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**MOLECULAR AND MESOSCALE  
SPECTROSCOPIC SIGNATURES OF BIOFLUIDS  
FOR CLINICAL APPLICATIONS**

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*Niente nella vita va temuto,  
Dev'essere solamente compreso.  
-Marie Curie*

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## RIASSUNTO

I biofluidi sono sempre più riconosciuti per la loro capacità di riflettere i processi fisiopatologici. La loro raccolta mediante procedure minimamente invasive ne aumenta l'applicabilità clinica, supportando il monitoraggio longitudinale della progressione della malattia e la valutazione completa delle risposte terapeutiche.

I biofluidi comprendono non solo cellule e molecole solubili, ma anche strutture mesoscalari (particelle da pochi nanometri a diversi micrometri) che costituiscono il cosiddetto secretoma nanostrutturato, che comprende sia particelle extracellulari non vescicolari sia Vescicole Extracellulari (EV). Le EV si sono dimostrate potenziali biomarcatori per diverse patologie, poiché trasportano molecole bioattive che riflettono la cellula di origine e, di conseguenza, la relativa condizione patologica.

Sfruttare questa architettura multiscale consente di accedere a un panorama biomolecolare più ampio, aumentando sia la diversità dei componenti rilevabili sia la quantità di informazioni ottenibili.

Nonostante tale potenziale, la traslazione clinica dei biofluidi interi e delle relative EV presenta ancora sfide significative:

1. Benché l'analisi dei biofluidi sia già stata esplorata in ambito oncologico, dimostrando un forte potenziale per la diagnosi precoce e il monitoraggio di diversi tipi di tumori, la sua applicazione come fonte affidabile di biomarcatori per le malattie non oncologiche resta complessa. Ciò è in parte dovuto ai costi elevati e alla limitata accessibilità degli strumenti analitici. Inoltre, i saggi progettati per misurare un singolo biomarcatore spesso non riescono a cogliere la complessità e l'eterogeneità delle malattie.
2. La traslazione clinica delle EV è ostacolata dalla loro eterogeneità, dalle dimensioni nanometriche e dalla bassa concentrazione, aspetti che rendono difficile la standardizzazione dei protocolli e dei flussi analitici.

In questo contesto, l'obiettivo della tesi è applicare tecniche spettroscopiche a livello molecolare e mesoscalare, con un focus specifico sulle EV. Infatti, generando impronte biochimiche dettagliate e riproducibili, queste tecniche potrebbero fornire biomarcatori utili per applicazioni diagnostiche e per il monitoraggio di trattamenti terapeutici o riabilitativi.

Le tecniche spettroscopiche vibrazionali, tra cui Spettroscopia Raman, Surface-Enhanced Raman Spectroscopy e Fourier Transform Infrared Spectroscopy, insieme alla spettroscopia di Risonanza Magnetica Nucleare, offrono caratteristiche analitiche uniche come approcci label-free, analisi non distruttive e la necessità di una manipolazione minima del campione.

Inoltre, la capacità delle spettroscopie vibrazionali di analizzare simultaneamente un elevato numero di campioni contribuisce a ridurre i costi, facilitando la traslazione di questi metodi nella diagnostica clinica per un'analisi multiscala.

Sfruttando tali vantaggi, questo lavoro si è focalizzato su diversi biofluidi: la saliva, per lo studio di malattie respiratorie quali Broncopneumopatia Cronica Ostruttiva, Asma, Sindrome delle Apnee Ostruttive del Sonno e COVID-19, e plasma o siero, per l'analisi della Intensive Care Unit-Acquired Weakness, della Leucemia Mieloide Cronica e della malattia di Parkinson, con l'obiettivo di valutare il potenziale clinico sia dei biofluidi interi sia delle loro EV nella diagnosi e nel monitoraggio di disturbi complessi e progressivi.

Questa strategia multiscala ha permesso l'identificazione di firme associate a specifiche condizioni patologiche e ha dimostrato la capacità dei biofluidi interi e delle loro EV di catturare processi biologici complessi e le alterazioni indotte dalla terapia.

In sintesi, questa tesi contribuisce ad affrontare bisogni clinici insoddisfatti attraverso l'impiego di tecniche spettroscopiche in grado di supportare diagnosi più precoci e di consentire ai clinici un monitoraggio più efficace della risposta terapeutica, orientando così le decisioni di trattamento e migliorando la gestione del paziente. Inoltre, l'approccio proposto offre nuove prospettive per l'utilizzo dei biofluidi interi e delle loro EV come biomarcatori affidabili in diverse patologie, rafforzando il loro potenziale ruolo nella medicina personalizzata e di precisione.

## SUMMARY

Biofluids are increasingly recognized for their ability to reflect pathophysiological processes. Their collection through minimally invasive procedures enhances their clinical applicability, supporting longitudinal monitoring of disease progression and a comprehensive assessment of therapeutic responses.

Biofluids comprise not only cells and soluble molecules but also mesoscale structures (particles ranging from a few nanometers to several micrometers) that refer to the so-called nanostructured secretome, which includes both non-vesicular extracellular particles and Extracellular Vesicles (EVs). EVs have been shown to be potential biomarkers for different diseases, as they transport bioactive molecules that mirror the cell of origin and, consequently, the associated pathology.

Exploiting this multiscale architecture enables access to a broader biomarker landscape, increasing both the diversity of detectable components and the information that can be obtained. Despite this potential, the clinical translation of whole biofluids and their EVs still faces significant challenges:

1. Although biofluid analysis has been explored in oncology, demonstrating strong potential for early diagnosis and monitoring of different types of tumors, its application as a reliable biomarker source for non-oncological diseases remains a major challenge. This is partly attributable to the high cost and limited accessibility of analytical instruments. In addition, assays designed to measure a single biomarker often fail to capture the complexity and heterogeneity of diseases.
2. The clinical translation of EVs is hindered due to their heterogeneity, nanoscale size, and low concentration, all of which complicate the standardization of protocols and analytical workflows.

Within this context, the thesis aims to apply spectroscopic techniques at both the molecular and mesoscale levels, focusing on EVs. Indeed, by generating detailed and reproducible biochemical fingerprints, it could serve as biomarkers for diagnostic applications and for monitoring therapeutic or rehabilitative treatments.

Vibrational spectroscopic techniques, including Raman Spectroscopy, Surface-Enhanced Raman Spectroscopy, and Fourier-Transform Infrared Spectroscopy, and Nuclear Magnetic Resonance, offer unique analytical features such as label-free approaches, non-destructive analysis, and require minimal sample manipulation.

Moreover, the ability of vibrational spectroscopies to analyze large numbers of samples simultaneously helps reduce costs, facilitating the translation of these methods into clinical diagnostics for multiscale analysis.

Exploiting these advantages, this work focused on a variety of biofluids: saliva, to investigate respiratory diseases such as Chronic Obstructive Pulmonary Disease, Asthma, Obstructive Sleep Apnea Syndrome, and COVID-19, and plasma or serum, for the study of Intensive Care Unit-Acquired Weakness, Chronic Myeloid Leukemia, and Parkinson's disease, with the aim of evaluating the clinical potential of both whole biofluids and their EV fractions in the diagnosis and monitoring of complex progressive disorders. This multiscale strategy led to the identification of signatures associated with specific pathological conditions. It demonstrated the ability of whole biofluids and their EV fractions to capture complex biological processes and alterations induced by the therapy.

In summary, this thesis addresses unmet clinical needs by using spectroscopic techniques that support earlier diagnosis and enable clinicians to monitor therapeutic response more effectively, thereby guiding treatment decisions and improving patient management. Moreover, the proposed approach offers new perspectives for using whole biofluids and their EV fractions as reliable biomarkers across different diseases, reinforcing their potential role in personalized and precision medicine.

## DISCLOSURE STATEMENT AND PUBLICATIONS

The results presented in this thesis have been obtained during my Ph.D. studies (December 2022 – November 2025) at the University of Brescia (Brescia, Italy), at Laboratory of Nanomedicine and Clinical Biophotonics, LABION (Milano, Italy) led by Dr. Marzia Bedoni, and at NMR Research Unit in University of Oulu, (Oulu, Finland) led by Prof. Ville-Veikko Telkki. Some of the experimental chapters, hereafter described, contain several unpublished data points.

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### Publications

During my Ph.D. studies, I contributed to several projects regarding Extracellular Vesicles (EVs) that led to the publication of the papers listed below:

1. **Biochemical Characterization of Human Salivary Extracellular Vesicles as a Valuable Source of Biomarkers.** Valentina Mangolini, Alice Gualerzi, Silvia Picciolini, Francesca Rodà, Angela Del Prete, Luana Forleo, Rudy Alexander Rossetto, Marzia Bedoni. 31 January 2023. *Biology*.
2. **Raman Spectroscopy Characterization of Multi-Functionalized Liposomes as Drug-Delivery Systems for Neurological Disorders.** Francesca Rodà, Silvia Picciolini, Valentina Mangolini, Alice Gualerzi, Pierfausto Seneci, Antonio Renda, Silvia Sesana, Francesca Re, Marzia Bedoni 11 February 2023. *Nanomaterials*.
3. **Multiplexing Biosensor for the Detection of Extracellular Vesicles as Biomarkers of Tissue Damage and Recovery after Ischemic Stroke.** Silvia Picciolini, Valentina Mangolini, Francesca Rodà, Angelo Montesano, Francesca Arnaboldi, Piergiuseppe Liuzzi, Andrea Mannini, Marzia Bedoni, Alice Gualerzi 27 April 2023. *International Journal of Molecular Sciences*.
4. **SPRi analysis of molecular interactions of mApoE-functionalized liposomes as drug delivery systems for brain diseases.** Silvia Picciolini, Francesca Rodà, Alice Gualerzi, Valentina Mangolini, Luana Forleo, Aurora Mangolini, Silvia Sesana, Antonia Antoniou, Francesca Re, Pierfausto Seneci, Marzia Bedoni. 31 October 2023. *Analyst*.

5. **Molecular Understanding of the Surface-Enhanced Raman Spectroscopy Salivary Fingerprint in People after Sars-COV-2 Infection and in Vaccinated Subjects.** Francesca Rodà, Alice Gualerzi, Silvia Picciolini, Luana Forleo, **Valentina Mangolini**, Roberta Mancuso, Simone Agostini, Rudy Alexander Rossetto, Paola Pierucci, Paolo Innocente Banfi, Marzia Bedoni. 11 July 2024. *Chemosensors*.
6. **Extracellular vesicles of different cellular origin feature distinct biomolecular corona dynamics.** Angelo Musicò, Andrea Zendrini, Santiago Gimenez Reyes, **Valentina Mangolini**, Lucia Paolini, Miriam Romano, Andrea Papait, Antonietta Rosa Silini, Paolo Di Gianvincenzo, Arabella Neva, Marina Cretich, Ornella Parolini, Camillo Almici, Sergio E Moya, Annalisa Radeghieri, Paolo Bergese. 7 November 2024. *Nanoscale Horizons*.
7. **MicroRNA-142-3p shuttling in extracellular vesicles marks regulatory T cell dysfunction in multiple sclerosis.** Giusy De Rosa, Claudia Russo, Silvia Garavelli, Dario Di Silvestre, Ilaria Spatocco, Giorgia Mele, Claudia La Rocca, Alessandra Colamatteo, Fortunata Carbone, Clorinda Fusco, Fabiana Passaro, Donatella Carpi, Elena Tagliabue, Francesco Prattichizzo, Francesca Brambilla, Pierluigi Mauri, Mirjam Hoxha, Valentina Bollati, Ilaria Giusti, Vincenza Dolo, Paola D'Antona, Paola Campomenosi, **Valentina Mangolini**, Annalisa Radeghieri, Paolo Bergese, Ivana Morabito, Alessandra Mandelli, Annamaria Finardi, Francesca Beretta, Edoardo Dalmato Schilke, Guido Cavaletti, Ettore Dolcetti, Fabio Buttari, Gianmarco Abbadessa, Simona Bonavita, Giacomo Lus, Elisabetta Signoriello, Roberta Lanzillo, Vincenzo Brescia Morra, Maria Mottola, Bruno Zuccarelli, Antonio Uccelli, Marco Salvetti, Diego Centonze, Roberto Furlan, Giuseppe Matarese, Claudio Procaccini, Paola de Candia. 28 May 2025. *Science Translational Medicine*.
8. **Circulating particles carotenoids are associated with rehabilitation recovery in Parkinson's disease.** Alice Gualerzi, Martina Gerli, Aurora Mangolini, Silvia Picciolini, Francesca Rodà, **Valentina Mangolini**, Stefano Doronzio, Diego Longo, Giulio Cherubini, Cristina Polito, Chiara De Santis, Silvia Ramat, Francesca Cecchi, Gemma Lombardi, Marzia Bedoni. 22 August 2025. *Redox Biology*.
9. **Assessment of chronic Myeloid Leukaemia in vitro models variability: insights into extracellular vesicles.** Silvia Mutti, Alessia Cavalleri, Stefania Federici, **Valentina Mangolini**, Lucia Paolini, Cristian Bonvicini, Rosalba Monica Ferraro, Elena Laura Mazzoldi, Luca Garuffo, Besjana Xhahysa, Alessandro Leoni, Federica Trenta, Federica Re, Silvia Clara Giliani, Daniele Avenoso, Mirko Farina, Michele Malagola,

Domenico Russo, Simona Bernardi. 22 October 2025. *Journal of Cellular and Molecular Medicine*.

10. **Tracking Leukemic Residuals: Dissecting the Inverse Relationship Between CD26<sup>+</sup> Stem Cells and Extracellular BCR::ABL1 in CML.** Silvia Mutti, Alessia Cavalleri, Anna Sicuranza, Paola Pacelli, Claudia Ielo, Lucia Paolini, **Valentina Mangolini**, Teresa Miracapillo, Camilla Turriziani, Elisabetta Abruzzese, Mirko Farina, Annalisa Radeghieri, Michele Malagola, Massimo Breccia, Monica Bocchia, Domenico Russo, and Simona Bernardi. 24 November 2025. *STEM CELLS Translational Medicine*.
11. **Red Blood Cell-derived Extracellular Vesicles as biomaterials: the opportunity of freezing-induced accelerated aging.** Paolini, Lucia; Romano, Miriam; **Mangolini, Valentina**; Tassoni, Selene; Jiang, Shuhan; Mazzoldi, Elena; Musicò, Angelo; Zendrini, Andrea; Kashkanova, Anna; Sandoghdar, Vahid; Berardi, Anna; Giliani, Silvia; Bergese, Paolo; Radeghieri, Annalisa. 25 November 2025. *Biomaterials Science*.
12. **Shared protocol to enrich and compare Extracellular Vesicles from human plasma and skeletal muscle biopsy.** **Valentina Mangolini**, Annalisa Radeghieri, Simone Piva, Stefano Cattaneo, Marco Brucale, Francesco Valle, Arianna Balestri, Costanza Montis, Nicola Latronico, Paolo Bergese, Lucia Paolini. 2025. Under peer review.

# **CHAPTER 1 – FROM MOLECULAR TO MESOSCALE DIMENSION: LIQUID BIOPSY AS A TOOL FOR CLINICAL DIAGNOSTICS AND FOLLOW-UP**

Biofluids are increasingly recognized for their potential in reflecting physiological and pathological processes. Furthermore, the ability to collect these samples using minimally invasive methods, particularly blood, saliva, and urine, enhances their utility in clinical settings. This practical aspect facilitates longitudinal monitoring of diseases, thereby enabling a comprehensive assessment of disease trajectories and responses to therapies<sup>[1,2]</sup>. In this context, liquid biopsies serve as promising diagnostic tools that can provide real-time insights into a patient's health status<sup>[3,4]</sup>.

One notable aspect of biofluids is the presence of mesoscale components, such as Extracellular Vesicles (EVs), which have attracted significant attention for their roles in intercellular communication and their biomarker potential. EVs transport molecules both inside and on their surface, forming as a biomolecular corona<sup>[5]</sup>, different types of molecules that mirror the molecular signatures of their cells of origin<sup>[6]</sup>, making them a promising focus of investigation within nanomedicine. Unfortunately, methodologies for EV analysis often lack standardization, underscoring the need for robust, reproducible workflows<sup>[7-9]</sup>.

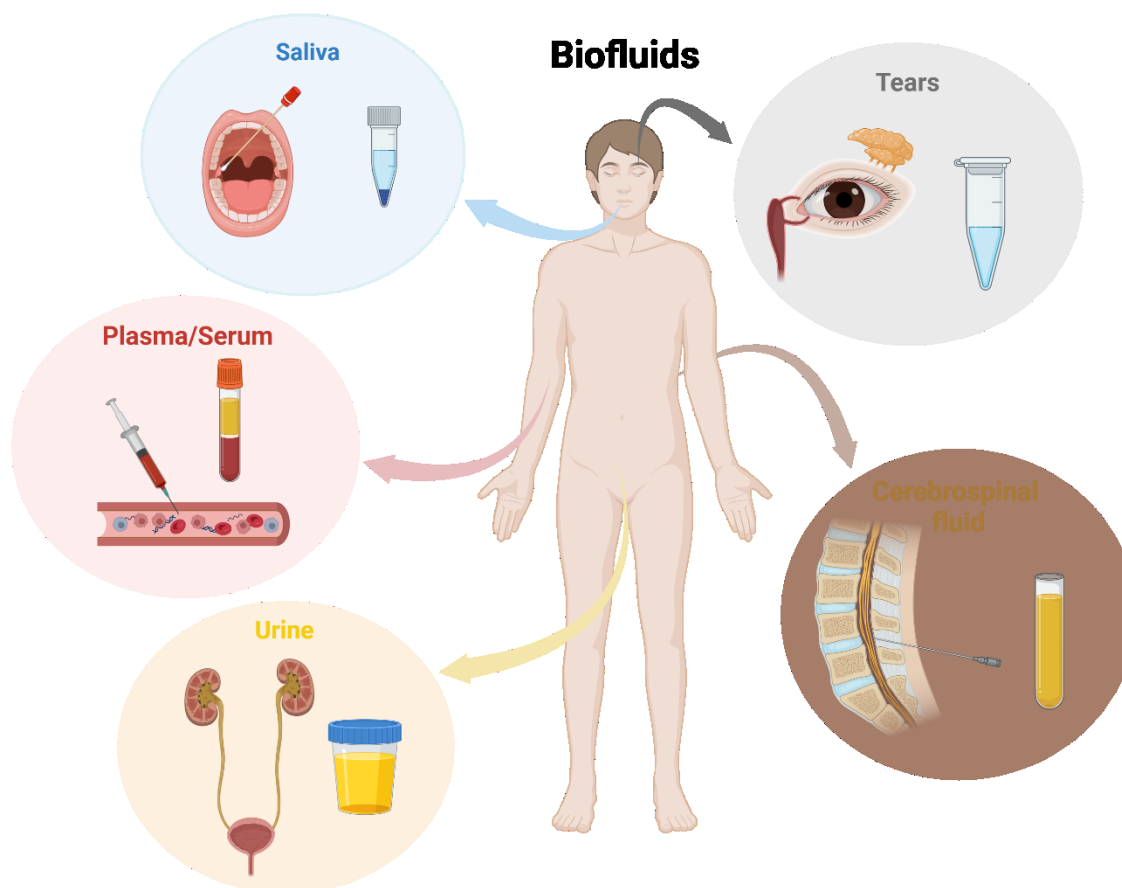
This thesis investigates molecular and mesoscale spectroscopic signatures of biofluids and their EV fractions, highlighting spectroscopic techniques as promising tools to overcome the limitations of conventional analytical methods. The principles and diagnostic potential of these techniques will be discussed in detail in Chapter 2.

This chapter introduces the clinical potential of biofluids and their EV fractions, reviewing their current applications and state of the art, and outlining the analytical and methodological barriers that still need to be addressed.

