of atherosclerosis in other regions and/or arterial stenosis greater than 50% are necessary.

Exclusion Criteria: Any acute conditions that may influence the severity of the patient's condition at the time of hospitalization or within the three months prior, including acute myocardial infarction, acute stroke, and acute pneumonia.

Chronic Debilitating Diseases, including:

- Type 1 diabetes mellitus (T1DM);
- Insulin-dependent type 2 diabetes mellitus (T2DM);
- Known oncological diseases;
- Chronic kidney disease (CKD) stage 4 or higher;
- Liver cirrhosis;
- Chronic obstructive pulmonary disease (COPD) stage 2 or higher;
- Hemoglobin levels below 90 g/L.

As illustrated in *Table* 1, the most significant difference between the main groups was the absence of clinical signs of chronic heart failure (CHF) in the multifocal atherosclerosis (MFA) group.

The comparison group comprised 75 patients of both sexes, aged 33 to 68 years, with no history of diseases associated with atherosclerosis, as confirmed by routine physical examinations and electrocardiograms (ECG). Cardiovascular risk was evaluated using the SCORE system, applicable to individuals aged 40 to 65 years. For patients older than 65 years, cardiovascular risk was classified as very high. Within the comparison group, 49 patients (65%) were classified as moderate risk, 16 patients (21%) as high risk, and 10 patients (14%) as very high risk (10% or more). Antihypertensive medication use and/or elevated blood pressure (≥140/90 mmHg) were observed in 24 patients (32%) within this group, qualifying them for a diagnosis of arterial hypertension (AH).

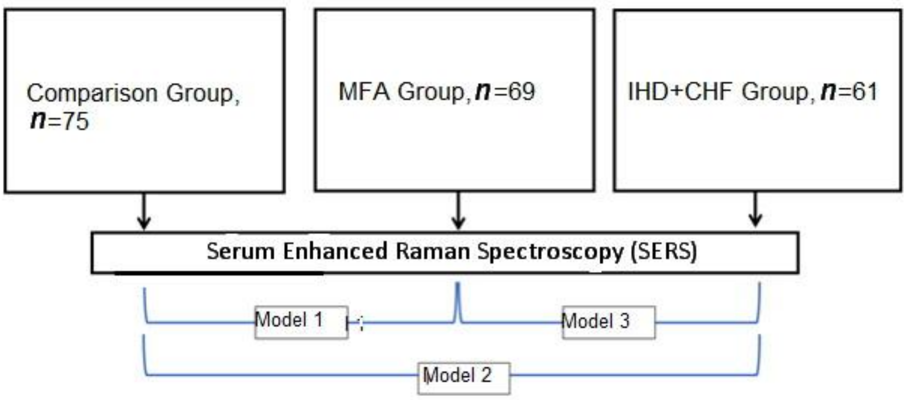

Figure 1. Study Design

Table 1. Frequency of Syndromes and Conditions in the Main Groups

Parameters	*MFA Group (n=69, 100%)*	*IHD+CHF Group (n=61, 100%)*	*p-value*
Arterial hypertension	67 (97%)	61 (100%)	0.235
History of myocardial infarction	41 (59%)	57 (93%)	0.000
History of stroke	7 (10%)	3 (5%)	0.334
History of CABG surgery	8 (11%)	4 (7%)	0.376
History of PCI surgery	23 (33%)	9 (15%)	0.015
Type 2 diabetes mellitus	12 (17%)	12 (20%)	0.822
Atrial fibrillation	16 (23%)	15 (24%)	0.999
Complete left bundle branch block (LBBB)	5 (7%)	6 (10%)	0.754
CHF Stage IIA (IIA/IIB)	0	31 (51%)	0.000
CHF Stage IIB	0	30 (49%)	0.000
CHF Functional Classes (NYHA)	8 (12%)	0	0.035
NYHA II	0	9 (15%)	0.006
NYHA III	0	49 (80%)	0.000
NYHA IV	0	3 (5%)	0.340

CABG, coronary artery bypass grafting; MI, myocardial infarction; Stroke, acute cerebrovascular accident; LBBB, left bundle branch block; CHF, chronic heart failure; PCI, percutaneous coronary intervention.