## **1.1 Liquid Biopsy**

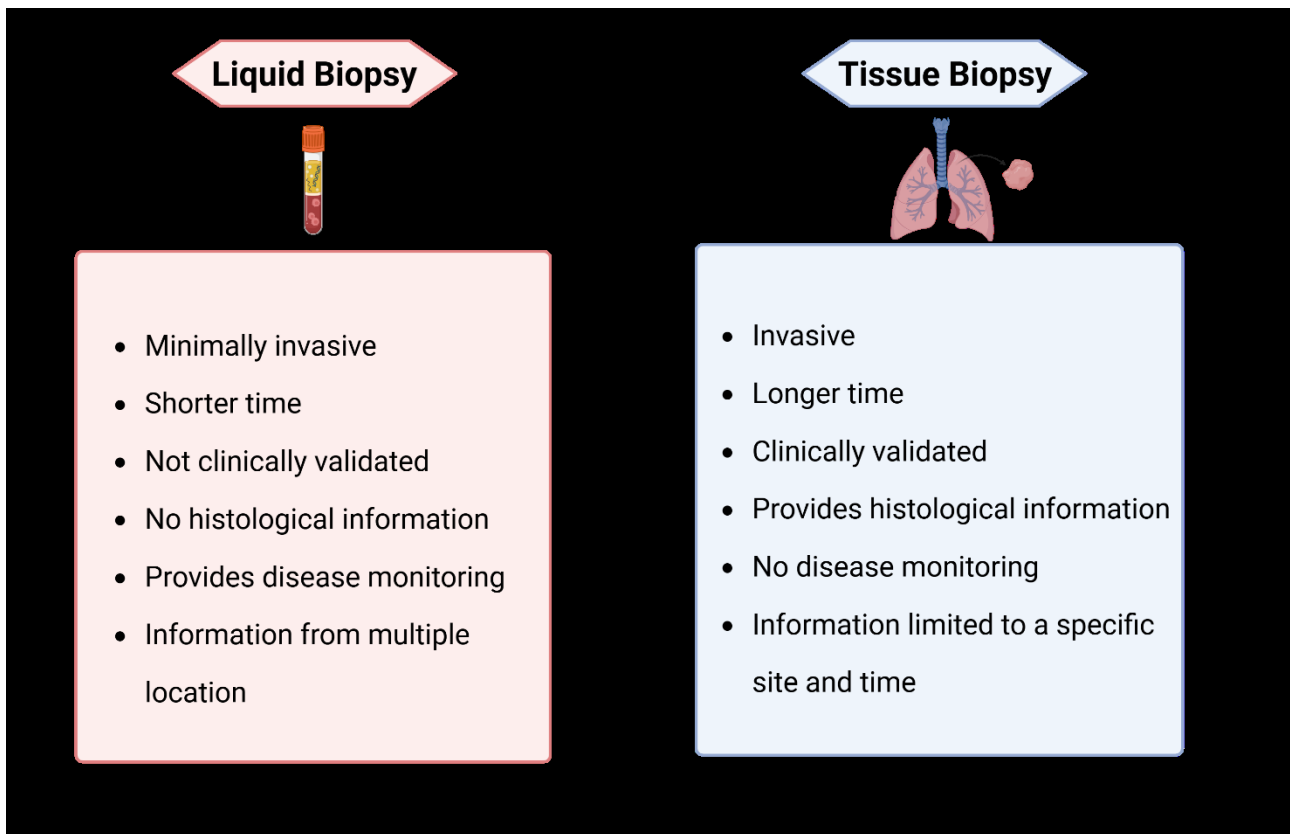
Human biofluids constitute an accessible and versatile source of information reflecting physiological and pathological states. Their collection is achievable through non-invasive procedures, such as liquid biopsy, avoiding the risks and limitations associated with tissue biopsies<sup>[10]</sup>. This makes biofluids a strategic resource for identifying disease-specific signatures, tracking disease progression, and evaluating the effects of therapeutic and rehabilitation responses<sup>[11]</sup>.

A variety of biofluids can be exploited for diagnostic purposes, including blood, plasma, serum, urine, saliva, cerebrospinal fluid (CSF), tears, and other compartment-specific fluids. Each matrix presents distinct compositional features, stability profiles, and sampling accessibility, which ultimately influence their suitability for specific analytical applications.



**Figure 1.1** Different types of biofluids used in a clinical setting. Created with Biorender.com

Thanks to the introduction of liquid biopsy, the clinical relevance of biofluid analysis has increased considerably within the framework of personalized medicine<sup>[12–14]</sup>. Although liquid biopsy applications extend across multiple disease domains, oncology has been at the front line of translating these methodologies into clinical practice<sup>[15–17]</sup>. Unlike tissue biopsies, which provide information limited to a specific site and time, a liquid biopsy carries signals from multiple locations in the body. Furthermore, compared to conventional tissue biopsies, liquid biopsies offer a non-invasive, faster, safer, and more feasible alternative<sup>[18]</sup>.



**Figure 1.2** Schematic overview of the differences between liquid biopsy and tissue biopsy, emphasizing the advantages and disadvantages. Created with Biorender.com

Traditionally, liquid biopsy has focused on the analysis of soluble or cellular components such as Circulating Tumor Cells (CTCs), cell-free DNA (cfDNA), and circulating RNA (cRNA), which provide valuable molecular information across a broad spectrum of pathological conditions<sup>[19]</sup>.

- **CTCs** → These are viable cancer cells shed from a primary tumor into the bloodstream and provide insights into tumor biology, metastasis, treatment response, and disease progression<sup>[20] [21]</sup>.
- **cfDNA** → Comprised mainly of fragments released from apoptotic and necrotic cells and carries tumor-related genetic alterations, making it particularly relevant for early cancer detection, treatment personalization, and monitoring minimal residual disease<sup>[22]</sup>.
- **cRNA** → Primarily consists of messenger RNA (mRNA), microRNA (miRNA), and long non-coding RNA (lncRNA). These RNA molecules can be released into circulation from tumor cells and other tissue types as biomarkers for diagnosis, prognosis, and treatment monitoring<sup>[23]</sup>.

### 1.1.1 Clinical Applications and Future Perspectives of Liquid Biopsy

Liquid biopsy has demonstrated remarkable potential in disease management, particularly in oncology, and is increasingly being explored in non-neoplastic conditions as well. In this context, real-time monitoring of cfDNA has demonstrated significant value for early relapse detection compared with conventional imaging techniques. The ability of cfDNA to anticipate disease recurrence provides critical opportunities for timely therapeutic interventions. Ji et al. demonstrated that plasma cfDNA analysis in non-Hodgkin lymphoma patients enables the prediction of relapse 1 to 6 months earlier than conventional methods<sup>[24]</sup>. Similarly, Bernard et al. reported that cfDNA detection in pancreatic cancer patients occurred on average 3.1 months after tumor resection, compared to 9.6 months using computed tomography, highlighting the potential of cfDNA for earlier clinical intervention<sup>[25]</sup>.

Additional evidence supports the clinical relevance of cfDNA across different oncological contexts. In pediatric medulloblastoma, whole-genome methylation sequencing of cfDNA from CSF enabled reliable tumor detection<sup>[26]</sup>, while in HER2-positive breast cancer with brain metastases, combined cfDNA analysis in plasma and CSF provided a more comprehensive molecular profile of the disease<sup>[27]</sup>.

Beyond oncology, liquid biopsy is gaining traction for diagnosing various diseases. Indeed, a marker of endothelial dysfunction called endocan has recently been investigated in plasma as a predictor of cardiovascular risk in patients with End-Stage Renal Disease (ESRD) undergoing hemodialysis. In a large cohort of subjects, plasma endocan levels were evaluated in relation to clinical parameters and cardiovascular outcomes. Patients with elevated plasma endocan showed a significantly higher incidence of cardiovascular events compared with those with lower levels, suggesting that plasma endocan may represent a promising biomarker for cardiovascular risk stratification in ESRD patients undergoing hemodialysis<sup>[28]</sup>.

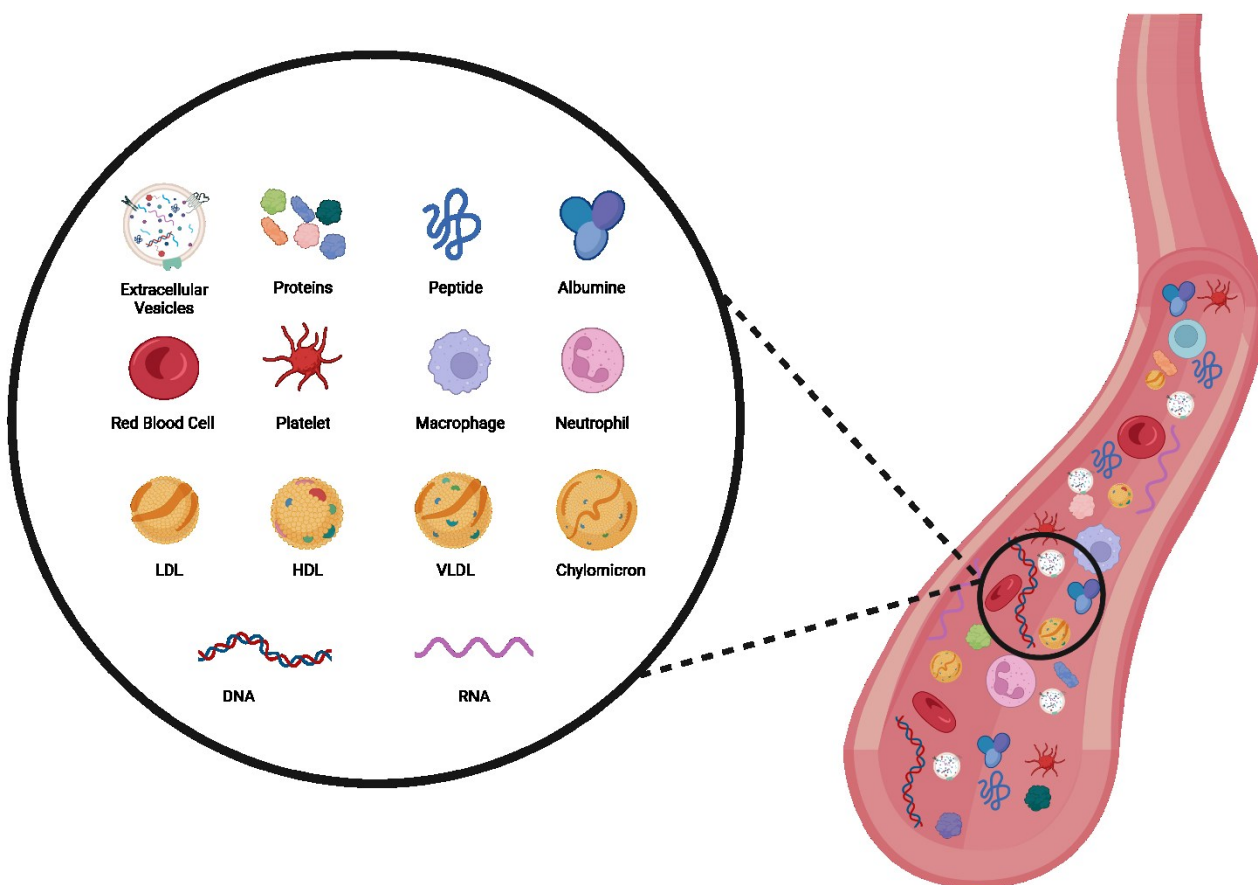
An additional study utilizes urine to investigate neonatal sepsis, a leading cause of mortality, through a metabolomics approach. By profiling 25 neonates, including septic and healthy controls, distinct metabolic signatures were identified, revealing alterations in glucose, lactate, ketone bodies, and Krebs cycle intermediates. These findings highlight the potential of liquid biopsies as early, non-invasive biomarkers, enabling timely diagnosis and targeted therapeutic interventions<sup>[29]</sup>.

Comprehensive proteomic profiling has further emphasized the potential of biofluids as reservoirs of clinically relevant biomarkers. A large-scale comparison of plasma, urine, CSF, saliva, and amniotic fluid using high-resolution mass spectrometry identified nearly 4700

nonredundant proteins, of which more than 3400 were quantitatively assessed. While over 500 proteins were shared across all biofluids, more than one-third of the proteins were unique to a single fluid, reflecting tissue-specific contributions. Interestingly, functional analysis revealed that beyond local signatures, biofluids collectively capture systemic physiological processes. These findings reinforce the value of liquid biopsies not only for disease-specific biomarker discovery but also for capturing integrative information on whole body functional states<sup>[30]</sup>. Despite these promising applications, liquid biopsy still faces significant challenges, including limited analytical sensitivity for low-abundance targets, technical variability, and the need for standardized pre-analytical procedures to ensure reproducibility across clinical settings<sup>[11]</sup>. Looking to the future, the integration of advanced analytical and spectroscopic techniques (discussed in Chapter 2), together with machine learning and AI-driven data analysis, is expected to enhance patient stratification, early relapse detection, and personalized therapy. Ongoing efforts toward cost reduction, automation, and point-of-care platforms are likely to further support clinical translation<sup>[31]</sup>. Taken together, liquid biopsy represents a versatile, minimally invasive platform with broad implications for precision medicine, with applications extending across multiple diseases and strong potential to improve patient management.

## **1.2 Biofluids Complexity and Their Mesoscale Dimension**

Biofluids are dynamic, complex systems composed of heterogeneous mixtures. Beyond their soluble fraction, biofluids contain a variety of particulate components, including cells, cell debris, and nanosized particles that span from lipid-enclosed vesicles to non-membranous nanoparticles. These mesoscale structures, typically ranging from a few nanometers to several micrometers, constitute the so-called nanostructured secretome<sup>[32]</sup>, which carries an immense amount of biochemical and biophysical information reflecting the physiological or pathological state of the organism.



**Figure 1.3** A schematic image for the representation of the complexity of biofluids.

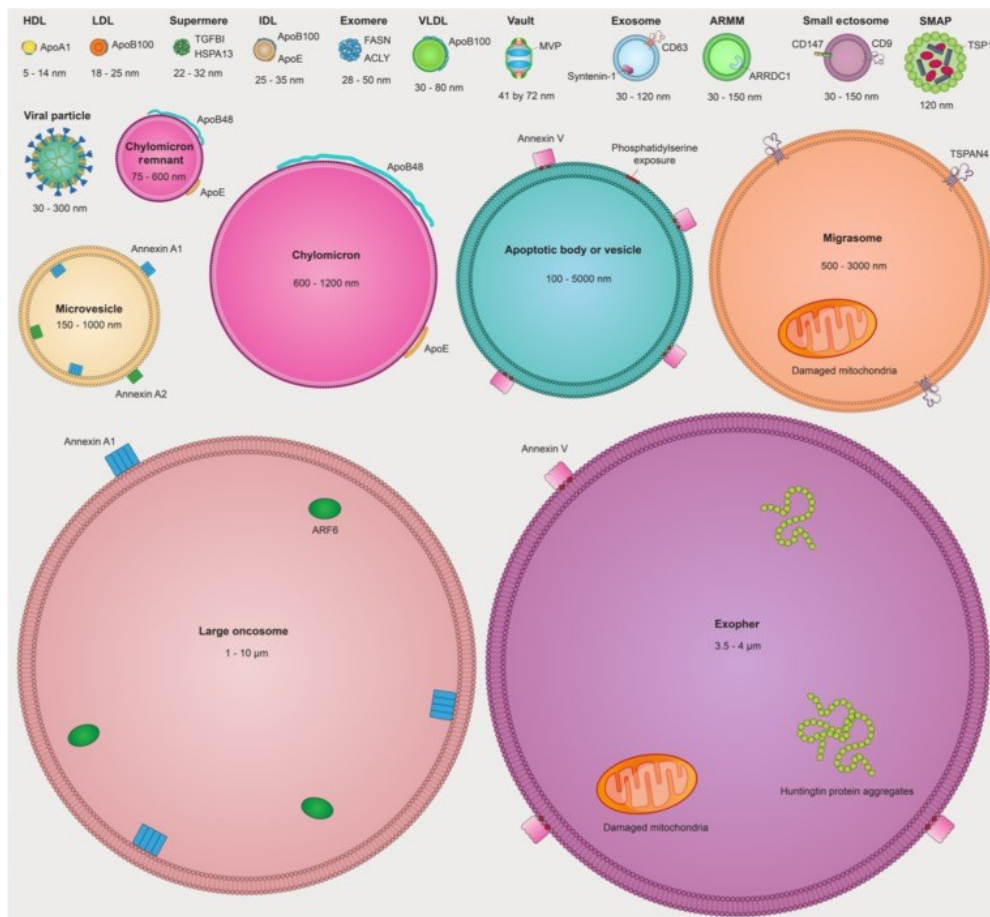
Among the most studied components of this secretome are EVs, lipid bilayer–enclosed vesicles released by all cell types under physiological and pathological conditions<sup>[33]</sup>. EVs are traditionally categorized based on their biogenesis and size into exosomes (30–150 nm), ectosomes or microvesicles (150–1000 nm), and apoptotic vesicles (100 nm to several micrometers)<sup>[34]</sup>. These vesicles transport a complex cargo of proteins, lipids, and nucleic acids, including messenger RNA (mRNA) and microRNA (miRNA), that can influence the phenotype and function of recipient cells<sup>[6]</sup>. Their presence in all major biological fluids, including plasma<sup>[35]</sup>, urine<sup>[36]</sup>, saliva<sup>[37]</sup>, cerebrospinal fluid<sup>[38]</sup>, and breast milk<sup>[39]</sup>, highlights their role in systemic signaling and their potential as diagnostic and therapeutic tools. Major information on EVs is explained in chapters 1.2.1 and 1.2.2.

In recent years, however, it has become evident that EVs represent only a portion of the extracellular nanoscale landscape. Non-vesicular extracellular particles (NVEPs), which lack a surrounding lipid bilayer, are also abundant in biofluids<sup>[40]</sup>. These include particles such as lipoproteins<sup>[41]</sup>, nucleosomes<sup>[42]</sup>, and vaults<sup>[43]</sup>, as well as recently identified nanoparticle

species such as exomeres and supermeres<sup>[44,45]</sup>. Lipoproteins (ranging from 5–1200 nm) are among the most abundant NVEPs in circulation and act as major carriers of lipids and small RNAs<sup>[46]</sup>. Nucleosomes, consisting of DNA wrapped around histone proteins, represent extracellular remnants of nuclear material and can be found either free or associated with vesicular fractions<sup>[47]</sup>. Vaults, large ribonucleoprotein particles (~70 nm), contain major vault protein (MVP) and small vault RNAs and are thought to be released through secretory amphisomes (hybrid vesicular arising from the fusion of multivesicular endosomes and autophagosomes) or cell death<sup>[48,49]</sup>. Other specialized NVEPs include supramolecular attack particles (SMAPs), secreted by cytotoxic T lymphocytes, which mediate targeted cell killing, and viral particles, which may exist independently or within EVs, further challenging the distinction between vesicular and non-vesicular entities<sup>[50,51]</sup>.

The discovery of exomeres (28–50 nm) and supermeres (15–25 nm) has further expanded the known complexity of extracellular nanoparticles. Both are amembranous particles enriched in metabolic enzymes, proteins, and RNA species, many of which were previously attributed to exosomes. Their exact biogenesis remains unknown, yet they are consistently detected in multiple biofluids, including plasma, suggesting conserved and biologically relevant secretion mechanisms<sup>[34,44]</sup>. Importantly, these NVEPs have been shown to participate in intercellular communication and to carry disease-related biomolecules, making them emerging targets for biomarker discovery and therapeutic applications<sup>[45]</sup>.

Together, these findings illustrate that biofluids are highly organized systems encompassing a wide spectrum of mesoscale entities, whose heterogeneity is essential for translational applications such as liquid biopsy and disease monitoring.

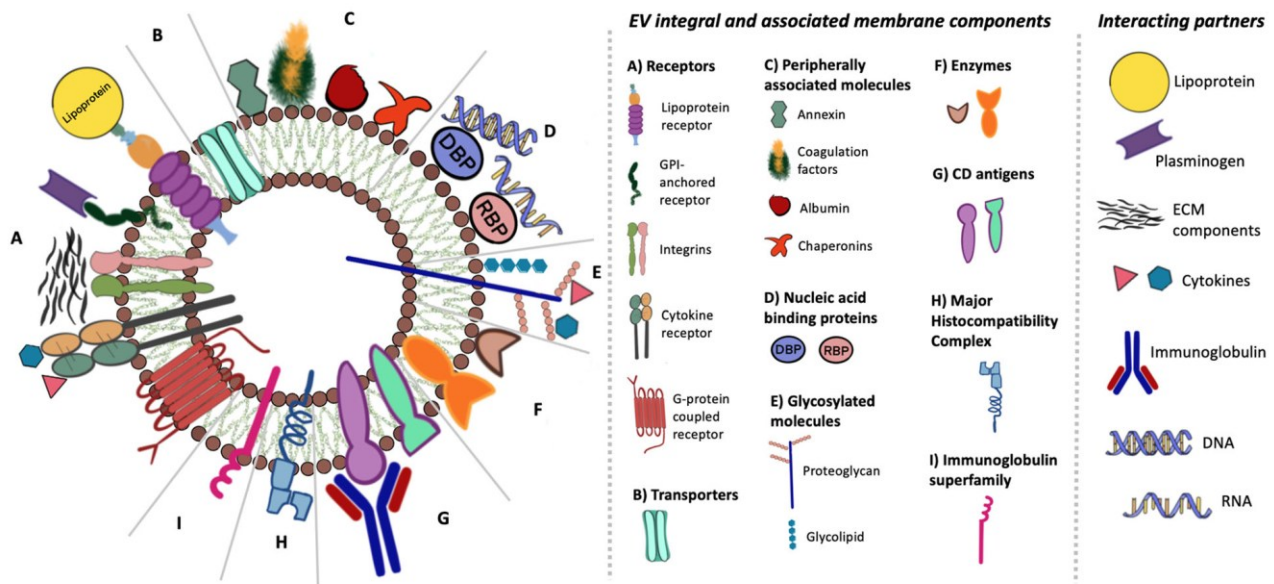


**Figure 1.4** The image illustrates the wide dimensional range and heterogeneity of extracellular components released by mammalian cells. They release a heterogeneous population of extracellular structures that span a broad size range, from nanoscale particles of approximately 5 nm to larger vesicular bodies exceeding 5  $\mu\text{m}$ . These secreted entities include both EVs and non-vesicular NVEPs, whose overlapping size distributions pose significant challenges for their complete separation and characterization<sup>[34]</sup>.

### 1.2.1 Extracellular Vesicles: Classification and Biogenesis

As mentioned earlier, EVs are the most-studied component of the nanostructured secretome. They are natural nanoparticles with a lipid bilayer released by all cell types without the capacity to replicate, as clarified in the MISEV2023 guidelines<sup>[52]</sup>. Because of their presence in all biological fluids, their analysis can be performed using liquid biopsy techniques. EVs typically range between 30 and 1000 nm and encapsulate a wide repertoire of proteins, lipids, metabolites, and nucleic acids<sup>[6]</sup>. By protecting these biomolecules from degradation and delivering them to recipient cells, EVs act as efficient mediators of bioactive signals. Initially regarded as cellular waste products, primarily involved in the disposal of unwanted or excess cellular components, EVs are now recognised as active mediators of intercellular communication<sup>[53]</sup>.

EVs are heterogeneous in size and composition, and their molecular cargo largely reflects the metabolic state, circadian rhythms, and cell type of origin. Despite this diversity, they are enriched in specific markers like tetraspanins (CD63, CD81, CD9) but also Alix, Flotillin, and TSG101 which are often used to validate their presence<sup>[52]</sup>.



**Figure 1.5** Molecular components associated with the surface of EVs. The image illustrates the diverse molecular components associated with the EV surface, reflecting their complex biological origin and multifunctionality. Extracellular vesicles display a heterogeneous molecular architecture composed of both integral membrane components and peripheral molecules. Integral membrane proteins include receptors such as G protein–coupled receptors, cytokine and lipoprotein receptors, integrins, GPI-anchored proteins, and members of the immunoglobulin superfamily, together with glycoproteins, transporters, enzymes, major histocompatibility complex (MHC) molecules, and various CD antigens. In addition, numerous peripheral molecules contribute to the overall surface composition by binding to these integral proteins or by interacting directly with the lipid bilayer. These include cytokines, extracellular matrix components, plasminogen, lipoproteins, and immunoglobulins. Other molecules reported to associate with the EV membrane comprise enzymes, nucleic acids (DNA and RNA), DNA/RNA-binding proteins, annexins, albumin, and components of the coagulation cascade<sup>[6]</sup>.

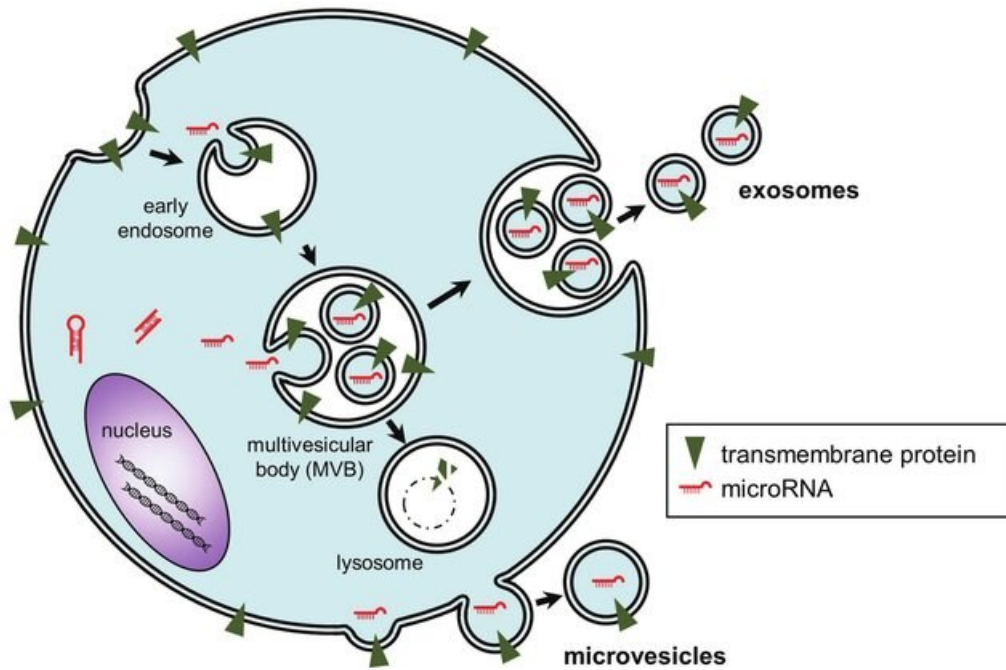
Traditionally, EVs have been divided into exosomes and microvesicles based on their biogenetic pathways. Because of the overlap in size and shared biomarkers between exosomes and microvesicles, a more recent classification categorizes exosome as small-EVs (sEVs), ranging from 30 to 200 nm, and large EVs (lEVs), ranging from 200 to 1000 nm<sup>[52]</sup>.

sEVs are produced through the endocytic pathway. Their formation begins with invagination of the plasma membrane, which generates intraluminal vesicles (ILVs) within multivesicular bodies (MVBs). During this process, both soluble proteins and membrane-associated molecules are incorporated into the vesicles. Cargo selection occurs during MVB formation, but the exact molecular mechanisms regulating it remain only partially elucidated<sup>[33]</sup>. To date, two alternative routes have been described: one dependent on the endosomal sorting complex required for transport (ESCRT) pathway<sup>[54]</sup> and one that is ESCRT-independent<sup>[55]</sup>. Proteins associated with the ESCRT pathway, including Alix and TSG101, have been identified as key regulators of MVB biogenesis<sup>[56,57]</sup>.

MVBs can subsequently fuse with lysosomes for degradation or merge with the plasma membrane to release sEVs into the extracellular space.

In contrast, IEVs originate directly from the plasma membrane through an outward budding process, which is driven by cytoskeletal rearrangements and changes in lipid composition at the budding site. This membrane protrusion progressively expands releasing the IEVs into the extracellular space<sup>[33]</sup>.

Although the molecular determinants of EV cargo selection are still only partially understood, emerging evidence indicates that certain EV subpopulations can selectively incorporate mitochondrial components. In this context, small-EVs enriched in mitochondrial-derived cargo, called mitoEVs, and larger mitochondrial-derived EVs (mdEVs) have been described, suggesting that mitochondrial content is not randomly packaged but rather reflects specialized pathways of EVs biogenesis and function<sup>[58,59]</sup>.



**Figure 1.6** Mechanisms of EVs release. sEVs, originate within MVBs, are secreted following their fusion with the plasma membrane. In contrast, lEVs arise through outward budding and subsequent shedding from the plasma membrane<sup>[60]</sup>.

### 1.2.2 The Power of Extracellular Vesicles as New Frontiers for Liquid Biopsy Tool

EVs have emerged as promising diagnostic tools due to their molecular cargo, which reflects the physiological or pathological state of the parental cell and supports minimally invasive strategies such as liquid biopsy<sup>[61]</sup>. Their lipid bilayer protects proteins, lipids, and nucleic acids from enzymatic degradation, ensuring molecular stability and detectability even in complex biological matrices<sup>[62]</sup>.

Beyond their intrinsic cargo, EVs acquire an additional layer of biological information through the adsorption of proteins, lipids, and other biomolecules from the surrounding biofluids, forming what is known as the biomolecular corona (BC). BC dynamically reflects the molecular environment in which EVs circulate and can strongly influence their biological identity, uptake, and functional effects. Importantly, the composition of the BC differs depending on the physiological or pathological state of the host, meaning that it may provide an additional source of diagnostic information beyond the EV content itself. In this context, characterizing the EV BC not only helps to better understand EVs–cell interactions but also opens new opportunities for the identification of highly specific disease-associated biomarkers, thereby enhancing the diagnostic power of EV-based liquid biopsy approaches<sup>[5]</sup>.

This dual source of information supports the use of EVs across different clinical settings. Indeed, in oncology tumor-derived EVs have been extensively studied for their role in tumor

initiation and progression, as they transport oncogenic proteins and nucleic acids that influence angiogenesis<sup>[63]</sup>, and prepare pre-metastatic niches at distant organs<sup>[64]</sup>. By transporting signaling molecules, they facilitate tumor cell proliferation, invasion, and resistance to apoptosis, while also modulating immune responses to evade recognition<sup>[65]</sup>. These multifaceted roles have generated strong interest in their potential use as diagnostic and prognostic biomarkers, as well as indicators of therapeutic response.

Beyond cancer, EVs have been implicated in neurodegenerative disorders such as Alzheimer's and Parkinson's disease, where they transport pathological proteins including amyloid- $\beta$  and  $\alpha$ -synuclein, contributing to disease progression and enabling early diagnostic strategies<sup>[66]</sup>. Notably, single-EV profiling studies have demonstrated that EV surface protein signatures in biofluids, particularly urine, can achieve high diagnostic accuracy in distinguishing Alzheimer's disease patients from healthy controls, highlighting the potential of EVs as non-invasive biomarkers for neurodegeneration<sup>[67]</sup>.

Large-scale studies have further demonstrated that plasma-derived EVs can reveal underlying proteinopathies in neurodegenerative disorders such as ALS and FTD. By quantifying EV-associated biomarkers, including TDP-43 and tau isoform ratios, researchers have been able to stratify patients according to distinct molecular pathologies. In particular, high EV TDP-43 levels distinguished ALS cases from other neurodegenerative conditions, while alterations in the tau isoform ratio allowed discrimination between FTD subtypes and progressive supranuclear palsy, supporting the use of EVs as minimally invasive tools for patient classification and precision medicine<sup>[68]</sup>.

In the cardiovascular field, EVs are increasingly recognized as active modulators of vascular homeostasis and disease. Recent studies have demonstrated that EVs released by M2 macrophages exert protective effects in abdominal aortic aneurysm by attenuating inflammation, preserving vascular smooth muscle cell integrity, and regulating macrophage polarization. These findings highlight EVs not only as biomarkers of vascular pathology but also as promising therapeutic vehicles in cardiovascular disease<sup>[69]</sup>.

Furthermore, EVs derived from adipose tissue have recently been implicated in obesity-related metabolic dysfunctions, acting as carriers of bioactive proteins linked to inflammation and insulin resistance. Proteomic profiling has revealed distinct cargo signatures between visceral and subcutaneous adipose tissue EVs, with visceral-derived EVs particularly enriched in adipokines and molecules associated with type 2 diabetes and obesity. These results underscore the usefulness of adipose tissue EVs as circulating biomarkers for monitoring metabolic status and obesity-associated comorbidities<sup>[70]</sup>.

In addition to molecular cargo, EV physical properties such as size, concentration, and mechanical stiffness are emerging as valuable diagnostic parameters, supported by advances in high-throughput analytical platforms<sup>[71]</sup>.

Despite this promise, the translation of EVs into routine clinical diagnostics is still a challenge. Overcoming these limitations is essential to fully harness EVs as robust, reproducible, and clinically actionable biomarkers<sup>[9]</sup>.

### **1.2.3 Limitations Hindering the Clinical Translation of Extracellular Vesicles for Diagnostic Applications**

As previously discussed, EVs are nanoscale particles ranging from approximately 30 to 1000 nm, placing them within the field of nanomedicine, the branch of medicine that exploits nanoscale systems for diagnostic and therapeutic innovation.

EV dimensions pose significant obstacles for their reliable detection and characterization. Indeed, conventional analytical tools are often unable to resolve particles within the full EV size range or to distinguish them from non-vesicular nanoparticles such as lipoproteins, protein aggregates, or viral particles. For example, Nanoparticle Tracking Analysis (NTA) is a widely used method that measures both the size distribution and concentration of EVs in suspension. By tracking particles through light scattering and Brownian motion, and with the option of fluorescence detection, it enables detailed and versatile EV characterization<sup>[72]</sup>. However, when operated in scatter mode, NTA detects all nanoparticles present in the sample, including co-isolated contaminants, which leads to an overestimation of EV counts and compromises measurement accuracy. Comparative studies have shown that in scatter mode NTA reports higher particle numbers than flow cytometry, while fluorescence-based NTA yields results more consistent with those obtained by flow cytometry. These findings indicate that both NTA and flow cytometry represent valuable tools for EV analysis, yet their limited sensitivity and resolution remain critical factors that can compromise reproducibility and standardization across laboratories<sup>[73]</sup>.

Furthermore, obtaining pure EV preparations remains a critical bottleneck as no single method provides an optimal balance between yield, purity, and scalability. Classical approaches such as ultracentrifugation risk co-isolating protein or lipoprotein contaminants<sup>[74]</sup>, while affinity-based techniques offer high specificity at the expense of throughput and cost<sup>[75]</sup>. Chromatographic and filtration methods preserve EV integrity but often dilute samples or require complex instrumentation<sup>[76,77]</sup>.

Many of the current EV isolation and characterization techniques require specialized equipment and expertise, making them expensive and impractical for large-scale clinical implementation. For instance, ultracentrifugation at high g-forces or nanoscale flow cytometry are not routinely accessible in hospital settings. Developing affordable and scalable platforms is therefore crucial to facilitate the integration of EVs into diagnostic and therapeutic settings<sup>[72]</sup>.

While guidelines such as MISEV<sup>[52]</sup> provide recommendations, variability across laboratories still results in low reproducibility and makes cross-study comparisons difficult. Furthermore, rapid and robust quality control assays to assess EV preparations before in vitro or in vivo applications are not yet available, limiting translational progress<sup>[9]</sup>.

In addition to analytical challenges, the storage of EVs represents another critical issue for their clinical translation. EV integrity and functional properties are highly sensitive to storage conditions, which can profoundly affect particle stability, and cargo preservation. Recent investigations have demonstrated that long-term preservation of EVs requires freezing at  $-80^{\circ}\text{C}$ , whereas short-term storage up to one week is feasible at  $4^{\circ}\text{C}$  without substantial loss of EVs integrity or function. Nevertheless, repeated freeze–thaw cycles and variations in storage protocols across laboratories remain major sources of variability, highlighting the urgent need for standardized handling procedures<sup>[78]</sup>.

In summary, the clinical translation of EVs remains hindered by their nanoscale dimensions, the difficulty of obtaining pure preparations, the high cost of current analytical platforms, and the lack of standardized protocols for isolation, characterization, and storage. These limitations explained why EVs, despite their promise as biomarkers and therapeutic vectors, are not yet fully integrated into clinical practice.

To address these challenges, innovative analytical strategies are needed. In the chapter 2, we will explore spectroscopic approaches, which are emerging as powerful, label-free, and reproducible methods for EV analysis, with the potential to facilitate their application in diagnostics and therapeutics.

### 1.3 References

- (1) Dunphy, K.; O'Mahoney, K.; Dowling, P.; O'Gorman, P.; Bazou, D. Clinical Proteomics of Biofluids in Haematological Malignancies. *International Journal of Molecular Sciences* **2021**, 22 (15), 8021. <https://doi.org/10.3390/ijms22158021>.
- (2) Nair, R. R.; An, J. M.; Kim, J.; Kim, D. Review: Recent Progress in Fluorescent Molecular Systems for the Detection of Disease-Related Biomarkers in Biofluids. *Coordination Chemistry Reviews* **2023**, 494, 215336. <https://doi.org/10.1016/j.ccr.2023.215336>.

- (3) Martins, I.; Ribeiro, I. P.; Jorge, J.; Gonçalves, A. C.; Sarmiento-Ribeiro, A. B.; Melo, J. B.; Carreira, I. M. Liquid Biopsies: Applications for Cancer Diagnosis and Monitoring. *Genes* **2021**, *12* (3), 349. <https://doi.org/10.3390/genes12030349>.
- (4) Casagrande, G. M. S.; Silva, M. de O.; Reis, R. M.; Leal, L. F. Liquid Biopsy for Lung Cancer: Up-to-Date and Perspectives for Screening Programs. *International Journal of Molecular Sciences* **2023**, *24* (3), 2505. <https://doi.org/10.3390/ijms24032505>.
- (5) Tassoni, S.; Bergese, P.; Radeghieri, A. The Extracellular Vesicle Biomolecular Corona: Current Insights and Diagnostic Potential. *Nanomedicine* **2025**, *20* (16), 2013–2021. <https://doi.org/10.1080/17435889.2025.2538426>.
- (6) Hallal, S.; Túzesi, Á.; Grau, G. E.; Buckland, M. E.; Alexander, K. L. Understanding the Extracellular Vesicle Surface for Clinical Molecular Biology. *Journal of Extracellular Vesicles* **2022**, *11* (10), e12260. <https://doi.org/10.1002/jev2.12260>.
- (7) Ciferri, M. C.; Quarto, R.; Tasso, R. Extracellular Vesicles as Biomarkers and Therapeutic Tools: From Pre-Clinical to Clinical Applications. *Biology* **2021**, *10* (5), 359. <https://doi.org/10.3390/biology10050359>.
- (8) *Extracellular vesicles as carriers of mRNA: Opportunities and challenges in diagnosis and treatment*. <https://www.thno.org/v14p2265.htm> (accessed 2025-10-25).
- (9) Gandham, S.; Su, X.; Wood, J.; Nocera, A. L.; Alli, S. C.; Milane, L.; Zimmerman, A.; Amiji, M.; Ivanov, A. R. Technologies and Standardization in Research on Extracellular Vesicles. *Trends Biotechnol* **2020**, *38* (10), 1066–1098. <https://doi.org/10.1016/j.tibtech.2020.05.012>.
- (10) Mannelli, C. Tissue vs Liquid Biopsies for Cancer Detection: Ethical Issues. *Bioethical Inquiry* **2019**, *16* (4), 551–557. <https://doi.org/10.1007/s11673-019-09944-y>.
- (11) Adhit, K. K.; Wanjari, A.; Menon, S.; K, S. Liquid Biopsy: An Evolving Paradigm for Non-Invasive Disease Diagnosis and Monitoring in Medicine. *Cureus* **2023**. <https://doi.org/10.7759/cureus.50176>.
- (12) Mallareddy, V.; Roy, R.; Cheng, Z.; Thej, C.; Benedict, C.; Truongcao, M.; Joladarashi, D.; Magadum, A.; Ibeti, J.; Cimini, M.; Gonzalez, C.; Garikipati, V. N. S.; Koch, W. J.; Kishore, R. Tipifarnib Reduces Extracellular Vesicles and Protects From Heart Failure. *Circ Res* **2024**, *135* (2), 280–297. <https://doi.org/10.1161/CIRCRESAHA.123.324110>.
- (13) Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Terenzi, F.; Ramat, S.; Sorbi, S.; Bedoni, M. Raman Profiling of Circulating Extracellular Vesicles for the Stratification of Parkinson's Patients. *Nanomedicine: Nanotechnology, Biology and Medicine* **2019**, *22*, 102097. <https://doi.org/10.1016/j.nano.2019.102097>.
- (14) *Liquid Biopsy Is a Promising Tool for Genetic Testing in Idiopathic Pulmonary Fibrosis*. <https://www.mdpi.com/2075-4418/11/7/1202> (accessed 2025-08-30).
- (15) Giunta, E. F.; Malapelle, U.; Russo, A.; De Giorgi, U. Blood-Based Liquid Biopsy in Advanced Prostate Cancer. *Critical Reviews in Oncology/Hematology* **2024**, *194*, 104241. <https://doi.org/10.1016/j.critrevonc.2023.104241>.
- (16) Yu, J.; Paterson, C.; Davies, P.; Palmer, J. C.; Higgins, J. P. T.; Kurian, K. M. Evaluating Liquid Biopsy Biomarkers for Early Detection of Brain Metastasis: A Systematic Review. *Neurooncol Pract* **2025**, *12* (4), 600–617. <https://doi.org/10.1093/nop/npaf032>.
- (17) Liao, W.; Yang, H.; Xu, H.; Wang, Y.; Ge, P.; Ren, J.; Xu, W.; Lu, X.; Sang, X.; Zhong, S.; Zhang, H.; Mao, Y. Noninvasive Detection of Tumor-Associated Mutations from Circulating Cell-Free DNA in Hepatocellular Carcinoma Patients by Targeted Deep Sequencing. *Oncotarget* **2016**, *7* (26), 40481–40490. <https://doi.org/10.18632/oncotarget.9629>.
- (18) Lone, S. N.; Nisar, S.; Masoodi, T.; Singh, M.; Rizwan, A.; Hashem, S.; El-Rifai, W.; Bedognetti, D.; Batra, S. K.; Haris, M.; Bhat, A. A.; Macha, M. A. Liquid Biopsy: A Step