Table 2. Main Parameters in the Examined Groups

Parameter	*Comparison Group (n=75) M±SD*	*MFA Group (n=69) M±SD*	*IHD+CHF Group (n=61) M±SD*
Age, years	61.97±9.52	63.26±6.64	71.34±9.45 ΔΔ
BMI, kg/m²	24.53±6.06	26.71±4.23	29.63±8.12 Δ
Systolic BP, mmHg	129.32±12.04	164.35±20.60 *	113.75±9.52 ΔΔΔ
Diastolic BP, mmHg	77.22±10.30	80.88±5.72	73.21±9.06 ΔΔ
Glucose, mmol/L	5.40±1.38	6.14±2.57	6.51±2.89 *
Total Cholesterol, mmol/L	5.42±0.60	4.42±1.49 **	3.52±1.01 ΔΔ
Creatinine, μmol/L	82.99±16.01	96.03±28.14	107.77±41.08 **
GFR, mL/min/m²	79.85±15.84	75.13±19.84	60.09±21.08 ΔΔ
Erythrocytes, 10^{12}/L	4.61±0.60	4.48±0.66	4.22±0.70 Δ
Hemoglobin, g/L	139.40±13.85	136.84±19.42	122.20±23.66 Δ
Leukocytes, 10^9/L	6.04±1.30	8.32±2.82 *	7.78±2.03 **
Platelets, 10^3/L	240.81±56.81	238.00±81.09	226.39±102.7

BMI, body mass index; systolic BP, systolic blood pressure; *GFR*, glomerular filtration rate. Statistical significance compared to the control group: * p<0.05, ** p<0.01, *** p<0.001. Statistical significance compared to the MFA group: Δ p<0.05, ΔΔ p<0.01, ΔΔΔ p<0.001.

Table 3. Characteristics of PLS-DA Discrimination by Age in the "IHD+CHF" Group Based on Multivariate Analysis of Serum Spectral Characteristics

Subsample	*Specificity, Mean (Min-Max)*	*Sensitivity, Mean (Min-Max)*	*Accuracy, Mean (Min-Max)*
Training	0.66 (0.50-0.76)	0.75 (0.57-0.83)	0.70 (0.54-0.77)
Cross-validation	0.62 (0.56-0.69)	0.65 (0.53-0.71)	0.64 (0.55-0.70)
Test	0.39 (0.18-0.55)	0.75 (0.42-1.00)	0.57 (0.48-0.71)

Statistical analyses were performed using SPSS version 25.0 (IBM Corporation, Armonk, New York, USA; license No. 5725-A54). The Shapiro-Wilk test was used to assess the distribution of quantitative variables. Data presented in *Table* 2 met the criteria for normal distribution and are expressed as mean ± standard deviation (M±SD). Differences between groups were evaluated using Student's t-test with Bonferroni correction. Categorical variables were analyzed using Pearson's chi-square test (χ^2), with Yates' correction or Fisher's exact test applied as appropriate.

Surface-Enhanced Raman Spectroscopy (SERS) of Blood Serum

The method for acquiring and analyzing spectral characteristics of blood serum using SERS follows the approach detailed in our previous studies [4, 5]. This technique enables the acquisition of surface-enhanced Raman spectra from blood serum samples applied onto a substrate composed of silver nanoparticles.

Blood serum samples were collected from patients in the morning after an overnight fast and placed into sterile tubes, then frozen at −30°C (−22°F). Prior to analysis, samples were thawed at room temperature. For spectral analysis, 1.5 µL of serum was applied onto a substrate coated with silver nanoparticles and allowed to dry for 30 minutes. Spectral analysis was performed using an experimental setup comprising a spectrometric system (EnSpectr R785, Spektr-M, Chernogolovka, Russia) and a microscope (ADF U300, ADF, China).

Spectra were excited in the near-infrared range using a laser module with a central wavelength of 785 nm. Each acquired spectrum consisted of a discrete set of 1700 parameters within the studied frequency range. Preprocessing of spectral data included noise smoothing, removal of autofluorescent background, and normalization. Noise smoothing was performed using the Savitzky–Golay filter with a window width of 15, applying a first-order polynomial and zero-order derivative (*i.e.*, no derivative). The smoothed spectra were subsequently subjected to autofluorescent background removal using a polynomial fitting method. Finally, spectral characteristics were normalized using the Standard Normal Variate (SNV) method based on standard deviation.

Model Construction for Classification

Each tested serum sample is associated with prior information regarding its membership in a specific group and the presence of certain biochemical characteristics. Thus, the data were analyzed using supervised learning techniques. To prevent overfitting, stability analyses of the constructed models and the selection of optimal parameters were conducted using 10-fold cross-validation (k=10). After cross-validation and determination of optimal model parameters, the entire dataset was randomly divided into training (80%) and test (20%) sets. It is important to note that when partitioning the original dataset into training and test samples (both during cross-validation and for final model construction), separation was performed by subject to prevent spectral characteristics from the same individual appearing in both sets, which could lead to overestimation of model performance. During model construction, predictor significance in the classification task was assessed using Variable Importance in Projection (VIP). Analysis of the VIP distribution enabled identification of spectral bands and corresponding serum components that differ between classification groups.

Classification of spectral data was performed using Discriminant Analysis with Projection to Latent Structures (PLS-DA). The SIMPLS algorithm was selected to implement the PLS-DA method. Multivariate analysis was conducted using the MDAtools package within R Studio software. The optimal number of loading vectors was determined based on the first local minimum of the root mean square error (RMSE) plot, calculated across varying numbers of components and cross-validation predictions.

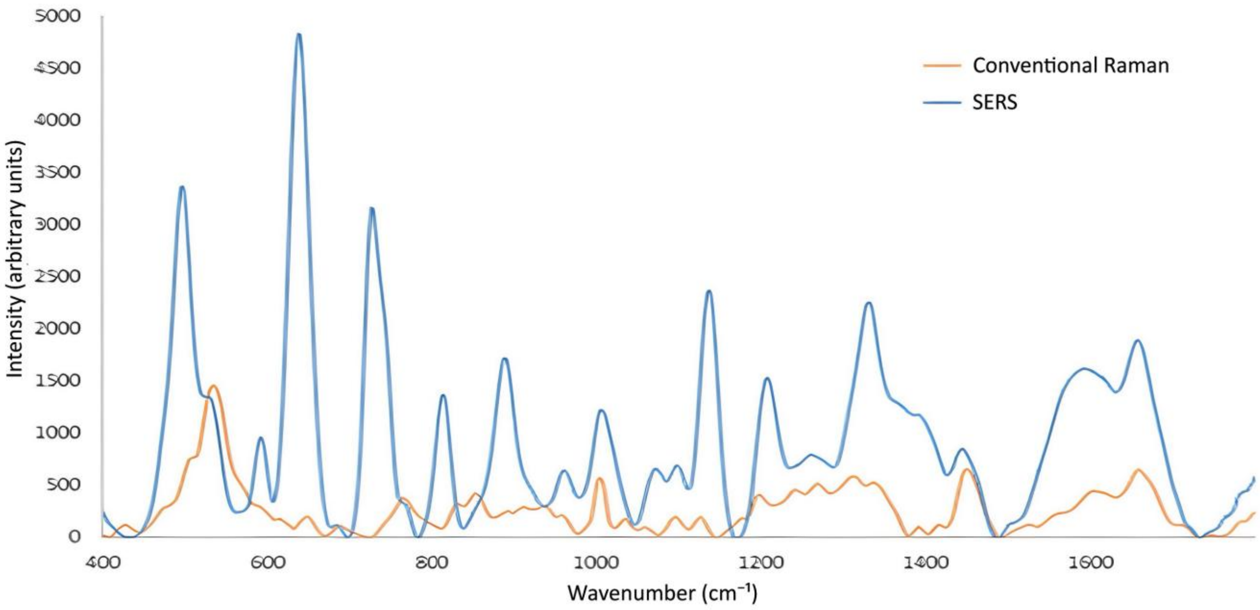

Figure 2. Comparison of spontaneous Raman spectrum and surface-enhanced Raman spectrum (SERS) of serum from a patient in the comparison group.