- Closer to Transform Diagnosis, Prognosis and Future of Cancer Treatments. *Molecular Cancer* **2022**, *21* (1), 79. <https://doi.org/10.1186/s12943-022-01543-7>.
- (19) Michela, B. Liquid Biopsy: A Family of Possible Diagnostic Tools. *Diagnostics* **2021**, *11* (8), 1391. <https://doi.org/10.3390/diagnostics11081391>.
  - (20) Ho, H.-Y.; Chung, K.-S. K.; Kan, C.-M.; Wong, S.-C. C. Liquid Biopsy in the Clinical Management of Cancers. *Int J Mol Sci* **2024**, *25* (16), 8594. <https://doi.org/10.3390/ijms25168594>.
  - (21) Qureshi, Z.; Altaf, F.; Khanzada, M.; Safi, A.; Asghar, Z.; Warraich, D.; Shah, S. Liquid Biopsies for Early Detection and Monitoring of Cancer: Advances, Challenges, and Future Directions. *Ann Med Surg (Lond)* **2025**, *87* (6), 3244–3253. <https://doi.org/10.1097/MS9.0000000000002776>.
  - (22) Aiyer, S.; Kim, T.-H.; Collier, K.; Pollock, R.; Verschraegen, C.; Stover, D. G.; Tinoco, G. Unlocking the Potential of ctDNA in Sarcomas: A Review of Recent Advances. *Cancers* **2025**, *17* (6), 1040. <https://doi.org/10.3390/cancers17061040>.
  - (23) Gao, Y.; Wang, J.-W.; Ren, J.-Y.; Guo, M.; Guo, C.-W.; Ning, S.-W.; Yu, S. Long Noncoding RNAs in Gastric Cancer: From Molecular Dissection to Clinical Application. *World Journal of Gastroenterology* **2020**, *26* (24), 3401–3412. <https://doi.org/10.3748/wjg.v26.i24.3401>.
  - (24) *Longitudinal Monitoring of Plasma Circulating Tumour DNA Enables the Prediction of Early Relapse in Patients with Non-Hodgkin Lymphoma: A Case Series.* <https://www.mdpi.com/2075-4418/11/11/2055> (accessed 2025-08-26).
  - (25) Bernard, V.; Kim, D. U.; Lucas, F. A. S.; Castillo, J.; Allenson, K.; Mulu, F. C.; Stephens, B. M.; Huang, J.; Semaan, A.; Guerrero, P. A.; Kamyabi, N.; Zhao, J.; Hurd, M. W.; Koay, E. J.; Taniguchi, C. M.; Herman, J. M.; Javle, M.; Wolff, R.; Katz, M.; Varadhachary, G.; Maitra, A.; Alvarez, H. A. Circulating Nucleic Acids Are Associated With Outcomes of Patients With Pancreatic Cancer. *Gastroenterology* **2019**, *156* (1), 108–118.e4. <https://doi.org/10.1053/j.gastro.2018.09.022>.
  - (26) Li, J.; Zhao, S.; Lee, M.; Yin, Y.; Li, J.; Zhou, Y.; Ballester, L. Y.; Esquenazi, Y.; Dashwood, R. H.; Davies, P. J. A.; Parsons, D. W.; Li, X.-N.; Huang, Y.; Sun, D. Reliable Tumor Detection by Whole-Genome Methylation Sequencing of Cell-Free DNA in Cerebrospinal Fluid of Pediatric Medulloblastoma. *Science Advances* **2020**, *6* (42), eabb5427. <https://doi.org/10.1126/sciadv.abb5427>.
  - (27) Siravegna, G.; Geuna, E.; Mussolin, B.; Crisafulli, G.; Bartolini, A.; Galizia, D.; Casorzo, L.; Sarotto, I.; Scaltriti, M.; Sapino, A.; Bardelli, A.; Montemurro, F. Genotyping Tumour DNA in Cerebrospinal Fluid and Plasma of a HER2-Positive Breast Cancer Patient with Brain Metastases. *ESMO Open* **2017**, *2* (4), e000253. <https://doi.org/10.1136/esmoopen-2017-000253>.
  - (28) Kim, J. S.; Ko, G. J.; Kim, Y. G.; Lee, S. Y.; Lee, D. Y.; Jeong, K. H.; Lee, S. H. Plasma Endocan as a Predictor of Cardiovascular Event in Patients with End-Stage Renal Disease on Hemodialysis. *J Clin Med* **2020**, *9* (12), 4086. <https://doi.org/10.3390/jcm9124086>.
  - (29) Fanos, V.; Caboni, P.; Corsello, G.; Stronati, M.; Gazzolo, D.; Noto, A.; Lussu, M.; Dessì, A.; Giuffrè, M.; Lacerenza, S.; Serraino, F.; Garofoli, F.; Serpero, L. D.; Liori, B.; Carboni, R.; Atzori, L. Urinary 1H-NMR and GC-MS Metabolomics Predicts Early and Late Onset Neonatal Sepsis. *Early Human Development* **2014**, *90*, S78–S83. [https://doi.org/10.1016/S0378-3782\(14\)70024-6](https://doi.org/10.1016/S0378-3782(14)70024-6).
  - (30) Zhao, M.; Yang, Y.; Guo, Z.; Shao, C.; Sun, H.; Zhang, Y.; Sun, Y.; Liu, Y.; Song, Y.; Zhang, L.; Li, Q.; Liu, J.; Li, M.; Gao, Y.; Sun, W. A Comparative Proteomics Analysis of Five Body Fluids: Plasma, Urine, Cerebrospinal Fluid, Amniotic Fluid, and Saliva. *PROTEOMICS – Clinical Applications* **2018**, *12* (6), 1800008. <https://doi.org/10.1002/prca.201800008>.

- (31) Pando-Caciano, A.; Trivedi, R.; Pauwels, J.; Nowakowska, J.; Cavina, B.; Falkman, L.; Debattista, J.; Belényesi, S.-K.; Radhakrishnan, P.; Molina, M. A. Unlocking the Promise of Liquid Biopsies in Precision Oncology. *The Journal of Liquid Biopsy* **2024**, *3*. <https://doi.org/10.1016/j.jlb.2024.100151>.
- (32) Busatto, S.; Zendrini, A.; Radeghieri, A.; Paolini, L.; Romano, M.; Presta, M.; Bergese, P. The Nanostructured Secretome. *Biomater. Sci.* **2019**, *8* (1), 39–63. <https://doi.org/10.1039/C9BM01007F>.
- (33) van Niel, G.; D'Angelo, G.; Raposo, G. Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat Rev Mol Cell Biol* **2018**, *19* (4), 213–228. <https://doi.org/10.1038/nrm.2017.125>.
- (34) Jeppesen, D. K.; Zhang, Q.; Franklin, J. L.; Coffey, R. J. Extracellular Vesicles and Nanoparticles: Emerging Complexities. *Trends in Cell Biology* **2023**, *33* (8), 667–681. <https://doi.org/10.1016/j.tcb.2023.01.002>.
- (35) Di Paolo, V.; Paolini, A.; Galardi, A.; Gasparini, P.; De Cecco, L.; Colletti, M.; Lampis, S.; Raieli, S.; De Stefanis, C.; Miele, E.; Russo, I.; Di Ruscio, V.; Casanova, M.; Alaggio, R.; Masotti, A.; Milano, G. M.; Locatelli, F.; Di Giannatale, A. Plasma-Derived Extracellular Vesicles miR-335–5p as Potential Diagnostic Biomarkers for Fusion-Positive Rhabdomyosarcoma. *J Exp Clin Cancer Res* **2024**, *43* (1), 282. <https://doi.org/10.1186/s13046-024-03197-3>.
- (36) Li, Y.; Fu, B.; Wang, M.; Chen, W.; Fan, J.; Li, Y.; Liu, X.; Wang, J.; Zhang, Z.; Lu, H.; Zhang, Y. Urinary Extracellular Vesicle N-Glycomics Identifies Diagnostic Glycosignatures for Bladder Cancer. *Nat Commun* **2025**, *16* (1), 2292. <https://doi.org/10.1038/s41467-025-57633-9>.
- (37) Rastogi, S.; Rani, K.; Rai, S.; Singh, R.; Bharti, P. S.; Sharma, V.; Sahu, J.; Kapoor, V.; Vishwakarma, P.; Garg, S.; Gholap, S. L.; Inampudi, K. K.; Modi, G. P.; Rani, N.; Tripathi, M.; Srivastava, A.; Rajan, R.; Nikolajeff, F.; Kumar, S. Fluorescence-Tagged Salivary Small Extracellular Vesicles as a Nanotool in Early Diagnosis of Parkinson's Disease. *BMC Med* **2023**, *21* (1), 335. <https://doi.org/10.1186/s12916-023-03031-1>.
- (38) Salviano-Silva, A.; Stevic, I.; Mende, C.; Reimer, R.; Maire, C. L.; Mader, M. M.; Dührsen, L.; Lamszus, K.; Westphal, M.; Eicker, S. O.; Ricklefs, F. L. Profiling Extracellular Vesicles from Cerebrospinal Fluid for Classification of Intradural Spinal Tumors. *Sci Rep* **2025**, *15* (1), 36479. <https://doi.org/10.1038/s41598-025-23190-w>.
- (39) Cho, Y.-E.; Chen, S.; Crouch, K.; Shutt, D.; Kaufman, J. W.; Singh, B. K. Human Breast Milk Extracellular Vesicles Mitigate Endothelial Dysfunction. *Nutrients* **2025**, *17* (18), 2953. <https://doi.org/10.3390/nu17182953>.
- (40) Jeppesen, D. K.; Zhang, Q.; Coffey, R. J. Extracellular Vesicles and Nanoparticles at a Glance. *J Cell Sci* **2024**, *137* (23), jcs260201. <https://doi.org/10.1242/jcs.260201>.
- (41) Li, K.; Wong, D. K.; Luk, F. S.; Kim, R. Y.; Raffai, R. L. Isolation of Plasma Lipoproteins as a Source of Extracellular RNA. In *Extracellular RNA: Methods and Protocols*; Patel, T., Ed.; Springer: New York, NY, 2018; pp 139–153. [https://doi.org/10.1007/978-1-4939-7652-2\\_11](https://doi.org/10.1007/978-1-4939-7652-2_11).
- (42) Wang, H.; Wang, Y.; Zhang, D.; Li, P. Circulating Nucleosomes as Potential Biomarkers for Cancer Diagnosis and Treatment Monitoring. *Int J Biol Macromol* **2024**, *262* (Pt 1), 130005. <https://doi.org/10.1016/j.ijbiomac.2024.130005>.
- (43) Routsias, J. G.; Marinou, D.; Mavrouli, M.; Tsakris, A.; Pitiriga, V. C. Major Vault Protein/Lung Resistance-Related Protein: A Novel Biomarker for Inflammation and Acute Infections. *Microorganisms* **2024**, *12* (9), 1762. <https://doi.org/10.3390/microorganisms12091762>.

- (44) Yu, L.; Shi, H.; Gao, T.; Xu, W.; Qian, H.; Jiang, J.; Yang, X.; Zhang, X. Exomeres and Supermeres: Current Advances and Perspectives. *Bioact Mater* **2025**, *50*, 322–343. <https://doi.org/10.1016/j.bioactmat.2025.04.012>.
- (45) Zhang, Q.; Jeppesen, D. K.; Higginbotham, J. N.; Graves-Deal, R.; Trinh, V. Q.; Ramirez, M. A.; Sohn, Y.; Neining, A. C.; Taneja, N.; McKinley, E. T.; Niitsu, H.; Cao, Z.; Evans, R.; Glass, S. E.; Ray, K. C.; Fissell, W. H.; Hill, S.; Rose, K. L.; Huh, W. J.; Washington, M. K.; Ayers, G. D.; Burnette, D. T.; Sharma, S.; Rome, L. H.; Franklin, J. L.; Lee, Y. A.; Liu, Q.; Coffey, R. J. Supermeres Are Functional Extracellular Nanoparticles Replete with Disease Biomarkers and Therapeutic Targets. *Nat Cell Biol* **2021**, *23* (12), 1240–1254. <https://doi.org/10.1038/s41556-021-00805-8>.
- (46) Vickers, K. C.; Palmisano, B. T.; Shoucri, B. M.; Shamburek, R. D.; Remaley, A. T. MicroRNAs Are Transported in Plasma and Delivered to Recipient Cells by High-Density Lipoproteins. *Nat Cell Biol* **2011**, *13* (4), 423–433. <https://doi.org/10.1038/ncb2210>.
- (47) Choi, D.; Montermini, L.; Jeong, H.; Sharma, S.; Meehan, B.; Rak, J. Mapping Subpopulations of Cancer Cell-Derived Extracellular Vesicles and Particles by Nano-Flow Cytometry. *ACS Nano* **2019**, *13* (9), 10499–10511. <https://doi.org/10.1021/acsnano.9b04480>.
- (48) Rome, L. H.; Kickhoefer, V. A. Development of the Vault Particle as a Platform Technology. *ACS Nano* **2013**, *7* (2), 889–902. <https://doi.org/10.1021/nn3052082>.
- (49) Carter, S. D.; Mamede, J. I.; Hope, T. J.; Jensen, G. J. Correlated Cryogenic Fluorescence Microscopy and Electron Cryo-Tomography Shows That Exogenous TRIM5 $\alpha$  Can Form Hexagonal Lattices or Autophagy Aggregates in Vivo. *Proceedings of the National Academy of Sciences* **2020**, *117* (47), 29702–29711. <https://doi.org/10.1073/pnas.1920323117>.
- (50) Chang, H.-F.; Schirra, C.; Ninov, M.; Hahn, U.; Ravichandran, K.; Krause, E.; Becherer, U.; Bálint, Š.; Harkiolaki, M.; Urlaub, H.; Valitutti, S.; Baldari, C. T.; Dustin, M. L.; Jahn, R.; Rettig, J. Identification of Distinct Cytotoxic Granules as the Origin of Supramolecular Attack Particles in T Lymphocytes. *Nat Commun* **2022**, *13* (1), 1029. <https://doi.org/10.1038/s41467-022-28596-y>.
- (51) Bálint, Š.; Müller, S.; Fischer, R.; Kessler, B. M.; Harkiolaki, M.; Valitutti, S.; Dustin, M. L. Supramolecular Attack Particles Are Autonomous Killing Entities Released from Cytotoxic T Cells. *Science* **2020**, *368* (6493), 897–901. <https://doi.org/10.1126/science.aay9207>.
- (52) Welsh, J. A.; Goberdhan, D. C. I.; O'Driscoll, L.; Buzas, E. I.; Blenkiron, C.; Bussolati, B.; Cai, H.; Di Vizio, D.; Driedonks, T. A. P.; Erdbrügger, U.; Falcon-Perez, J. M.; Fu, Q.-L.; Hill, A. F.; Lenassi, M.; Lim, S. K.; Mahoney, M. G.; Mohanty, S.; Möller, A.; Nieuwland, R.; Ochiya, T.; Sahoo, S.; Torrecilhas, A. C.; Zheng, L.; Zijlstra, A.; Abuelreich, S.; Bagabas, R.; Bergese, P.; Bridges, E. M.; Brucal, M.; Burger, D.; Carney, R. P.; Cocucci, E.; Crescitelli, R.; Hanser, E.; Harris, A. L.; Haughey, N. J.; Hendrix, A.; Ivanov, A. R.; Jovanovic-Talman, T.; Kruh-Garcia, N. A.; Ku'ulei-Lyn Faustino, V.; Kyburz, D.; Lässer, C.; Lennon, K. M.; Lötvall, J.; Maddox, A. L.; Martens-Uzunova, E. S.; Mizenko, R. R.; Newman, L. A.; Ridolfi, A.; Rohde, E.; Rojalin, T.; Rowland, A.; Saftics, A.; Sandau, U. S.; Saugstad, J. A.; Shekari, F.; Swift, S.; Ter-Ovanesyan, D.; Tosar, J. P.; Useckaite, Z.; Valle, F.; Varga, Z.; van der Pol, E.; van Herwijnen, M. J. C.; Wauben, M. H. M.; Wehman, A. M.; Williams, S.; Zendrini, A.; Zimmerman, A. J.; MISEV Consortium; Théry, C.; Witwer, K. W. Minimal Information for Studies of Extracellular Vesicles (MISEV2023): From Basic to Advanced Approaches. *J Extracell Vesicles* **2024**, *13* (2), e12404. <https://doi.org/10.1002/jev2.12404>.

- (53) Doyle, L. M.; Wang, M. Z. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* **2019**, *8* (7), 727. <https://doi.org/10.3390/cells8070727>.
- (54) Juan, T.; Fürthauer, M. Biogenesis and Function of ESCRT-Dependent Extracellular Vesicles. *Seminars in Cell & Developmental Biology* **2018**, *74*, 66–77. <https://doi.org/10.1016/j.semcdb.2017.08.022>.
- (55) van Niel, G.; Carter, D. R. F.; Clayton, A.; Lambert, D. W.; Raposo, G.; Vader, P. Challenges and Directions in Studying Cell–Cell Communication by Extracellular Vesicles. *Nat Rev Mol Cell Biol* **2022**, *23* (5), 369–382. <https://doi.org/10.1038/s41580-022-00460-3>.
- (56) *ALIX- and ESCRT-III-dependent sorting of tetraspanins to exosomes* | *Journal of Cell Biology* | Rockefeller University Press. <https://rupress.org/jcb/article/doi/10.1083/jcb.201904113/133723/ALIX-and-ESCRT-III-dependent-sorting-of> (accessed 2025-08-30).
- (57) *Oncogene-regulated release of extracellular vesicles: Developmental Cell*. [https://www.cell.com/developmental-cell/fulltext/S1534-5807\(21\)00443-3?\\_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS1534580721004433%3Fshowall%3Dtrue](https://www.cell.com/developmental-cell/fulltext/S1534-5807(21)00443-3?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS1534580721004433%3Fshowall%3Dtrue) (accessed 2025-08-30).
- (58) Iorio, R.; Petricca, S.; Di Emidio, G.; Falone, S.; Tatone, C. Mitochondrial Extracellular Vesicles (mitoEVs): Emerging Mediators of Cell-to-Cell Communication in Health, Aging and Age-Related Diseases. *Ageing Res Rev* **2024**, *101*, 102522. <https://doi.org/10.1016/j.arr.2024.102522>.
- (59) Picca, A.; Guerra, F.; Calvani, R.; Coelho-Júnior, H. J.; Landi, F.; Bucci, C.; Marzetti, E. Mitochondrial-Derived Vesicles: The Good, the Bad, and the Ugly. *International Journal of Molecular Sciences* **2023**, *24* (18), 13835. <https://doi.org/10.3390/ijms241813835>.
- (60) Matsuzaki, J.; Ochiya, T. Circulating microRNAs: Next-Generation Cancer Detection. *The Keio Journal of Medicine* **2020**, *69*. <https://doi.org/10.2302/kjm.2019-0011-OA>.
- (61) Bernardi, S.; Balbi, C. Extracellular Vesicles: From Biomarkers to Therapeutic Tools. *Biology* **2020**, *9* (9), 258. <https://doi.org/10.3390/biology9090258>.
- (62) Liang, Y.; Lehrich, B. M.; Zheng, S.; Lu, M. Emerging Methods in Biomarker Identification for Extracellular Vesicle-Based Liquid Biopsy. *Journal of Extracellular Vesicles* **2021**, *10* (7), e12090. <https://doi.org/10.1002/jev2.12090>.
- (63) Ateeq, M.; Broadwin, M.; Sellke, F. W.; Abid, M. R. Extracellular Vesicles' Role in Angiogenesis and Altering Angiogenic Signaling. *Medical Sciences* **2024**, *12* (1), 4. <https://doi.org/10.3390/medsci12010004>.
- (64) Li, Y.; Zheng, Y.; Tan, X.; Du, Y.; Wei, Y.; Liu, S. Extracellular Vesicle-Mediated Pre-Metastatic Niche Formation via Altering Host Microenvironments. *Front. Immunol.* **2024**, *15*. <https://doi.org/10.3389/fimmu.2024.1367373>.
- (65) Meehan, K.; Vella, L. J. The Contribution of Tumour-Derived Exosomes to the Hallmarks of Cancer. *Critical Reviews in Clinical Laboratory Sciences* **2016**, *53* (2), 121–131. <https://doi.org/10.3109/10408363.2015.1092496>.
- (66) Vella, L. J.; Hill, A. F.; Cheng, L. Focus on Extracellular Vesicles: Exosomes and Their Role in Protein Trafficking and Biomarker Potential in Alzheimer's and Parkinson's Disease. *International Journal of Molecular Sciences* **2016**, *17* (2), 173. <https://doi.org/10.3390/ijms17020173>.
- (67) Cai, Y.; Chen, T.; Cai, Y.; Liu, J.; Yu, B.; Fan, Y.; Su, J.; Zeng, Y.; Xiao, X.; Ren, L.; Tang, Y. Surface Protein Profiling and Subtyping of Extracellular Vesicles in Body Fluids Reveals Non-CSF Biomarkers of Alzheimer's Disease. *Journal of Extracellular Vesicles* **2024**, *13* (4), e12432. <https://doi.org/10.1002/jev2.12432>.