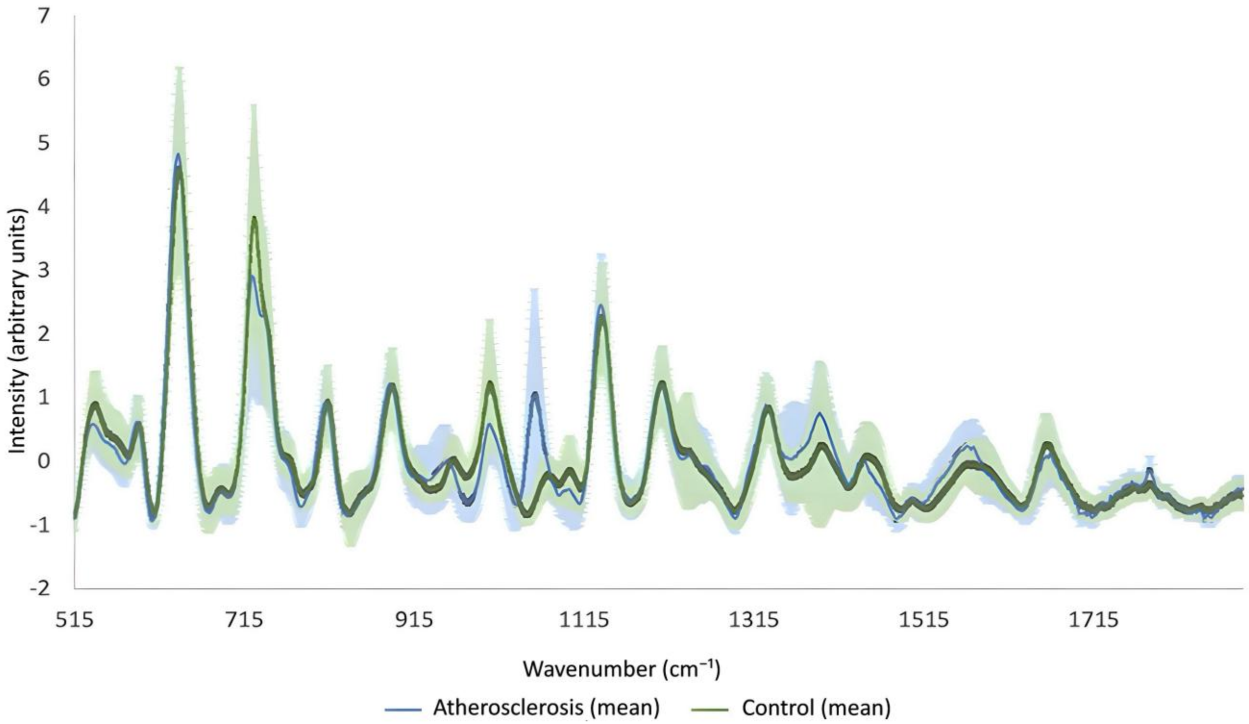

Figure 3. Averaged spectral characteristics of serum in the comparison group and the MFA group.

Results

As shown in *Table* 2, patients with MFA did not differ from the comparison group in age; however, they exhibited increased *body mass index* (*BMI*), higher smoking prevalence, and significantly elevated blood pressure, glycemia, and leukocyte counts. Paradoxically, the lower total cholesterol levels may be attributed to the use of cholesterol-lowering medications, primarily statins. Patients with chronic heart failure (CHF) were significantly older than both the comparison group and those with MFA, necessitating investigation into the effect of age on SERS characteristics.

Since the median and mean age in the "IHD+CHF" group was 71 years, this group was divided by age into those younger than 71 years (n=30) and those aged 71 years or older (n=30). The resulting dataset was then randomly split, with 80% of subjects allocated for model training (training sample) and 20% for model testing (test sample). The model was trained on the training sample and subsequently applied to classify the test sample. This division was

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repeated four additional times. *Table* 3 presents the characteristics of discrimination based on age within the "IHD+CHF" group, derived from multivariate analysis of serum spectral characteristics. The receiver operating characteristic (ROC) curves for discriminating serum spectral characteristics by age in patients with IHD+CHF exhibited minimum and maximum *area under the curve* (*AUC*) values of 0.65 and 0.76, respectively, across five iterations of model construction.

The obtained data suggest that age induces subtle changes in the SERS parameters of serum in patients with chronic heart failure (CHF). Furthermore, patients with CHF exhibited a significantly higher *body mass index* (*BMI*) attributable to obesity, a lower *glomerular filtration rate* (*GFR*) indicative of chronic kidney disease (CKD), and reduced concentrations of erythrocytes and hemoglobin. The observed hypotension in these patients can be attributed to the diminished contractile function of the heart. Additionally, the marked reduction in total cholesterol is associated with hepatic metabolic dysfunction, a characteristic feature of congestive CHF.

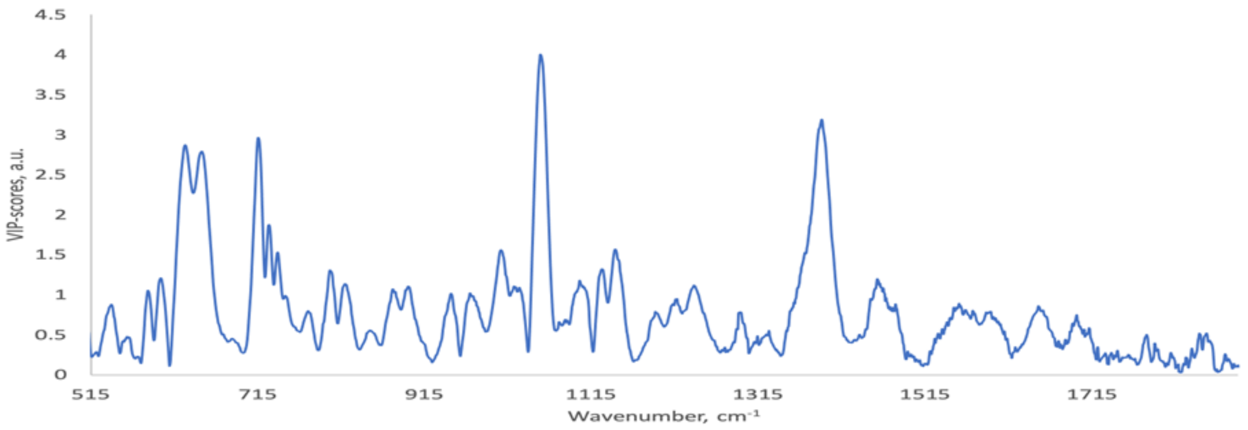

Figure 4. Very important parameters (VIP) of Raman scattering for Mathematical Model #1: separating the comparison group and the MFA group. VIP were identified at 670-680, 718, 1004, 1073, 1146 и 1439 см$^{-1}$.