- (68) Chatterjee, M.; Özdemir, S.; Fritz, C.; Möbius, W.; Kleineidam, L.; Mandelkow, E.; Biernat, J.; Doğdu, C.; Peters, O.; Cosma, N. C.; Wang, X.; Schneider, L.-S.; Priller, J.; Spruth, E.; Kühn, A. A.; Krause, P.; Klockgether, T.; Vogt, I. R.; Kimmich, O.; Spottke, A.; Hoffmann, D. C.; Fließbach, K.; Miklitz, C.; McCormick, C.; Weydt, P.; Falkenburger, B.; Brandt, M.; Guenther, R.; Dinter, E.; Wiltfang, J.; Hansen, N.; Bähr, M.; Zerr, I.; Flöel, A.; Nestor, P. J.; Düzel, E.; Glanz, W.; Incesoy, E.; Bürger, K.; Janowitz, D.; Perneczky, R.; Rauchmann, B. S.; Hopfner, F.; Wagemann, O.; Levin, J.; Teipel, S.; Kilimann, I.; Goerss, D.; Prudlo, J.; Gasser, T.; Brockmann, K.; Mengel, D.; Zimmermann, M.; Synofzik, M.; Wilke, C.; Selma-González, J.; Turon-Sans, J.; Santos-Santos, M. A.; Alcolea, D.; Rubio-Guerra, S.; Fortea, J.; Carbayo, Á.; Lleó, A.; Rojas-García, R.; Illán-Gala, I.; Wagner, M.; Frommann, I.; Roeske, S.; Bertram, L.; Heneka, M. T.; Brosseron, F.; Ramirez, A.; Schmid, M.; Beschorner, R.; Halle, A.; Herms, J.; Neumann, M.; Barthélemy, N. R.; Bateman, R. J.; Rizzu, P.; Heutink, P.; Dols-Icardo, O.; Höglinger, G.; Hermann, A.; Schneider, A. Plasma Extracellular Vesicle Tau and TDP-43 as Diagnostic Biomarkers in FTD and ALS. *Nat Med* **2024**, *30* (6), 1771–1783. <https://doi.org/10.1038/s41591-024-02937-4>.
- (69) Ma, Y.; Ding, X.; Lu, S.; Huang, X.; Hu, Y.; Liu, H.; Liu, B.; Liu, K.; Zhang, M.; Wang, H.; Xu, F.; Qin, W. M2 Macrophage-Derived Extracellular Vesicles Protect against Abdominal Aortic Aneurysm by Modulating Macrophage Polarization through miR221-5p. *Cellular & Molecular Biology Letters* **2025**, *30* (1), 96. <https://doi.org/10.1186/s11658-025-00768-w>.
- (70) Camino, T.; Lago-Baameiro, N.; Bravo, S. B.; Molares-Vila, A.; Sueiro, A.; Couto, I.; Baltar, J.; Casanueva, E. F.; Pardo, M. Human Obese White Adipose Tissue Sheds Depot-Specific Extracellular Vesicles and Reveals Candidate Biomarkers for Monitoring Obesity and Its Comorbidities. *Translational Research* **2022**, *239*, 85–102. <https://doi.org/10.1016/j.trsl.2021.01.006>.
- (71) *Lab-on-Chip for Exosomes and Microvesicles Detection and Characterization*. <https://www.mdpi.com/1424-8220/18/10/3175> (accessed 2025-08-17).
- (72) Wang, Z.; Zhou, X.; Kong, Q.; He, H.; Sun, J.; Qiu, W.; Zhang, L.; Yang, M. Extracellular Vesicle Preparation and Analysis: A State-of-the-Art Review. *Advanced Science* **2024**, *11* (30), 2401069. <https://doi.org/10.1002/advs.202401069>.
- (73) George, S. K.; Lauková, L.; Weiss, R.; Semak, V.; Fendl, B.; Weiss, V. U.; Steinberger, S.; Allmaier, G.; Tripisciano, C.; Weber, V. Comparative Analysis of Platelet-Derived Extracellular Vesicles Using Flow Cytometry and Nanoparticle Tracking Analysis. *International Journal of Molecular Sciences* **2021**, *22* (8), 3839. <https://doi.org/10.3390/ijms22083839>.
- (74) Cvjetkovic, A.; Lötvall, J.; Lässer, C. The Influence of Rotor Type and Centrifugation Time on the Yield and Purity of Extracellular Vesicles. *Journal of Extracellular Vesicles* **2014**, *3* (1), 23111. <https://doi.org/10.3402/jev.v3.23111>.
- (75) Ströhle, G.; Gan, J.; Li, H. Affinity-Based Isolation of Extracellular Vesicles and the Effects on Downstream Molecular Analysis. *Anal Bioanal Chem* **2022**, *414* (24), 7051–7067. <https://doi.org/10.1007/s00216-022-04178-1>.
- (76) Clos-Sansalvador, M.; Monguió-Tortajada, M.; Roura, S.; Franquesa, M.; Borràs, F. E. Commonly Used Methods for Extracellular Vesicles' Enrichment: Implications in Downstream Analyses and Use. *Eur J Cell Biol* **2022**, *101* (3), 151227. <https://doi.org/10.1016/j.ejcb.2022.151227>.
- (77) Franco, C.; Ghirardello, A.; Bertazza, L.; Gasparotto, M.; Zanatta, E.; Iaccarino, L.; Valadi, H.; Doria, A.; Gatto, M. Size-Exclusion Chromatography Combined with Ultrafiltration Efficiently Isolates Extracellular Vesicles from Human Blood Samples in

- Health and Disease. *International Journal of Molecular Sciences* **2023**, *24* (4), 3663. <https://doi.org/10.3390/ijms24043663>.
- (78) Ahmadian, S.; Jafari, N.; Tamadon, A.; Ghaffarzadeh, A.; Rahbarghazi, R.; Mahdipour, M. Different Storage and Freezing Protocols for Extracellular Vesicles: A Systematic Review. *Stem Cell Research & Therapy* **2024**, *15* (1), 453. <https://doi.org/10.1186/s13287-024-04005-7>.

## **CHAPTER 2 – SPECTROSCOPIC STRATEGIES FOR BIOFLUIDS AND THEIR EXTRACELLULAR VESICLES TO ADVANCE IN CLINICAL TRANSLATION**

In current clinical practice, the analysis of biofluids typically relies on the detection of single biomarkers, often neglecting the complex molecular interplay that characterizes these biological matrices<sup>[1]</sup>. However, as previously discussed, biofluids represent a highly heterogeneous system <sup>[2]</sup>, therefore, a comprehensive analytical approach able to capture the global molecular landscape of biofluids is essential to better reflect the physiological and pathological states of the organism.

In this context, spectroscopic techniques have emerged as powerful tools for the analysis of biofluids and their EV fractions<sup>[3]</sup>. In general, spectroscopies exploit the interaction between light and matter to generate unique molecular fingerprints that encode the biochemical composition of complex samples in a rapid, label-free, and non-destructive manner. These techniques can be directly applied to unprocessed biofluids, such as plasma, serum, urine, or saliva, minimizing sample handling and preserving the native molecular architecture<sup>[4]</sup>.

Importantly, spectroscopic approaches can also be specifically employed to analyze EVs<sup>[5]</sup>. Compared with whole-biofluid analysis, EV-targeted spectroscopy provides higher molecular specificity and may yield insights into the underlying biological pathways of disease progression.

Furthermore, the use of spectroscopic methods offers several practical advantages that address key barriers to the clinical translation of EV-based diagnostics. These include high sensitivity and resolution, minimal sample requirements, and the absence of labeling procedures<sup>[3]</sup>. By combining these capabilities with advanced computational analysis, spectroscopy paves the way for the development of robust, reproducible, and clinically applicable diagnostic platforms that bridge the gap between research and clinical applications.

### **2.1 Vibrational Spectroscopy for Clinical Applications**

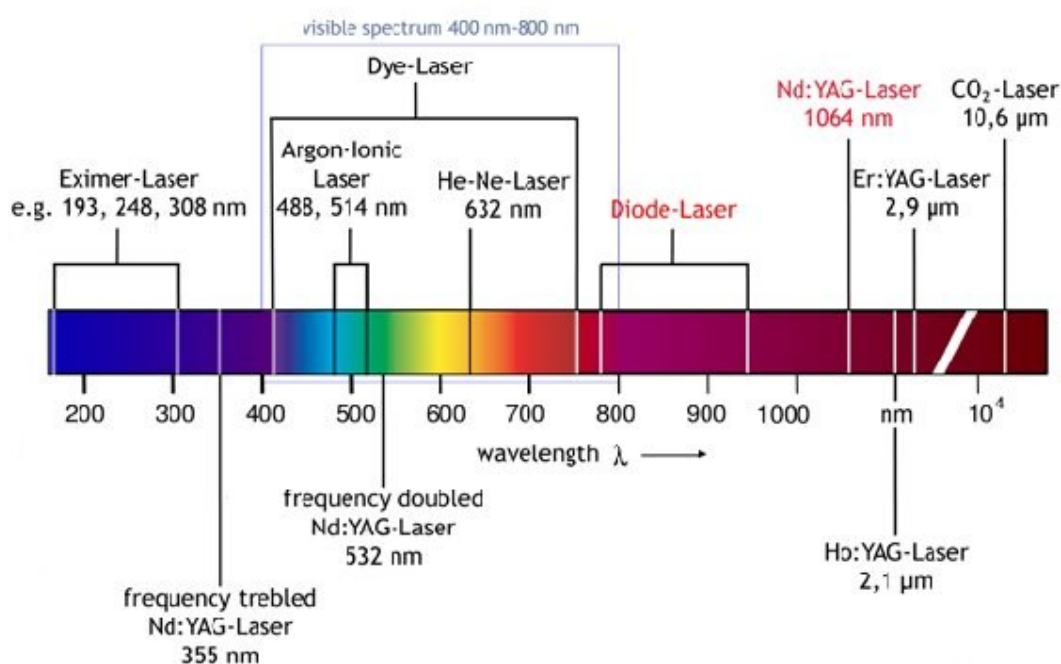
Vibrational spectroscopies are powerful analytical techniques that probe the intrinsic molecular vibrations of a sample, providing detailed information on its chemical composition and structure<sup>[3]</sup>. Among these, infrared (IR) and Raman Spectroscopy (RS) are the most widely used, enabling label-free, non-destructive, and rapid analysis of complex biological samples<sup>[6]</sup>. By generating characteristic molecular fingerprints, vibrational spectroscopies allow the simultaneous detection of multiple biomolecules, including proteins, lipids, nucleic acids, and

metabolites. This capacity makes them particularly suitable for the comprehensive analysis of biofluids<sup>[7]</sup> and their EV fractions<sup>[8,9]</sup>, offering insights into disease-related biochemical alterations while minimizing sample preparation and preserving native molecular information.

### 2.1.1 Raman Spectroscopy

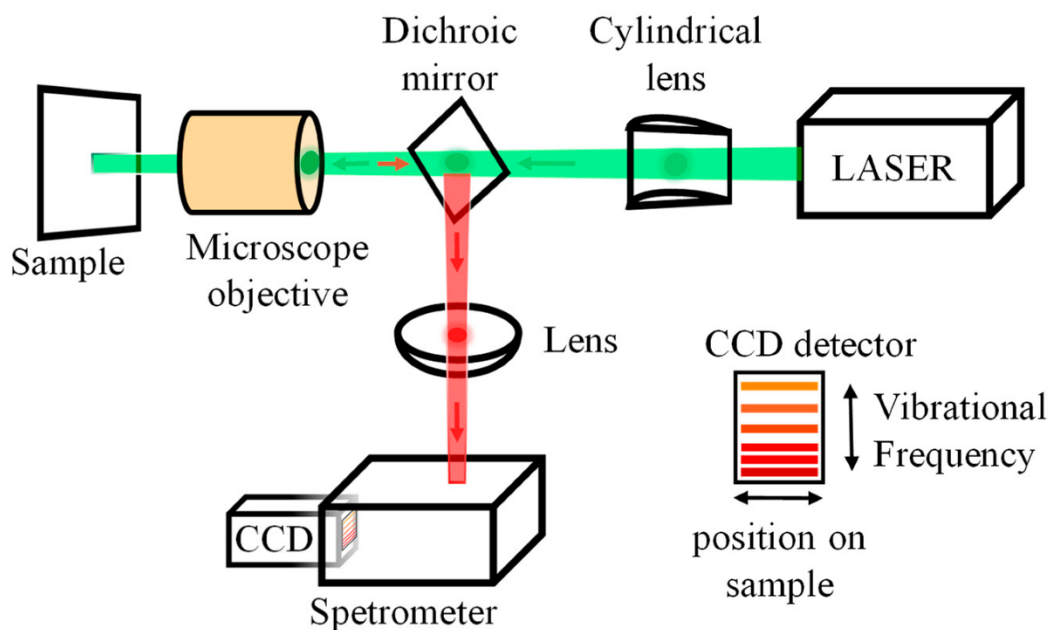
Raman spectroscopy (RS) is a vibrational spectroscopic technique based on the inelastic scattering of light, first described by C.V. Raman in 1928<sup>[10]</sup>.

A typical Raman spectrometer consists of four key elements: a laser source, a sample compartment, a spectrograph, and a detector. The laser provides a narrow, monochromatic beam that excites molecular vibrations, generating scattered photons with shifted energies. Excitation wavelengths commonly span from the ultraviolet to the near-infrared region; widely employed options include argon-ionic (488–514 nm), helium-neon (632 nm), diode lasers (780–940 nm), and Nd:YAG sources (532 and 1064 nm).



**Figure 2.1** Representative laser types across the optical spectrum<sup>[11]</sup>.

The sample holder ensures stability and minimizes environmental perturbations during acquisition. Scattered light is then dispersed by the spectrometer according to its energy shifts, and the resulting spectrum is recorded by sensitive detectors, such as charge-coupled devices (CCDs), enabling detailed molecular fingerprinting<sup>[12]</sup>.

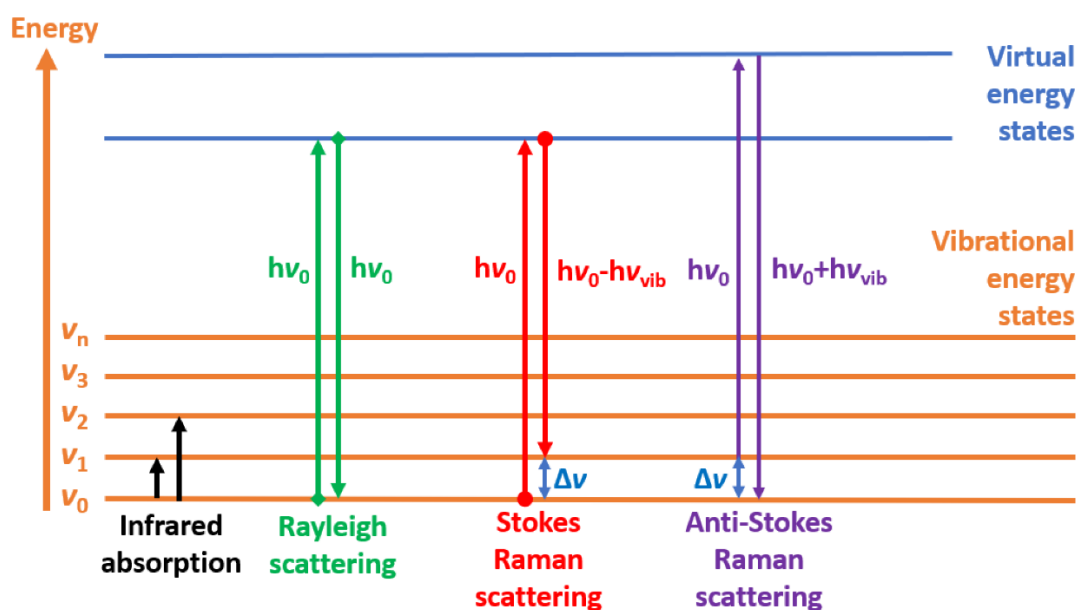


**Figure 2.2** Schematic of a standard Raman spectroscopy system<sup>[13]</sup>.

When monochromatic light interacts with a sample, most photons undergo elastic scattering (Rayleigh scattering), in which their energy and wavelength remain unchanged. However, a small fraction is scattered inelastically, resulting in a shift in photon energy that reflects the vibrational states of the molecules. This phenomenon, known as the Raman effect, provides molecular information that enables the identification of chemical bonds. The vibrational energy levels follow the quantum relation:

$$E_v = h\nu \left( n + \frac{1}{2} \right)$$

where  $\nu$  is the vibrational frequency,  $h$  is Planck's constant, and  $n$  is the vibrational quantum number<sup>[14]</sup>.



**Figure 2.3** Illustration of scattering processes when a molecule interacts with monochromatic light. Rayleigh scattering occurs without change in vibrational energy, whereas Stokes Raman scattering involves energy transfer from the photon to the molecule (lower scattered frequency), and anti-Stokes Raman scattering reflects energy transfer from the molecule to the photon (higher scattered frequency). Raman scattering differs from infrared absorption in that the scattered photon does not correspond directly to the energy difference between initial and final states<sup>[12]</sup>.

From a physical point of view, Raman scattering arises due to the modulation of a molecule's polarizability during vibration. When the oscillating electric field of the incident light interacts with the electron cloud, it induces a temporary dipole. If the vibration of the molecule alters the polarizability, the scattered light exhibits an energy shift corresponding to the vibrational mode. The energy difference between the incident and scattered photons is directly related to the vibrational frequency of the molecular bond and is conventionally expressed in wavenumbers ( $\text{cm}^{-1}$ )<sup>[15]</sup>.

The resulting Raman spectrum is typically represented as a plot of intensity versus Raman shift. Each peak corresponds to a specific vibrational mode, providing characteristic molecular information.

Importantly, RS is label-free and non-destructive, enabling real-time characterization of samples without the need for additional reagents or extensive preparation<sup>[16]</sup>.

In the biomedical context, RS has gained considerable attention due to its ability to provide detailed biochemical information from complex matrices such as tissues<sup>[17]</sup>, cells<sup>[18]</sup>, and biofluids<sup>[19,20]</sup>. Unlike conventional biochemical assays, Raman spectra capture molecular signatures that reflect proteins, lipids, nucleic acids, and metabolites simultaneously. This

intrinsic multiplexing capacity makes the technique particularly valuable in precision medicine, where subtle molecular differences can indicate early disease onset, progression, or therapeutic response<sup>[21,22]</sup>.

Indeed, the so-called fingerprint region (600–1800  $\text{cm}^{-1}$ ) contains most of the characteristic peaks associated with proteins, nucleic acids, lipids, and carbohydrates, thereby enabling a comprehensive assessment of cellular and extracellular components. Within this range, the amide I band ( $\sim 1650 \text{ cm}^{-1}$ ), dominated by C=O stretching, provides insights into protein secondary structures, while the amide III region (1200–1300  $\text{cm}^{-1}$ ) reflects contributions from C–N stretching and N–H bending. Peaks around 1000  $\text{cm}^{-1}$  are attributed to phenylalanine ring breathing, serving as robust protein markers, whereas phosphate vibrations ( $\sim 1090 \text{ cm}^{-1}$ ) are indicative of nucleic acid backbones<sup>[23]</sup>. Lipid-associated bands are commonly found in the high wavenumber region (2850–2950  $\text{cm}^{-1}$ ), corresponding to  $\text{CH}_2$  and  $\text{CH}_3$  stretching modes, and can be used to evaluate membrane composition and metabolic alterations<sup>[24]</sup>. Taken together, these spectral windows provide a molecular fingerprint that can sensitively capture biochemical changes underlying both physiological and pathological processes.

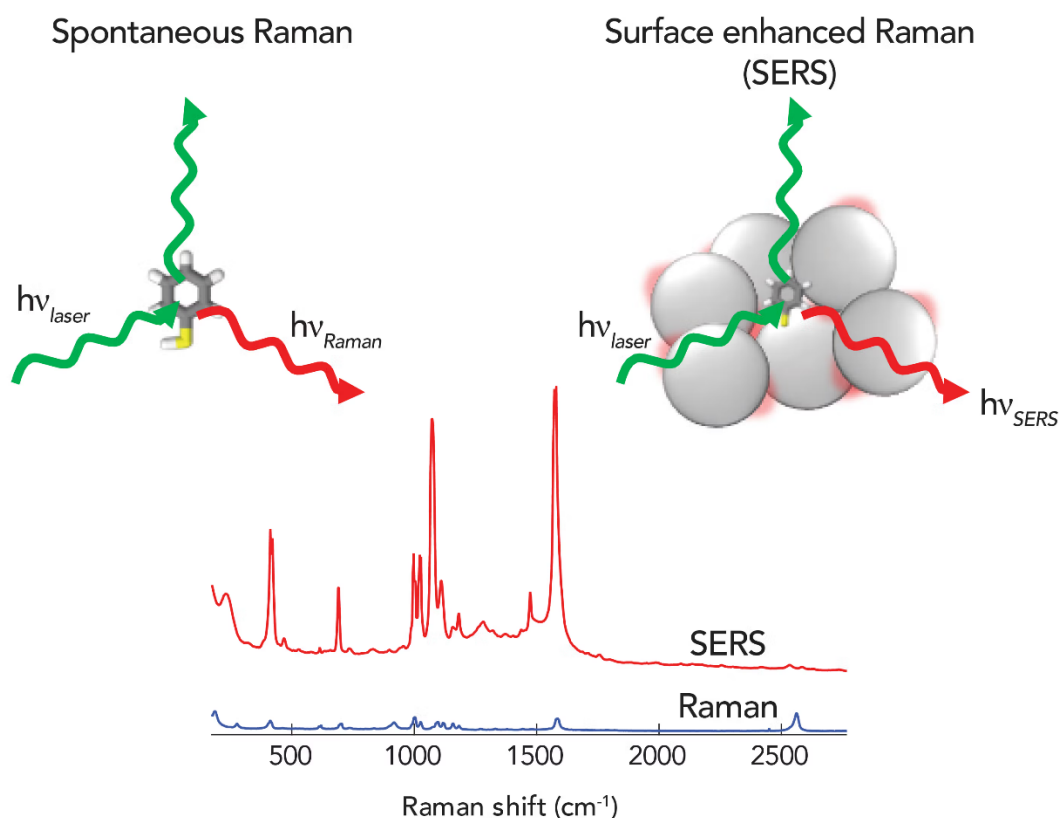
Despite its promise, spontaneous RS suffers from inherently weak signal intensity, as only about one in  $10^6$ – $10^8$  photons undergo Raman scattering. This limitation has led to the development of signal-enhancement strategies, most notably Surface-Enhanced Raman Scattering (SERS), which exploits metallic nanostructures to amplify Raman signals by several orders of magnitude<sup>[25]</sup>. The principle underlying SERS is that when molecules are adsorbed onto or located near metallic nanostructures, typically composed of gold or silver, the local electromagnetic field generated by the excitation of surface plasmons drastically increases the Raman scattering efficiency. This enhancement can reach factors of  $10^6$  to  $10^{10}$ , allowing the detection of molecules at very low concentrations. Due to its remarkable capability for detecting analytes at ultralow concentrations, SERS has emerged as a critical analytical tool in catalysis, biosensing, pharmaceutical monitoring, optoelectronic applications<sup>[26]</sup>, and in clinical fields<sup>[27]</sup>. Current understanding suggests that SERS performance is intimately linked to the interplay between Localized Surface Plasmon Resonance (LSPR) phenomena and charge transfer mechanisms intrinsic to the material.