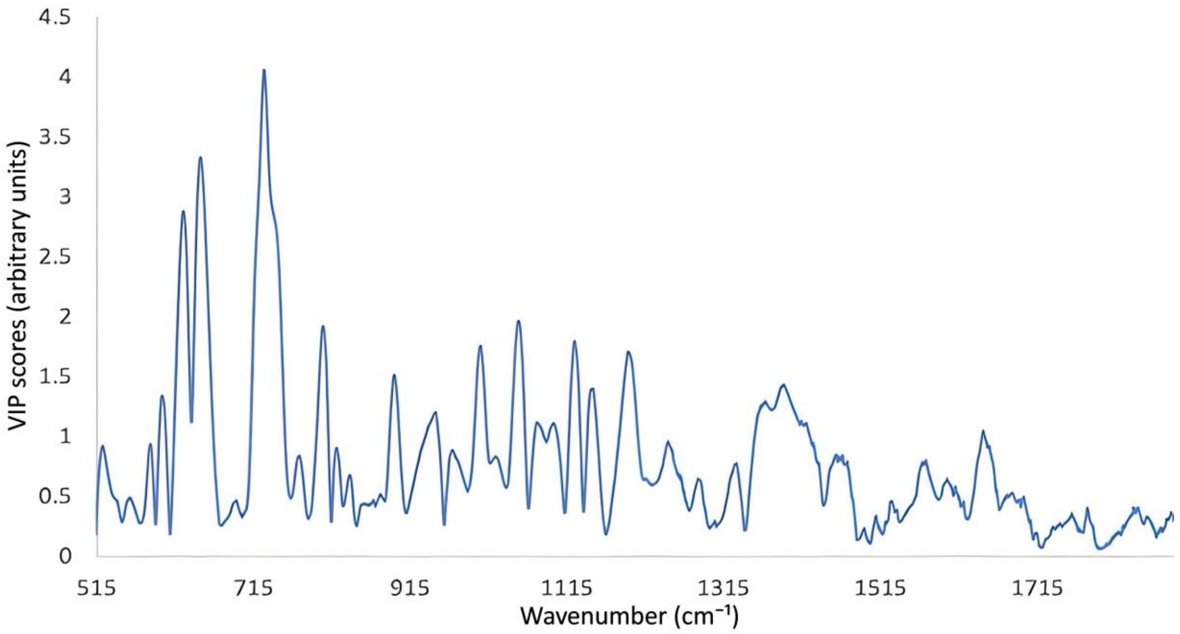

Figure 5. Very important parameters (VIP) of Raman scattering for Mathematical Model #2: separating the comparison group and the IHD+CHF group. The most significant frequencies were identified at 665, 681, 720, 785, 1080, and 1208 cm^{-1}.

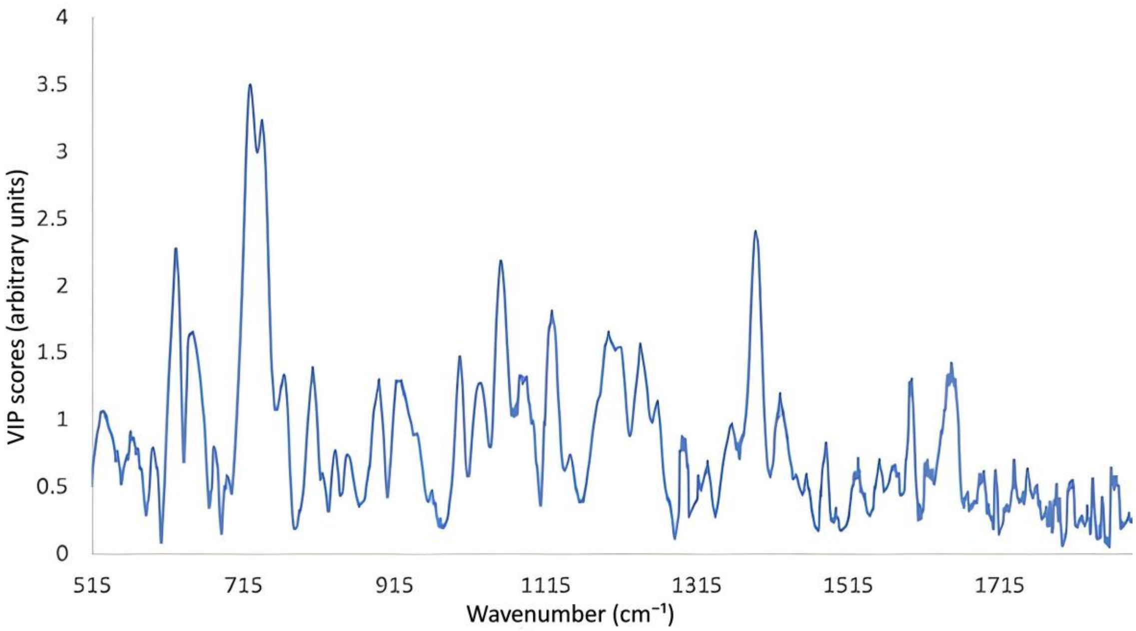

Figure 6. Very important parameters (VIP) of Raman scattering for Mathematical Model #3: separating the MFA and IHD+CHF groups. Significant frequencies for discrimination were identified at 672, 728, 1077, 1123, 1214, 1284, and 1402 cm^{-1}

Table 4. Discriminant characteristics of the constructed classification models

Model	Comparison Groups	Sensitivity	Specificity	Accuracy
1	MFA *vs.* Control Group (*n*=144)	1.00	1.00	1.00
2	IHD+CHF *vs.* Control Group (*n*=136)	1.00	1.00	1.00
3	IHD+CHF vs MFA (*n*=130)	0.96	0.91	0.93

The smoking frequency in the MFA group (0.78) was significantly higher than in both the comparison group (0.28) and the IHD+CHF group (0.17), with a significance level of p<0.030. These intergroup differences are expected, as they reflect the relationship among established risk factors – smoking, excess weight, and lipid and glucose metabolism disorders – associated with MFA which also manifested concomitantly with type 2 diabetes mellitus (see *Table* 1) in both primary groups. The systemic inflammatory response, as indicated by leukocyte levels, is likewise characteristic of IHD and its complications with CHF. The importance of this preliminary analysis lies in elucidating the primary biochemical alterations that serum Raman spectroscopy (RS) may reflect.

Figure 2 illustrates the comparison between spontaneous Raman spectra and surface-enhanced Raman spectra (SERS). Notably, SERS provides a significantly higher signal amplitude as well as a variable enhancement factor depending on the Raman frequency. This variability accounts for the differences observed when employing various nanosubstrate fabrication methods for SERS. *Figure* 3 presents the averaged spectral SERS characteristics of blood serum from the control group and the MFA group.

Figures 4, 5, and 6 display the most significant frequencies identified in the mathematical discrimination models, using a criterion of at least 1.5 arbitrary units.

For Mathematical Model #1, which separates the MFA group from the comparison group, the most significant frequencies based on Variable Importance in Projection (VIP) scores were observed at 670–680, 718, 1004, 1073, 1146, and 1439 cm^{-1}.

According to our data, the SERS method demonstrates high accuracy in discriminating between patients of investigated groups in the comparison group and those in the MFA group (see *Table* 4).

Discussion

The pathophysiology of atherosclerosis, IHD and CHF is intricately linked to the metabolic profile of blood. The Human Metabolome Database comprises a comprehensive list of 220,845 metabolites – molecules with a mass less than 1.5 kilodaltons – including carbohydrates, lipids, amino acids, and ketones [7]. Concurrently, approximately 90% of the total mass of proteins circulating in the blood is attributed to six high-molecular-weight proteins, each exceeding 50 kDa in mass [8]. Furthermore, a vast array of chemical elements, whose mass and concentration in blood serum can vary by several hundredfold, cannot be adequately accounted for using Raman spectroscopy (RS). It is also important to note that a single chemical substance possesses multiple vibrational centers, resulting in multiple peaks in RS spectra. Moreover, the spatial configuration of molecules significantly influences RS measurements. Consequently, accurately determining the chemical structures responsible for vibrations at specific frequencies presents considerable challenges. Although extensive spectral libraries have been developed and are actively updated for this purpose, their accuracy remains unvalidated. While RS is fundamentally an analytical technique, at the current stage of surface-enhanced Raman spectroscopy (SERS) development, it is more appropriate to consider the spectral output as a "portrait" – a spectral configuration corresponding to a particular disease or phenotype.

Interest in SERS as a diagnostic and prognostic tool has increased substantially in recent years. For example, to evaluate the potential of SERS in identifying patients with three forms of IHD – stable angina and two types of myocardial infarction (MI) distinguished by the presence or absence of ST elevation on electrocardiogram (ECG) – extracellular vesicles ranging from 20 nm to several microns were isolated from plasma via ultracentrifugation. These vesicles, secreted by all cell types from both normal and pathologically altered tissues, encompass various types, including exosomes, apoptotic bodies, ectosomes, and microvesicles that contain lipids, nucleic acids, and metabolites. The most effective mathematical approach was the support vector machine (SVM) method, which achieved 99.7% sensitivity and 95.7% specificity for discrimination. The authors note that data obtained from spectroscopic measurements are semi-quantitative, indicating that analyses based solely on a single RS peak associated with pathology are unlikely to yield reliable diagnoses or phenotype classifications. The optimal approach involves applying mathematical modeling techniques to extract diagnostic or classification information from the entire spectral profile, represented by the nine most informative frequencies [9].