In detail, two main mechanisms contribute to this enhancement:

- 1) **Electromagnetic enhancement**, which arises from the excitation of localized surface plasmons on the metal surface. When incident light resonates with the collective oscillation of conduction electrons, intense electromagnetic fields are generated in specific regions

known as “hot spots,” leading to a dramatic amplification of the Raman signal from nearby molecules<sup>[28]</sup>.

- 2) **Chemical enhancement**, which involves direct electronic interactions between the adsorbed molecule and the metal surface. Although its contribution is typically smaller than that of the electromagnetic effect, it further modulates the Raman intensity and can provide additional selectivity<sup>[29,30]</sup>.



**Figure 2.4** Illustration of a laser interacting with a molecule to produce spontaneous Raman scattering, compared to a laser interacting with a molecule adsorbed on plasmonic nanoparticles to generate SERS. The figure shows the SERS spectrum of molecule on silver nanostructures and the spontaneous Raman spectrum of pure molecule. The localized electric field at the nanoparticle surface significantly amplifies the Raman signal in the SERS measurement<sup>[31]</sup>.

This ability to combine molecular specificity with ultra-high sensitivity has positioned SERS as a promising approach in biomedicine, bridging the gap between fundamental vibrational spectroscopy and diagnostic applications. Nevertheless, when applied to complex biological samples, the overlapping signals of multiple chemical bonds can complicate spectral interpretation and potentially lead to misinterpretations. To address this challenge, chemometric

analyses are commonly employed to reliably extract meaningful information while preserving the advantages of SERS in clinical applications<sup>[12,27]</sup>.

### **2.1.2 Fourier-Transform Infrared Spectroscopy**

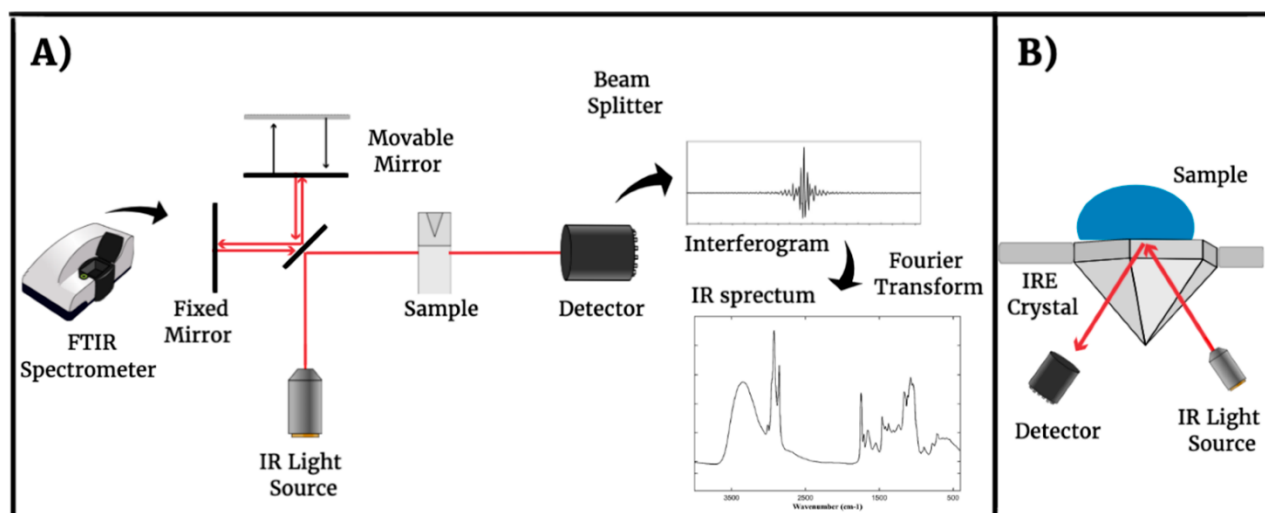
Fourier Transform Infrared Spectroscopy (FTIR) is a vibrational spectroscopic technique that exploits the interaction between infrared radiation and molecular bonds to probe chemical composition and structural organization. When molecules absorb IR radiation, they undergo vibrational transitions corresponding to stretching, bending, or twisting of their chemical bonds. Each functional group absorbs at a characteristic frequency, generating a spectral fingerprint that reflects the overall biochemical composition of the sample<sup>[32]</sup>.

Unlike Raman spectroscopy, which probes changes in molecular polarizability, FTIR is based on variations in dipole moment during vibrational transitions<sup>[33]</sup>. This complementary mechanism makes FTIR particularly sensitive to polar functional groups such as carbonyls, hydroxyls, and amides. The technique is able to detect a wide range of biomolecules including proteins, lipids, nucleic acids, and carbohydrates, thereby providing an integrated overview of the molecular landscape of cells, tissues, and biofluids<sup>[34]</sup>.

The core principle of FTIR spectroscopy relies on the use of an interferometer, typically a Michelson interferometer, to modulate the IR beam before it interacts with the sample. The interferogram generated by the optical path differences is then mathematically converted into a spectrum through Fourier transformation. This design allows simultaneous acquisition of a broad spectral range with high signal-to-noise ratio and short acquisition times compared to dispersive IR spectroscopy<sup>[35]</sup>.

The application of FTIR in biomedical research dates back to the 1970s, with its diagnostic potential in biological systems recognized about a decade later<sup>[36]</sup>. However, a major limitation of conventional FTIR lies in the strong absorbance of water, which restricts analysis of aqueous samples and reduces reproducibility due to path length variability. To address these challenges, Attenuated Total Reflectance (ATR)-FTIR was developed<sup>[37]</sup>. In this approach, infrared light undergoes internal reflection within a high-refractive-index crystal (commonly diamond, ZnSe, Ge, or Si) allowing efficient spectral acquisition with minimal sample preparation. This

configuration enhances reproducibility, reduces the impact of water absorption, and has greatly expanded the applicability of FTIR in biological and clinical contexts<sup>[32]</sup>.



**Figure 2.5** (A) Schematic of a double-mirror Michelson interferometer as employed in FTIR spectroscopy. (B) Illustration of the principle of internal reflection in infrared spectroscopy<sup>[32]</sup>.

The biological relevance of FTIR spectra is largely concentrated in two spectral windows. The mid-infrared region ( $4000\text{--}400\text{ cm}^{-1}$ ) encompasses most fundamental vibrations of biomolecules, with the so-called “fingerprint region” ( $1800\text{--}900\text{ cm}^{-1}$ ) being especially informative for identifying proteins, nucleic acids, and carbohydrates. Within this range, the amide I ( $\sim 1650\text{ cm}^{-1}$ ) and amide II ( $\sim 1550\text{ cm}^{-1}$ ) bands provide structural information on protein secondary structures, while phosphate vibrations ( $\sim 1080\text{ cm}^{-1}$  and  $\sim 1230\text{ cm}^{-1}$ ) are markers for nucleic acids. The high wavenumber region ( $3000\text{--}2800\text{ cm}^{-1}$ ) is dominated by CH stretching vibrations, typically used to evaluate lipid content and membrane composition<sup>[38]</sup>.

The strengths of FTIR lie in its label-free and non-destructive nature, rapid acquisition, and the potential to extract complex biochemical information. However, limitations include spectral overlap between different molecular groups, strong absorption of water in the mid-IR region, and the need for robust computational and chemometric methods to deconvolute the rich spectral data.

Overall, FTIR spectroscopy provides a powerful and complementary tool to Raman-based methods for the characterization of biological materials. By offering a molecular fingerprint sensitive to both compositional and structural features, FTIR has become increasingly

attractive for biomedical and clinical applications, including liquid biopsy and EVs profiling<sup>[5,12]</sup>.

### **2.1.3 Raman, SERS and FTIR Spectroscopy as Advanced Tools for Diagnostic Purposes and Follow-up**

RS and SERS have increased interest in the clinical field thanks to their ability to provide rapid, label-free, low-cost and highly specific molecular information from complex biological samples with no interference of water. In contrast to conventional biochemical assays that require extensive sample preparation or the use of external markers, these vibrational spectroscopies enable direct molecular fingerprinting of biofluids and their EV fractions using small quantities of samples<sup>[5,39]</sup>. This feature positions them as powerful candidates for liquid biopsy approaches, capable of complementing traditional diagnostic techniques.

For instance, serum Raman spectra combined with machine learning have been shown to distinguish benign from malignant pulmonary nodules with high diagnostic accuracy, outperforming conventional clinical approaches while avoiding invasive procedures, thus highlighting the translational potential of RS in lung cancer screening<sup>[40]</sup>.

Similar evidence has emerged in colorectal cancer (CRC). In a study analyzing dried plasma samples, RS revealed distinct spectral signatures linked to proteins, lipids, nucleic acids, and aromatic amino acids, enabling the discrimination of CRC patients from individuals with inflammatory or diverticular diseases. Notably, longitudinal sampling before and after surgical resection showed that spectral alterations normalized in the postoperative phase, directly linking the Raman fingerprint to tumor presence. These findings highlight the dual role of RS not only as a diagnostic tool but also as a means for monitoring disease progression and therapeutic efficacy<sup>[41]</sup>.

In the context of respiratory and neurodegenerative disorders, recent pilot studies have highlighted the potential of salivary SERS spectra to provide disease-specific biochemical fingerprints. In both Chronic Obstructive Pulmonary Disease (COPD)<sup>[19]</sup> and Parkinson's disease (PD)<sup>[42]</sup>, SERS analysis revealed alterations in proteins, lipids, saccharides, and nucleic acids, consistent with the underlying metabolic and pathological processes. The adoption of multivariate statistical approaches, together with advanced machine and deep learning algorithms, enabled highly accurate discrimination between patients, healthy controls, and additional pathological groups. Beyond diagnostic capability, SERS-based saliva profiling demonstrated correlations with established clinical scales in PD and showed promise for patient stratification, therapy monitoring, and rehabilitation assessment in both conditions. These

studies collectively suggest that analysis of saliva by SERS could emerge as a minimally invasive, cost-effective tool for precision diagnostics and longitudinal monitoring across diverse clinical domains<sup>[19,42]</sup>.

As anticipated, several studies have focused on using spectroscopy techniques not only on whole biofluid, but also on their EV fractions in order to perform a finer biochemical characterization. For instance, a study explored the use of SERS to profile EVs in CSF for the diagnosis and monitoring of pediatric medulloblastoma. Researchers used Silver nanocube-based three-dimensional SERS substrates to acquire spectra from CSF, in order to capture distinct molecular fingerprint of tumor-derived EVs. Stacking machine learning frameworks applied to the spectral data achieved high accuracy in differentiating medulloblastoma from other intracranial tumors. Furthermore, dynamic changes in EV Raman profiles reflected therapeutic response, highlighting the potential of this approach for the disease monitoring<sup>[43]</sup>.

Extending the application of label-free RS to neurodegenerative disorders, circulating EVs from PD patients have been shown to carry disease-related biochemical signatures. Analysis of serum-derived EVs using an optimized RS protocol reproducible biochemical signatures correlated with disease severity measured by clinical scales. These spectral variations likely arise from changes in brain-derived EV populations, modifications in lipid composition, and altered glycosylation or glycation of EV cargo, including  $\alpha$ -synuclein oligomers<sup>[44]</sup>.

EV Raman fingerprint from breast cancer have been studied by Bonizzi et al. This study explored the application of RS for EV biochemical characterization isolated from the serum of breast cancer patients, with a specific focus on differentiating them from circulating lipoproteins. By combining Raman spectral profiling with multivariate statistical analyses, the authors identified distinct molecular signatures that enabled effective separation of EVs from lipoproteins, as well as between breast cancer patients and healthy controls. Key differences were attributed to lipid and protein-related vibrational modes, reflecting pathological alterations associated with tumor-derived EVs. Overall, these findings support the potential of Raman-based methodologies as a robust, minimally invasive tool for liquid biopsy, offering both diagnostic insights and a better understanding of disease-specific EVs composition<sup>[45]</sup>.

In clinical applications, FTIR spectroscopy has emerged as a complementary tool due to its label-free, non-destructive nature, minimal sample requirements, rapid acquisition, and high reproducibility, making it attractive for routine biomedical and diagnostic use. Thanks to all these abilities, FTIR enables the acquisition of detailed molecular fingerprints with strong reproducibility, positioning it as an attractive candidate for translation into routine biomedical and diagnostic applications<sup>[46]</sup>.

For example, in breast cancer, FTIR spectroscopy has been applied to serum analysis to detect structural alterations in circulating proteins. Through curve-fitting of the amide I band and evaluation of absorbance ratios in the amide II and III regions, the study revealed significant changes in secondary structure, particularly in the  $\alpha$ -helix and  $\beta$ -sheet content. Importantly, extending the analysis to the nucleic acid and carbohydrate spectral windows further enhanced the discrimination between cancer patients and healthy controls.<sup>[47]</sup>

Another study explored myocardial damage induced by diabetes using murine myocardium tissue and murine biofluids (plasma, urine, and saliva) and analyzed by FTIR spectroscopy. The results showed that mice exhibited significant metabolic alterations in lipids, proteins, nucleic acids, and carbohydrates in both myocardium tissue and biofluids. Importantly, plasma was most informative for early stages of diabetic cardiomyopathy, while saliva better reflected later disease progression, and their combined analysis improved diagnostic accuracy. These findings highlight the clinical potential of FTIR-based biofluid profiling as a non-invasive tool for monitoring diseases<sup>[48]</sup>.

In EV field, Imola Cs Szigyártó et al. have optimized a protocol to analysed EVs by ATR-FTIR with complementary methodologies to investigate biochemical changes in blood and pancreatic tumor-derived EVs. The use of ATR-FTIR enabled direct insight into the molecular composition of EVs, providing a reliable basis for their biochemical characterization<sup>[49]</sup>.

Another study, used ATR-FTIR as method for EV protein quantification. The approach was based on measuring the integrated area of the Amide I band, which directly correlates with protein content. Calibration was first performed with bovine serum albumin (BSA) standards in the range of 0.08–1 mg/mL, showing excellent linearity. To verify the robustness of the method in complex matrices, the authors conducted EV dilution experiments and performed a spike-in assay, where defined amounts of BSA were deliberately added to EV preparations. The proportional increase in the Amide I signal confirmed that the method reliably detected variations in protein concentration even within a complex matrix as EV samples. Importantly, the protein quantification obtained by ATR-FTIR was comparable to conventional colorimetric assays, while avoiding reagents and additional sample manipulation. These findings support the use of ATR-FTIR as a straightforward, reproducible, and clinically applicable approach for EV protein analysis<sup>[50]</sup>.

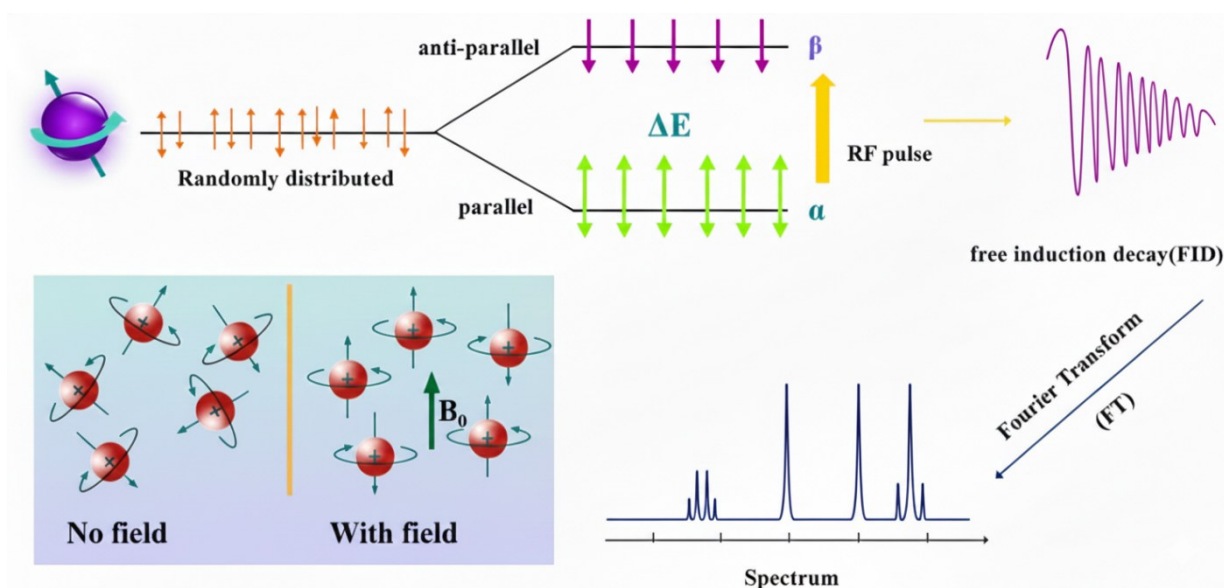
Recent investigations have also applied FTIR spectroscopy to salivary EVs in the context of smoking-related oral carcinogenesis. In this study, EVs isolated from saliva of non-smokers, smokers, and oral cancer patients displayed distinct molecular fingerprints, with FTIR detecting changes in protein and amino acid content that reflected altered metabolic activity. These

findings were complemented by biochemical analyses, which revealed elevated EV protein levels, significant alterations in amino acid profiles, and increased concentrations of pro-inflammatory cytokines IL-6 and IL-8. Importantly, IL-6 levels showed a progressive increase from smokers to oral cancer patients, suggesting its involvement in tumor initiation and progression. Collectively, these results underscore the potential of EV-based FTIR analysis, in combination with conventional biochemical assays, as a non-invasive strategy for early detection and monitoring of oral cancer also<sup>[51]</sup>.

Taken together, these findings highlight how vibrational spectroscopy approaches, particularly when applied to biofluids and their EV fractions, can capture disease-related biochemical alterations with high sensitivity, supporting their translation into minimally invasive tools for early diagnosis, and patient stratification.

## **2.2 Nuclear Magnetic Resonance Spectroscopy for Innovative Biofluid Analysis in Clinical applications**

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical method that utilizes the magnetic characteristics of certain nuclei. When a sample is exposed to a strong magnetic field, nuclei such as hydrogen ( $^1\text{H}$ ) and carbon ( $^{13}\text{C}$ ) adopt specific spin orientations. The application of a radiofrequency pulse promotes transitions between these spin states, and as the nuclei return to equilibrium, they release distinctive signals. These signals are converted into spectra, which provide detailed insights into the chemical environment, atomic connectivity, and molecular dynamics, thereby enabling the determination of both molecular structure and composition<sup>[52]</sup>.



**Figure 2.6** Fundamental Principle of Nuclear Magnetic Resonance (NMR) and Signal Acquisition. The image illustrates the sequence of events that occur when an external static magnetic field ( $B_0$ ) is applied to an NMR-active nucleus (e.g., a proton). In the absence of  $B_0$  (center, low-energy state), the nuclear magnetic moments are randomly oriented. Upon application of the strong external magnetic field ( $B_0$  applied vertically), the spin energy levels split into two distinct states: the lower-energy  $\alpha$  state (spin aligned parallel to  $B_0$ ) and the higher-energy  $\beta$  state (spin aligned anti-parallel to  $B_0$ ). The energy separation ( $\Delta E$ ) between these two states is directly proportional to the magnetic field strength, according to the Larmor equation:  $\Delta E = \gamma(h/2\pi)B_0$ . Resonance occurs when the system absorbs radiofrequency (RF) energy that matches  $\Delta E$  exactly, promoting a transition (flip) of spins from the  $\alpha$  state to the  $\beta$  state. After the RF pulse, the excited nuclei relax back toward equilibrium, generating a complex time-domain signal known as the Free Induction Decay (FID). The final NMR spectrum, which displays signal intensity as a function of frequency, is obtained by applying a Fourier Transform (FT) to the FID<sup>53</sup>. Image modified by AI.

NMR spectroscopy is not limited to providing fundamental structural data but also serves as a powerful tool for investigating interactions within biomolecules such as proteins and nucleic acids. Advances in methodologies, including hyperpolarization strategies and isotopic enrichment, have markedly improved both the sensitivity and the scope of NMR in macromolecular research. These developments allow scientists to gain comprehensive insights into the conformational dynamics and intermolecular interactions of large biological systems<sup>[53,54]</sup>.

Moreover, NMR has gained significant relevance in the biological field for metabolomics applications. Indeed, in a recent study, lipoprotein profiling by NMR, combined with advanced multivariate analysis, was employed to distinguish patients with PD from individuals with AD

and healthy controls. The approach demonstrated excellent classification performance, achieving complete separation between groups as well as between early-stage PD and PD-related dementia. Rather than focusing on single biomarkers, this methodology leveraged disease- and stage-specific lipoprotein signatures, highlighting the potential of NMR-based lipidomics<sup>[55]</sup>.

In addition to neurodegenerative disorders, NMR-based metabolomics has been explored in inflammatory muscle diseases. Anupam Guleria and collaborators investigated serum and muscle samples from patients with idiopathic inflammatory myopathies (IIM) using high-field NMR spectroscopy, comparing them with healthy controls. The metabolic profiles revealed marked alterations, including enhanced anaerobic metabolism, impaired oxidative pathways, reduced muscle mass, and lipid imbalances. Importantly, these metabolic signatures were able to differentiate active from inactive disease states independently of clinical subtype<sup>[56]</sup>.

Similarly, another study investigated metabolic alterations in the serum of sepsis patients using <sup>1</sup>H NMR spectroscopy, comparing septic patients with age- and gender-matched healthy controls. Significant changes were observed in metabolites linked to energy metabolism and inflammation, including increased glucose, glycine, 3-hydroxybutyrate, creatinine, and glycoprotein acetyls, alongside decreased citrate and histidine levels. Researchers also discovered that non-survivors exhibited higher lactate and citrate concentrations, suggesting these metabolites reflect disease severity and prognosis. The findings highlight <sup>1</sup>H NMR as a robust, non-invasive tool for simultaneous quantification of multiple serum metabolites, offering potential for early sepsis diagnosis and patient stratification<sup>[57]</sup>.

To date, the application of NMR to the study of EVs remains largely unexplored. However, one study applied <sup>1</sup>H diffusion-ordered spectroscopy (DOSY) to characterize different subpopulations of extracellular nanoparticles. Thanks to this approach, researchers were able to non-invasively determine particle size distributions spanning 1–500 nm, capturing both conventional small-EVs (50-150 nm) and smaller nanoparticles such as exomers, which are undetectable by NTA. EVs derived from milk, embryonic kidney, and renal carcinoma cells showed distinct size profiles, validated by TEM and the expression of EV markers. This NMR analysis provides sensitive, label-free characterization of particle heterogeneity, purity, and composition, offering valuable tools for both basic research and future clinical applications in EV field also<sup>[58]</sup>.

Building on these findings, NMR spectroscopy presents several distinct advantages that enhance its clinical utility. It provides high-resolution analysis and enables the study of samples directly in solution, eliminating the need for crystallization. Unlike techniques such as X-ray

crystallography, NMR requires minimal sample preparation, allowing rapid and reproducible measurements. Moreover, it can capture dynamic processes in biomolecules, offering insights into functional and metabolic changes in real time. These features make NMR particularly well-suited for investigating complex pathophysiological conditions, where monitoring multiple metabolites simultaneously can inform early diagnosis and prognostic assessment<sup>[59]</sup>. Despite its advantages, NMR spectroscopy also presents certain limitations. A primary concern is its relatively low sensitivity compared to techniques such as mass spectrometry, often requiring higher sample concentrations or isotopic labeling to achieve adequate signal detection<sup>[60]</sup>. Additionally, the interpretation of NMR spectra can be challenging, particularly in regions with overlapping signals, complicating data analysis<sup>[61]</sup>. High-field NMR instruments, although offering improved resolution, are costly and demand essential infrastructure, which may restrict their accessibility in some research or clinical settings<sup>[62]</sup>.

Nevertheless, NMR represents a valuable approach to conventional methodologies, with the potential applications in elucidating disease mechanisms and in the diagnosis of conditions that remain challenging to detect.

## **2.3 Aim of the Thesis**

Biofluids are increasingly recognised as highly informative sources in clinical field due to their ability to reflect pathophysiological processes. Collected through minimally invasive procedures, they provide an opportunity for early diagnosis, disease monitoring and evaluation of therapeutic responses. Beyond cells and soluble molecules, biofluids contain mesoscale structures, ranging from a few nanometers to several micrometers, which constitute the nanostructured secretome. Among these, non-vesicular extracellular particles and EVs are present. In particular, EVs are carriers of different bioactive molecules, reflecting cells of origin and, therefore, becoming interesting as potential new biomarkers of diseases.

The clinical translation of whole biofluids and their EVs remain hindered by technical challenges. While the analysis of whole biofluids is relatively easier respect to EVs analysis, it still requires expensive instrumentation, and time-consuming procedures. In contrast, EVs pose additional complexities due to their nanoscale dimensions, heterogeneity, low concentration, and the lack of standardized separation and characterization protocols, which limit their reproducibility and clinical applicability.

These limitations underscore the need for analytical strategies able to capture both the molecular and mesoscale spectroscopic signatures, addressing the multiscale complexity of biofluids.

Within this framework, the aim of this PhD thesis is to investigate the potential of different spectroscopic methods, alone or in combination, for the characterization of biofluids and their EV fractions, with a focus on developing reproducible and clinically relevant protocols that could accelerate their translation into diagnostics. By targeting both molecular and mesoscale signatures, this work seeks to provide a comprehensive spectroscopic approach to the biochemical landscape of biofluids.

In detail:

- Chapter 3 explores saliva as a model biofluid for clinical applications, highlighting both whole saliva and salivary EVs as sources of molecular and mesoscale information respectively. A patented Raman-based workflow is presented as proof-of-concept for the diagnostic use of whole saliva, demonstrating its ability to discriminate between different respiratory conditions based on their spectroscopic signatures. In addition, RS and SERS analysis are applied to salivary EVs and whole saliva respectively, in order to show how this accessible and non-invasive biofluid can serve as a valuable diagnostic platform and support biomarker discovery within a multiscale framework;
- Chapter 4 focuses on blood, one of the most widely used biofluids in clinical diagnostics. In this chapter, different spectroscopic techniques are applied to whole plasma, as well as to plasma- and serum-derived EVs, to explore their diagnostic and translational potential across both the molecular and mesoscale levels. RS is combined with NMR to analyze whole plasma, with NMR providing complementary molecular information that enables the assignment of Raman peaks and the identification of metabolites relevant for disease discrimination.

In parallel, vibrational spectroscopies, including RS and FTIR, are employed to investigate plasma- and serum-derived EVs in different pathological and clinical contexts. These nanostructured components are analyzed to uncover their mesoscale spectroscopic signatures, including conditions such as Intensive Care Unit-Acquired Weakness (ICU-AW), hematological malignancies, and rehabilitation interventions in Parkinson's disease. This integrated and multiscale approach enhances the interpretability of spectroscopic data, providing deeper insights into the biochemical complexity of blood and its nanoscale structures, while reinforcing the potential of EVs as translational biomarkers for patient monitoring, therapeutic evaluation, and precision diagnostics.

Altogether, this thesis aims to address unmet clinical needs using spectroscopies to analyze both whole biofluids and their EVs fractions. By developing analytical workflows capturing

molecular and mesoscale spectroscopic signatures, this work explores the multiscale architecture to support patient stratification, biomarker discovery, and the monitoring of therapeutic or rehabilitative outcomes in precision medicine.

## 2.4 References

- (1) Dunphy, K.; O'Mahoney, K.; Dowling, P.; O'Gorman, P.; Bazou, D. Clinical Proteomics of Biofluids in Haematological Malignancies. *International Journal of Molecular Sciences* **2021**, *22* (15), 8021. <https://doi.org/10.3390/ijms22158021>.
- (2) Jeppesen, D. K.; Zhang, Q.; Franklin, J. L.; Coffey, R. J. Extracellular Vesicles and Nanoparticles: Emerging Complexities. *Trends in Cell Biology* **2023**, *33* (8), 667–681. <https://doi.org/10.1016/j.tcb.2023.01.002>.
- (3) Baker, M. J.; Hussain, S. R.; Lovergne, L.; Untereiner, V.; Hughes, C.; Lukaszewski, R. A.; Thiéfin, G.; Sockalingum, G. D. Developing and Understanding Biofluid Vibrational Spectroscopy: A Critical Review. *Chem. Soc. Rev.* **2016**, *45* (7), 1803–1818. <https://doi.org/10.1039/C5CS00585J>.
- (4) Aitekenov, S.; Sultangaziyev, A.; Abdirova, P.; Yussupova, L.; Gaipov, A.; Utegulov, Z.; Bukasov, R. Raman, Infrared and Brillouin Spectroscopies of Biofluids for Medical Diagnostics and for Detection of Biomarkers. *Critical Reviews in Analytical Chemistry* **2023**, *53* (7), 1561–1590. <https://doi.org/10.1080/10408347.2022.2036941>.
- (5) Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Rodà, F.; Bedoni, M. Biophotonics for Diagnostic Detection of Extracellular Vesicles. *Advanced Drug Delivery Reviews* **2021**, *174*, 229–249. <https://doi.org/10.1016/j.addr.2021.04.014>.
- (6) Zhang, S.; Qi, Y.; Tan, S. P. H.; Bi, R.; Olivo, M. Molecular Fingerprint Detection Using Raman and Infrared Spectroscopy Technologies for Cancer Detection: A Progress Review. *Biosensors (Basel)* **2023**, *13* (5), 557. <https://doi.org/10.3390/bios13050557>.
- (7) Mayer, R.; Carney, R.; Chiu, K. L. Preanalytical Optimization of Raman Biofluid Measurements toward the Automation of Cancer Detection. In *Optical Diagnostics and Sensing XXIV: Toward Point-of-Care Diagnostics*; SPIE, 2024; Vol. PC12850, p PC1285007. <https://doi.org/10.1117/12.3003212>.
- (8) Paolini, L.; Federici, S.; Consoli, G.; Arceri, D.; Radeghieri, A.; Alessandri, I.; Bergese, P. Fourier-Transform Infrared (FT-IR) Spectroscopy Fingerprints Subpopulations of Extracellular Vesicles of Different Sizes and Cellular Origin. *Journal of Extracellular Vesicles* **2020**, *9* (1), 1741174. <https://doi.org/10.1080/20013078.2020.1741174>.
- (9) Carney, R. P.; Mizenko, R. R.; Bozkurt, B. T.; Lowe, N.; Henson, T.; Arizzi, A.; Wang, A.; Tan, C.; George, S. C. Harnessing Extracellular Vesicle Heterogeneity for Diagnostic and Therapeutic Applications. *Nat. Nanotechnol.* **2025**, *20* (1), 14–25. <https://doi.org/10.1038/s41565-024-01774-3>.
- (10) Raman, C. V.; Krishnan, K. S. A New Type of Secondary Radiation. *Nature* **1928**, *121* (3048), 501–502. <https://doi.org/10.1038/121501c0>.
- (11) Gourzoulidis, G.; Tsilikas, I.; Serafetinides, A.; Sianoudis, I.; Stasinopoulou, P.; Achtipis, A.; Kappa, C.; Makropoulou, M. THE IDENTIFICATION OF OCCUPATIONAL EXPOSURE TO LASER RADIATION IN GREECE. *e-Journal of Science & Technology, (e-JST)* **2017**, *12*, 1–18.
- (12) *Molecular Fingerprint Detection Using Raman and Infrared Spectroscopy Technologies for Cancer Detection: A Progress Review*. <https://www.mdpi.com/2079-6374/13/5/557> (accessed 2025-08-29).

- (13) Deng, J.; Zhao, M.; Jiang, H. Research Progress in the Detection of Mycotoxins in Cereals and Their Products by Vibrational Spectroscopy. *Foods* **2025**, *14* (15), 2688. <https://doi.org/10.3390/foods14152688>.
- (14) Jones, R. R.; Hooper, D. C.; Zhang, L.; Wolverson, D.; Valev, V. K. Raman Techniques: Fundamentals and Frontiers. *Nanoscale Res Lett* **2019**, *14* (1), 231. <https://doi.org/10.1186/s11671-019-3039-2>.
- (15) *Raman Spectroscopy: Theory - Keresztury - 2001 - Major Reference Works - Wiley Online Library*. <https://onlinelibrary.wiley.com/doi/abs/10.1002/0470027320.s0109> (accessed 2025-08-31).
- (16) *Raman Spectroscopy for Chemical Biology Research | Journal of the American Chemical Society*. <https://pubs.acs.org/doi/10.1021/jacs.2c05359> (accessed 2025-08-31).
- (17) Yamamoto, T.; Minamikawa, T.; Harada, Y.; Yamaoka, Y.; Tanaka, H.; Yaku, H.; Takamatsu, T. Label-Free Evaluation of Myocardial Infarct in Surgically Excised Ventricular Myocardium by Raman Spectroscopy. *Sci Rep* **2018**, *8* (1), 14671. <https://doi.org/10.1038/s41598-018-33025-6>.
- (18) Li, M.; Liao, H.-X.; Bando, K.; Nawa, Y.; Fujita, S.; Fujita, K. Label-Free Monitoring of Drug-Induced Cytotoxicity and Its Molecular Fingerprint by Live-Cell Raman and Autofluorescence Imaging. *Anal. Chem.* **2022**, *94* (28), 10019–10026. <https://doi.org/10.1021/acs.analchem.2c00293>.
- (19) Carlomagno, C.; Gualerzi, A.; Picciolini, S.; Rodà, F.; Banfi, P. I.; Lax, A.; Bedoni, M. Characterization of the COPD Salivary Fingerprint through Surface Enhanced Raman Spectroscopy: A Pilot Study. *Diagnostics* **2021**, *11* (3), 508. <https://doi.org/10.3390/diagnostics11030508>.
- (20) Carlomagno, C.; Bertazioli, D.; Gualerzi, A.; Picciolini, S.; Banfi, P. I.; Lax, A.; Messina, E.; Navarro, J.; Bianchi, L.; Caronni, A.; Marengo, F.; Monteleone, S.; Arienti, C.; Bedoni, M. COVID-19 Salivary Raman Fingerprint: Innovative Approach for the Detection of Current and Past SARS-CoV-2 Infections. *Sci Rep* **2021**, *11* (1), 4943. <https://doi.org/10.1038/s41598-021-84565-3>.
- (21) Becker, L.; Lu, C.-E.; Montes-Mojarro, I. A.; Layland, S. L.; Khalil, S.; Nsair, A.; Duffy, G. P.; Fend, F.; Marzi, J.; Schenke-Layland, K. Raman Microspectroscopy Identifies Fibrotic Tissues in Collagen-Related Disorders via Deconvoluted Collagen Type I Spectra. *Acta Biomater* **2023**, *162*, 278–291. <https://doi.org/10.1016/j.actbio.2023.03.016>.
- (22) Cutshaw, G.; Joshi, N.; Wen, X.; Quam, E.; Bogatcheva, G.; Hassan, N.; Uthaman, S.; Waite, J.; Sarkar, S.; Singh, B.; Bardhan, R. Metabolic Response to Small Molecule Therapy in Colorectal Cancer Tracked with Raman Spectroscopy and Metabolomics. *Angewandte Chemie International Edition* **2024**, *63* (43), e202410919. <https://doi.org/10.1002/anie.202410919>.
- (23) Rygula, A.; Majzner, K.; Marzec, K. M.; Kaczor, A.; Pilarczyk, M.; Baranska, M. Raman Spectroscopy of Proteins: A Review. *Journal of Raman Spectroscopy* **2013**, *44* (8), 1061–1076. <https://doi.org/10.1002/jrs.4335>.
- (24) De Gelder, J.; De Gussem, K.; Vandenabeele, P.; Moens, L. Reference Database of Raman Spectra of Biological Molecules. *Journal of Raman Spectroscopy* **2007**, *38* (9), 1133–1147. <https://doi.org/10.1002/jrs.1734>.
- (25) Serebrennikova, K. V.; Berlina, A. N.; Sotnikov, D. V.; Zherdev, A. V.; Dzantiev, B. B. Raman Scattering-Based Biosensing: New Prospects and Opportunities. *Biosensors* **2021**, *11* (12), 512. <https://doi.org/10.3390/bios11120512>.
- (26) Jin, S.; Zhang, D.; Yang, B.; Guo, S.; Chen, L.; Jung, Y. M. Noble Metal-Free SERS: Mechanisms and Applications. *Analyst* **2023**, *149* (1), 11–28. <https://doi.org/10.1039/d3an01669b>.

- (27) Mahmoud, A. Y. F.; Teixeira, A.; Aranda, M.; Relvas, M. S.; Quintero, S.; Sousa-Silva, M.; Chícharo, A.; Chen, M.; Hashemi, M.; King, J. B.; Tunnell, J. W.; Morasso, C.; Piccotti, F.; Corsi, F.; Henriksen-Lacey, M.; de Aberasturi, D. J.; Méndez-Merino, D.; Rodríguez-Patón, A.; Abalde-Cela, S.; Diéguez, L. Will Data Analytics Revolution Finally Bring SERS to the Clinic? *TrAC Trends in Analytical Chemistry* **2023**, *169*, 117311. <https://doi.org/10.1016/j.trac.2023.117311>.
- (28) Ding, S.-Y.; You, E.-M.; Tian, Z.-Q.; Moskovits, M. Electromagnetic Theories of Surface-Enhanced Raman Spectroscopy. *Chem. Soc. Rev.* **2017**, *46* (13), 4042–4076. <https://doi.org/10.1039/C7CS00238F>.
- (29) Campion, A.; Ivanecy, J. E. I.; Child, C. M.; Foster, M. On the Mechanism of Chemical Enhancement in Surface-Enhanced Raman Scattering. *J. Am. Chem. Soc.* **1995**, *117* (47), 11807–11808. <https://doi.org/10.1021/ja00152a024>.
- (30) Ling, X.; Xie, L.; Fang, Y.; Xu, H.; Zhang, H.; Kong, J.; Dresselhaus, M. S.; Zhang, J.; Liu, Z. Can Graphene Be Used as a Substrate for Raman Enhancement? *Nano Lett.* **2010**, *10* (2), 553–561. <https://doi.org/10.1021/nl903414x>.
- (31) Schultz, Z. SERS and TERS. **2020**, 7–11.
- (32) Neves, M. M.; Guerra, R. F.; De Lima, I. L.; Arrais, T. S.; Guevara-Vega, M.; Ferreira, F. B.; Rosa, R. B.; Vieira, M. S.; Fonseca, B. B.; Sabino Da Silva, R.; Silva, M. V. D. Perspectives of FTIR as Promising Tool for Pathogen Diagnosis, Sanitary and Welfare Monitoring in Animal Experimentation Models: A Review Based on Pertinent Literature. *Microorganisms* **2024**, *12* (4), 833. <https://doi.org/10.3390/microorganisms12040833>.
- (33) *Fourier transform infrared (FTIR) spectroscopy | Photosynthesis Research*. <https://link.springer.com/article/10.1007/s11120-009-9439-x> (accessed 2025-08-21).
- (34) Al-Kelani, M.; Buthelezi, N. Advancements in Medical Research: Exploring Fourier Transform Infrared (FTIR) Spectroscopy for Tissue, Cell, and Hair Sample Analysis. *Skin Res Technol* **2024**, *30* (6), e13733. <https://doi.org/10.1111/srt.13733>.
- (35) Larkin, P. Chapter 3 - Instrumentation and Sampling Methods. In *Infrared and Raman Spectroscopy*; Larkin, P., Ed.; Elsevier: Oxford, 2011; pp 27–54. <https://doi.org/10.1016/B978-0-12-386984-5.10003-5>.
- (36) Fadlelmoula, A.; Pinho, D.; Carvalho, V. H.; Catarino, S. O.; Minas, G. Fourier Transform Infrared (FTIR) Spectroscopy to Analyse Human Blood over the Last 20 Years: A Review towards Lab-on-a-Chip Devices. *Micromachines* **2022**, *13* (2), 187. <https://doi.org/10.3390/mi13020187>.
- (37) Grdadolnik, J. ATR-FTIR SPECTROSCOPY: ITS ADVANTAGES AND LIMITATIONS. *Acta Chim. Slov.* **2002**.
- (38) Ricciardi, V.; Portaccio, M.; Perna, G.; Lasalvia, M.; Capozzi, V.; Cammarata, F. P.; Pisciotta, P.; Petringa, G.; Delfino, I.; Manti, L.; Lepore, M. FT-IR Transfection Micro-Spectroscopy Study on Normal Human Breast Cells after Exposure to a Proton Beam. *Applied Sciences* **2021**, *11* (2), 540. <https://doi.org/10.3390/app11020540>.
- (39) Qi, Y.; Chen, E. X.; Hu, D.; Yang, Y.; Wu, Z.; Zheng, M.; Sadi, M. A.; Jiang, Y.; Zhang, K.; Chen, Z.; Chen, Y. P. Applications of Raman Spectroscopy in Clinical Medicine. *Food Frontiers* **2024**, *5* (2), 392–419. <https://doi.org/10.1002/fft2.335>.
- (40) Luo, H.; Zu, R.; Li, L.; Deng, Y.; He, S.; Yin, X.; Zhang, K.; He, Q.; Yin, Y.; Yin, G.; Yao, D.; Wang, D. Serum Laser Raman Spectroscopy as a Potential Diagnostic Tool to Discriminate the Benignancy or Malignancy of Pulmonary Nodules. *iScience* **2023**, *26* (5), 106693. <https://doi.org/10.1016/j.isci.2023.106693>.
- (41) Morasso, C.; Daveri, E.; Bonizzi, A.; Truffi, M.; Colombo, F.; Danelli, P.; Albasini, S.; Rivoltini, L.; Mazzucchelli, S.; Sorrentino, L.; Corsi, F. Raman Spectroscopy on Dried Blood Plasma Allows Diagnosis and Monitoring of Colorectal Cancer. *MedComm* **2024**, *5* (11), e774. <https://doi.org/10.1002/mco2.774>.