The literature also describes a method for identifying patients with chronic forms of IHD through RS analysis of urine. Authors have identified nine peaks upon which a developed mathematical model can accurately classify IHD patients [10].

In the present study, high accuracy in identifying group membership was achieved using SERS, as detailed in *Table* 4. This finding is particularly significant because patients in the comparison group, due to their age and associated hypertension (which is present in 32% of cases), may exhibit subclinical manifestations of atherosclerosis. This is further supported by the observed prevalence of high or very high risk in 31% of these patients. Therefore, it is highly probable that the comparison group differed from the MFA group not qualitatively but quantitatively. This suggests a lower degree of atherosclerotic arterial damage rather than a complete absence of such damage. Evidence supporting the widespread prevalence of subclinical atherosclerosis, as measured by the calcium index via computed tomography (CT), has been obtained from large population-based studies focused on primary prevention [11, 12]. Furthermore, Models 2 and 3 demonstrated a very high discriminative ability.

A key question arises as to whether the spectral regions most critical for distinguishing patients in the comparison group from those with IHD complicated by congestive heart failure (CHF) overlap with those that differentiate patients with severe chronic kidney disease (CKD) from the comparison group. This issue is pertinent because patients with IHD complicated by CHF exhibited significantly lower estimated glomerular filtration rate (eGFR) compared to both the MFA and comparison groups, as shown in *Table* 2. To investigate this, we compared the significant spectral regions identified in Model 2 (665, 681, 720, 785, 1080, and 1208 cm^{-1}) with the frequencies that distinguished patients with severe CKD from those with preserved or mildly reduced eGFR in our previously conducted study (637, 724, 1001, 1095, 1238, and 1393 cm^{-1}) [13]. As shown, overlap occurred only within the spectral frequency range of 720-724 cm^{-1}, suggesting that metabolites associated with kidney dysfunction do not substantially affect the ability to identify patients with IHD complicated by CHF.

The methodology employed in this study is multimodal, integrating distinct technologies: optical scattering detection on one hand and mathematical transformation and analysis methods on the other. Consequently, the demonstrated high performance reflects the adequacy of all technologies employed in this study, providing a novel solution for diagnostic and disease phenotyping tasks.

Limitations

Several differences were observed between the comparison group and the group undergoing MFA with IHD complicated by congestive heart failure, as detailed in *Table* 3. Consequently, a question arises regarding how the differences in SERS spectra correlate with variations in routine biochemical parameters. The relatively small sample size limits our ability to provide a comprehensive answer to this inquiry.

Patients in the primary groups were administered multi-component therapy in accordance with current clinical guidelines, which could not be discontinued due to ethical considerations. Consequently, the extent to which medications present in the serum of patients in the primary groups influenced the SERS parameters in this study could not be accounted for.

Conclusions

Mathematical modeling utilizing SERS blood serum data guarantees precise differentiation between patients exhibiting clinical manifestations of atherosclerosis and those without such manifestations. Considering the relative simplicity of the SERS method and its high discriminative capacity, it is promising to pursue research aimed at detecting subclinical stages of diseases associated with atherosclerosis.

The SERS method facilitates the identification of a blood metabolic profile associated with clinically expressed congestive heart failure syndrome, classified as class II-III, complicating IHD. This capability of SERS offers an opportunity to stratify the risk of this syndrome and can also be employed to monitor the effectiveness of therapeutic programs in patients with CHF.

The age of patients with IHD complicated by CHF does not significantly influence the characteristics of SERS blood serum.

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Conflict of Interests

The authors declare that there are no conflicts of interest.

Ethical Compliance

All procedures involving human participants were conducted in accordance with the ethical standards set forth by the institutional research committee, as well as the 1964 Declaration of Helsinki and its subsequent amendments or comparable ethical standards.

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