- (42) Carlomagno, C.; Bertazioli, D.; Gualerzi, A.; Picciolini, S.; Andrico, M.; Rodà, F.; Meloni, M.; Banfi, P. I.; Verde, F.; Ticozzi, N.; Silani, V.; Messina, E.; Bedoni, M. Identification of the Raman Salivary Fingerprint of Parkinson's Disease Through the Spectroscopic–Computational Combinatory Approach. *Frontiers in Neuroscience* **2021**, *15*.
- (43) Shao, G.; Chen, R.; Li, M.; Liu, Y.; Zhang, K.; Zhan, Q. Direct SERS Profiling of Small Extracellular Vesicles in Cerebrospinal Fluid for Pediatric Medulloblastoma Detection and Treatment Monitoring. *Anal Bioanal Chem* **2025**. <https://doi.org/10.1007/s00216-025-05970-5>.
- (44) Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Terenzi, F.; Ramat, S.; Sorbi, S.; Bedoni, M. Raman Profiling of Circulating Extracellular Vesicles for the Stratification of Parkinson's Patients. *Nanomedicine: Nanotechnology, Biology and Medicine* **2019**, *22*, 102097. <https://doi.org/10.1016/j.nano.2019.102097>.
- (45) Bonizzi, A.; Signati, L.; Grimaldi, M.; Truffi, M.; Piccotti, F.; Gagliardi, S.; Dotti, G.; Mazzucchelli, S.; Albasini, S.; Cazzola, R.; Bhowmik, D.; Narayana, C.; Corsi, F.; Morasso, C. Exploring Breast Cancer-Related Biochemical Changes in Circulating Extracellular Vesicles Using Raman Spectroscopy. *Biosensors and Bioelectronics* **2025**, *278*, 117287. <https://doi.org/10.1016/j.bios.2025.117287>.
- (46) Faelelbom, K. M.; Saleh, A.; Al-Tabakha, M. M. A.; Ashames, A. A. Recent Applications of Quantitative Analytical FTIR Spectroscopy in Pharmaceutical, Biomedical, and Clinical Fields: A Brief Review. *Reviews in Analytical Chemistry* **2022**, *41* (1), 21–33. <https://doi.org/10.1515/revac-2022-0030>.
- (47) Ghimire, H.; Garlapati, C.; Janssen, E. A. M.; Krishnamurti, U.; Qin, G.; Aneja, R.; Perera, A. G. U. Protein Conformational Changes in Breast Cancer Sera Using Infrared Spectroscopic Analysis. *Cancers* **2020**, *12* (7), 1708. <https://doi.org/10.3390/cancers12071708>.
- (48) Lin, H.; Wang, Z.; Luo, Y.; Lin, Z.; Hong, G.; Deng, K.; Huang, P.; Shen, Y. Non/Mini-Invasive Monitoring of Diabetes-Induced Myocardial Damage by Fourier Transform Infrared Spectroscopy: Evidence from Biofluids. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **2022**, *1868* (9), 166445. <https://doi.org/10.1016/j.bbadis.2022.166445>.
- (49) Szigyártó, I. Cs.; Singh, P.; Sonallya, T.; Románszki, L.; Mihály, J.; Wiener, Z.; Bebesi, T.; Mészáros, G.; Keresztes, Z.; Thompson, M.; Varga, Z.; Beke-Somfai, T. ATR-Infrared Spectroscopy and Acoustic Sensing in Characterization of Blood and Pancreatic Ductal Adenocarcinoma-Derived Extracellular Vesicles. In *The Surfaceome: Methods and Protocols*; Matta, C., Mobasheri, A., Takács, R., Eds.; Springer US: New York, NY, 2025; pp 225–238. [https://doi.org/10.1007/978-1-0716-4434-8\\_15](https://doi.org/10.1007/978-1-0716-4434-8_15).
- (50) Szentirmai, V.; Wacha, A.; Németh, C.; Kitka, D.; Rácz, A.; Héberger, K.; Mihály, J.; Varga, Z. Reagent-Free Total Protein Quantification of Intact Extracellular Vesicles by Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy. *Anal Bioanal Chem* **2020**, *412* (19), 4619–4628. <https://doi.org/10.1007/s00216-020-02711-8>.
- (51) Bano, A.; Vats, R.; Yadav, P.; Kamboj, M.; Bhardwaj, R. Smoking-Induced Shifts in Salivary Exosomal Cytokines and Amino Acid Profiles as Potential Early Biomarkers for Oral Cancer. *Cytokine* **2025**, *187*, 156857. <https://doi.org/10.1016/j.cyto.2025.156857>.
- (52) Tognarelli, J. M.; Dawood, M.; Shariff, M. I. F.; Grover, V. P. B.; Crossey, M. M. E.; Cox, I. J.; Taylor-Robinson, S. D.; McPhail, M. J. W. Magnetic Resonance Spectroscopy: Principles and Techniques: Lessons for Clinicians. *Journal of Clinical and Experimental Hepatology* **2015**, *5* (4), 320–328. <https://doi.org/10.1016/j.jceh.2015.10.006>.
- (53) Hu, Y.; Liu, L.; Yan, G.; Bai, L.; Yang, L.; Kong, L.; Sun, H.; Liu, C.; Sun, Y.; Han, Y.; Wang, X. Nuclear Magnetic Resonance Based Metabolomics—A Rising Star in

- Traditional Chinese Medicine Research. *Pharmaceuticals* **2025**, *18* (8), 1186. <https://doi.org/10.3390/ph18081186>.
- (54) Yang, L.; Liu, D.; Wüthrich, K. GPCR Structural Characterization by NMR Spectroscopy in Solution. *ABBS* **2022**, *54* (9), 1207–1212. <https://doi.org/10.3724/abbs.2022106>.
  - (55) Kerfah, R.; Plevin, M. J.; Sounier, R.; Gans, P.; Boisbouvier, J. Methyl-Specific Isotopic Labeling: A Molecular Tool Box for Solution NMR Studies of Large Proteins. *Curr Opin Struct Biol* **2015**, *32*, 113–122. <https://doi.org/10.1016/j.sbi.2015.03.009>.
  - (56) Pizarro, C.; Esteban-Díez, I.; Espinosa, M.; Rodríguez-Royo, F.; González-Sáiz, J.-M. An NMR-Based Lipidomic Approach to Identify Parkinson's Disease-Stage Specific Lipoprotein–Lipid Signatures in Plasma. *Analyst* **2019**, *144* (4), 1334–1344. <https://doi.org/10.1039/C8AN01778F>.
  - (57) Guleria, A.; Kumar, U.; Kumar, D.; R, N.; Anuja, A. K.; Singh, M. K.; Sharma, P.; Agarwal, V.; Misra, R.; Gupta, L. NMR-Based Serum and Muscle Metabolomics for Diagnosis and Activity Assessment in Idiopathic Inflammatory Myopathies. *Analytical Science Advances* **2021**, *2* (11–12), 515–526. <https://doi.org/10.1002/ansa.202000171>.
  - (58) Jaurila, H.; Koivukangas, V.; Koskela, M.; Gäddnäs, F.; Myllymaa, S.; Kullaa, A.; Salo, T.; Ala-Kokko, T. I. <sup>1</sup>H NMR Based Metabolomics in Human Sepsis and Healthy Serum. *Metabolites* **2020**, *10* (2), 70. <https://doi.org/10.3390/metabo10020070>.
  - (59) Ullah, M. S.; Zhivonitko, V. V.; Samoylenko, A.; Zhyvolozhnyi, A.; Viitala, S.; Kankaanpää, S.; Komulainen, S.; Schröder, L.; Vainio, S. J.; Telkki, V.-V. Identification of Extracellular Nanoparticle Subsets by Nuclear Magnetic Resonance. *Chem. Sci.* **2021**, *12* (24), 8311–8319. <https://doi.org/10.1039/D1SC01402A>.
  - (60) *NMR and Metabolomics—A Roadmap for the Future*. <https://www.mdpi.com/2218-1989/12/8/678> (accessed 2025-08-31).
  - (61) Emwas, A.-H. M. The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research. In *Metabonomics: Methods and Protocols*; Bjerrum, J. T., Ed.; Springer: New York, NY, 2015; pp 161–193. [https://doi.org/10.1007/978-1-4939-2377-9\\_13](https://doi.org/10.1007/978-1-4939-2377-9_13).
  - (62) Mónico, A.; Martínez-Senra, E.; Cañada, F. J.; Zorrilla, S.; Pérez-Sala, D. Drawbacks of Dialysis Procedures for Removal of EDTA. *PLoS One* **2017**, *12* (1), e0169843. <https://doi.org/10.1371/journal.pone.0169843>.
  - (63) Emwas, A.-H.; Roy, R.; McKay, R. T.; Tenori, L.; Saccenti, E.; Gowda, G. A. N.; Raftery, D.; Alahmari, F.; Jaremko, L.; Jaremko, M.; Wishart, D. S. NMR Spectroscopy for Metabolomics Research. *Metabolites* **2019**, *9* (7), 123. <https://doi.org/10.3390/metabo9070123>.

## CHAPTER 3 – SALIVA

In this chapter, I present works using whole saliva and salivary-derived EVs as biomarkers for clinical applications in different diseases. Indeed, saliva represents an attractive biofluid in clinical field due to its easy, non-invasive and low-cost collection, as well as its rich biochemical content. Exploiting the presence of a repertoire of informative molecules, (e.g secreted and serum-derived immunoglobulins, cytokines, proteins, and nucleic acids) saliva could be a valid alternative to blood and traditional swab, reducing patient discomfort.

In the following sections, three independent studies are presented in order to explore the diagnostic relevance of whole saliva and their salivary EVs through RS and SERS.

In detail:

- Sub-chapter 3.1 describes a patented Raman method developed for the study of whole saliva, aimed at the differential diagnosis of respiratory diseases, with a specific focus on COPD, asthma, and OSA;
- Sub-chapter 3.2 reports the application of SERS for the molecular characterization of whole saliva in subjects previously infected with SARS-CoV-2 or vaccinated against COVID-19. SERS, with its high sensitivity and ability to provide detailed molecular fingerprints, offers a promising approach to identify biochemical changes associated with viral infection and the immune response;
- Sub-chapter 3.3 presents the development and optimization of a protocol for the separation of salivary EVs, specifically for RS analysis. This workflow was compared with an established serum-derived EV protocol, confirming the feasibility of using salivary EVs as a minimally invasive alternative for mesoscale biomarker discovery.

### 3.1 Whole Saliva Fingerprint by Raman Spectroscopy for Diagnostic and Monitoring of Respiratory Diseases

This sub-chapter presents data related to Patent with the international publication number WO 2025/032430 A1

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#### 3.1.1 Introduction

Chronic respiratory diseases represent a major global health burden, with Chronic Obstructive Pulmonary Disease (COPD) standing among the most prevalent and debilitating conditions. Indeed, COPD is currently the fourth leading cause of death worldwide, with over three million deaths annually<sup>1,2</sup>. COPD is characterized by persistent airflow limitation, progressive structural remodelling of the airways, and parenchymal destruction leading to emphysema<sup>3</sup>. All these problems cause chronic cough, excessive mucus production, and dyspnea<sup>4</sup>. As previously described, COPD is extremely complex and as the disease progresses, different disease phenotypes can occur: asthma-COPD overlap (aCOPD), non-exacerbating (neCOPD), frequently exacerbating with pulmonary emphysema (eeCOPD), and frequently exacerbating with chronic bronchitis (ebCOPD)<sup>5</sup>. The four phenotypes can be diagnosed based on a follow-up where the clinician evaluates the number of exacerbations, the results of High-Resolution Computed Tomography (HRCT), the presence of chronic bronchitis and  $\alpha$ -1 antitrypsin deficiency, in addition to classic pulmonary exams such as dyspnea, low FEV<sub>1</sub> and PaO<sub>2</sub><sup>6</sup>. To understand the severity of the disease, clinicians rely on exacerbations (acute episodes of clinical instability) because they provide information regarding morbidity and mortality. However, predicting the onset of exacerbations requires approximately 2 or 3 years of longitudinal evaluation<sup>7</sup>.

Current diagnostic and therapeutic determination in COPD are based on spirometry, radiological evaluation, symptom scoring, and prolonged clinical monitoring<sup>8</sup>. To date, the Global Initiative For Chronic Obstructive Lung Disease (GOLD) has approved a combination drug therapy using Inhaled Bronchodilator Steroids (IBS) and long-acting  $\beta$ 2-agonists/long-acting muscarinic antagonists (LABA/LAMA) to prevent or reduce symptoms<sup>9</sup>. Another problem is the non-adherence to the therapy by the patient is quite common in COPD both for pharmacological one and personalized respiratory rehabilitation. The non-adherence to the therapy leads to deteriorations in health status, increased mortality risk, and the failure of the

therapy<sup>10</sup>. Unfortunately, there is no biomarkers for a quantitative evaluation of the efficacy rehabilitation process. Indeed, to date clinicians use the so-called 6 Minute Walk Test. Consequently, there is a need for rapid, sensitive, and non-invasive diagnostic tools able to support early phenotyping, predicting exacerbation risk, and enabling longitudinal patient monitoring<sup>11</sup>.

Other prevalent respiratory disorders such as asthma and Obstructive Sleep Apnea (OSA) share overlapping symptoms, inflammatory features, and clinical presentations. Asthma is characterized by reversible airway obstruction and chronic airway inflammation<sup>12</sup>, while OSA is a sleep-related breathing disorder that involves a decrease or collapse during sleep of upper airflow, leading to intermittent hypoxia and a continuous effort to breathe<sup>13</sup>. These conditions often coexist or mimic COPD, complicating clinical assessment<sup>14</sup>.

For this reason, a diagnostic method able to discriminate between COPD, asthma, and OSA is urgently needed.

Within this context, Raman spectroscopy (RS) has emerged as a powerful analytical platform for biomedical applications<sup>15</sup>. RS is based on the vibrational modes of molecules, generating a highly specific biochemical fingerprint that reflects of the presence of proteins, lipids, nucleic acids, metabolites, and exogenous compounds in the sample under analysis. Moreover, its non-destructive, label-free nature, minimal sample preparation requirements, and potential for rapid measurement make RS an attractive candidate for clinical diagnostics. Importantly, even subtle biochemical alterations, often invisible using conventional assays, can be detected with high sensitivity, enabling the identification of a specific fingerprint relative to pathology and molecular changes induced by treatment<sup>16</sup>.

Saliva represents an ideal matrix for RS in diagnostics. Indeed, saliva is easily accessible, non-invasively collected, and rich in biomolecular information, including proteins, lipids, nucleic acids, hormones, and metabolites<sup>17</sup>. These characteristics make it optimal for longitudinal sampling. Furthermore, there are different evidences in literature that show that salivary RS can differentiate between healthy and diseased states in oncology<sup>18</sup>, neurodegeneration<sup>19</sup>, and pulmonary disorders<sup>20</sup>, supporting its translational potential.

Building on this information, the patented method described in this chapter aims to develop a saliva-based Raman spectroscopic approach able to discriminate among major respiratory diseases, including COPD, asthma, and OSA, and providing biochemical indicators related to disease phenotype, exacerbation risk, adherence to the therapy, and rehabilitation progress.

The second aim is to present a method that can be translated to a portable Raman in order to obtain a point of care instrument.

By generating molecular fingerprints from whole saliva, the method wants to overcome the limitations of current diagnostic problems with respiratory diseases, creating a rapid, reproducible, and actionable tool useful to clinical diagnostic.

### **3.1.2 Patented Method**

#### *3.1.2.1 Saliva Collection and Preparation*

Whole saliva was collected using commercial swabs (Salivette® - Sarstedt AG & CO, Numbrecht, Germany), from 70 COPD patients, 77 ASTHMA patients, 33 Healthy Controls (CTRL) subjects, and 25 OSA patients. The swabs were chewed for approximately one minute. Samples were obtained at least two hours after the last meal and one hour after tooth brushing to reduce variability and the presence of contaminants. Furthermore, any recent oral infections (e.g., candidiasis, gingivitis), dental procedures, smoking status (current or former), respiratory comorbidities, ongoing or recent medications, clinical scales, and rehabilitation treatments were registered.

Swabs could be stored at 4°C for a short term (up to 72 hours) or frozen at -20°C for longer conservation. Then, they were centrifuged (2 min at 1000 × g) to recover saliva. The empty swab has a weight of 6.5 g; if the collected sample weighed ≤ 6.5 g, the centrifugation step was repeated with increased speed and duration. In rare cases where no saliva could be recovered, 400 µL of deionized water was added to the swab to facilitate elution. The resulting dilution was documented, although samples obtained in this way were generally excluded due to insufficient biological material.

For empty samples (with a swab weight equal to or less than its empty weight of 6.5 g), the centrifugation step is repeated at a higher speed and duration. If no saliva is recovered after further centrifugation, 400 µL of deionized water is added to facilitate elution. The resulting dilution is documented due to possible insufficient biological material. Finally, saliva was stored at -80°C until use.

#### *3.1.2.2 Raman Spectra Acquisitions*

About 3 µL of saliva were spotted onto aluminum slides (2×4 cm commercial foil for portable Raman instruments, or 1”×3” slides coated with about 100 nm aluminum, Deposition Research Lab. Inc., for benchtop instruments) and allowed to dry for 10-15 minutes under sterile conditions using biological hood.

Spectra were acquired using both a benchtop Raman microspectroscope (Aramis, Horiba Jobin-Yvon) and a portable Raman system (iRaman Plus, B&W Tek). In both tools, the

monochromatic light used was a 785 nm laser, and spectra were acquired in the 400-1600  $\text{cm}^{-1}$  spectral range.

For benchtop Raman, the parameters were:

- Acquisition time: 30 seconds
- Accumulation: 2
- Delay time: 2 seconds
- Grating: 600 gr/mm
- Hole: 400  $\mu\text{m}$
- Object: 50 $\times$  (Olympus, Japan)

For portable Raman, the parameters were:

- Acquisition time: 30 seconds
- Accumulation: 2
- Laser Power: 100%

To maximize biochemical information, spectra were collected at the border of the drop; the so-called coffee ring. For each sample, 10 spectra were acquired excluding saturated signals.

For portable Raman, a dark spectrum was collected to record the background signal from light, and then subtracted from all sample spectra to eliminate environmental light interference.

Finally, for each patient the average spectrum was generated as a representative spectrum for each subject, and all spectra were exported in .txt format.

### *3.1.2.3 Post-processing of the Raman Spectra*

Raw Raman spectra data were processed using a standardized digital processing pipeline, which included:

1. Spike removal using a Savitzky–Golay despiking algorithm (grade 1 to 3);
2. Baseline correction using a 4th–5th degree polynomial fit with 60-80 max points for its construction and with 20-30 noise points;
3. Wavenumber alignment to a reference peak at 1001  $\text{cm}^{-1}$ ;
4. Normalization via unit-vector scaling to account for intensity fluctuations;
5. Resolution equalization and spectral cropping were performed to standardize datasets across acquisitions;
6. For each experimental group, the average spectrum and the calculation of standard deviation were performed;
7. To highlight the main differences between the experimental groups, the subtraction spectrum was performed;

8. Calculation of the area under the curve (AUC) in the 898-947  $\text{cm}^{-1}$  region to highlight the contribution of lactate in the saliva spectrum<sup>21</sup>;
9. Principal Component Analysis (PCA) was applied to reduce dimensionality and capture the major spectral variance;
10. Linear Discriminant Analysis (LDA) was trained using PCA scores to create classification models.

All of these corrections were performed by LabSpec6 and OriginPro.

### 3.1.3 Results

#### *3.1.3.1 Development of Two Raman-Based Classification Models for Respiratory Diseases*

To establish a Raman-based diagnostic workflow able to discriminate between major respiratory disorders, two independent PCA-LDA models were developed:

- Method 1: to classify CTRL, ASTHMA, and COPD.
- Method 2: to classify OSA from CTRL.

Both models were generated using post-processed saliva Raman spectra, using PCA for dimensionality reduction and LDA for supervised classification.

Normalized spectra were used for multivariate analysis in OriginPro, where each spectrum was assigned to the corresponding ID name and diagnostic class. PCA was performed on the wavenumber, extracting at least 15 principal components to capture meaningful biochemical variation across samples. Finally, scores were obtained and used for LDA classification.

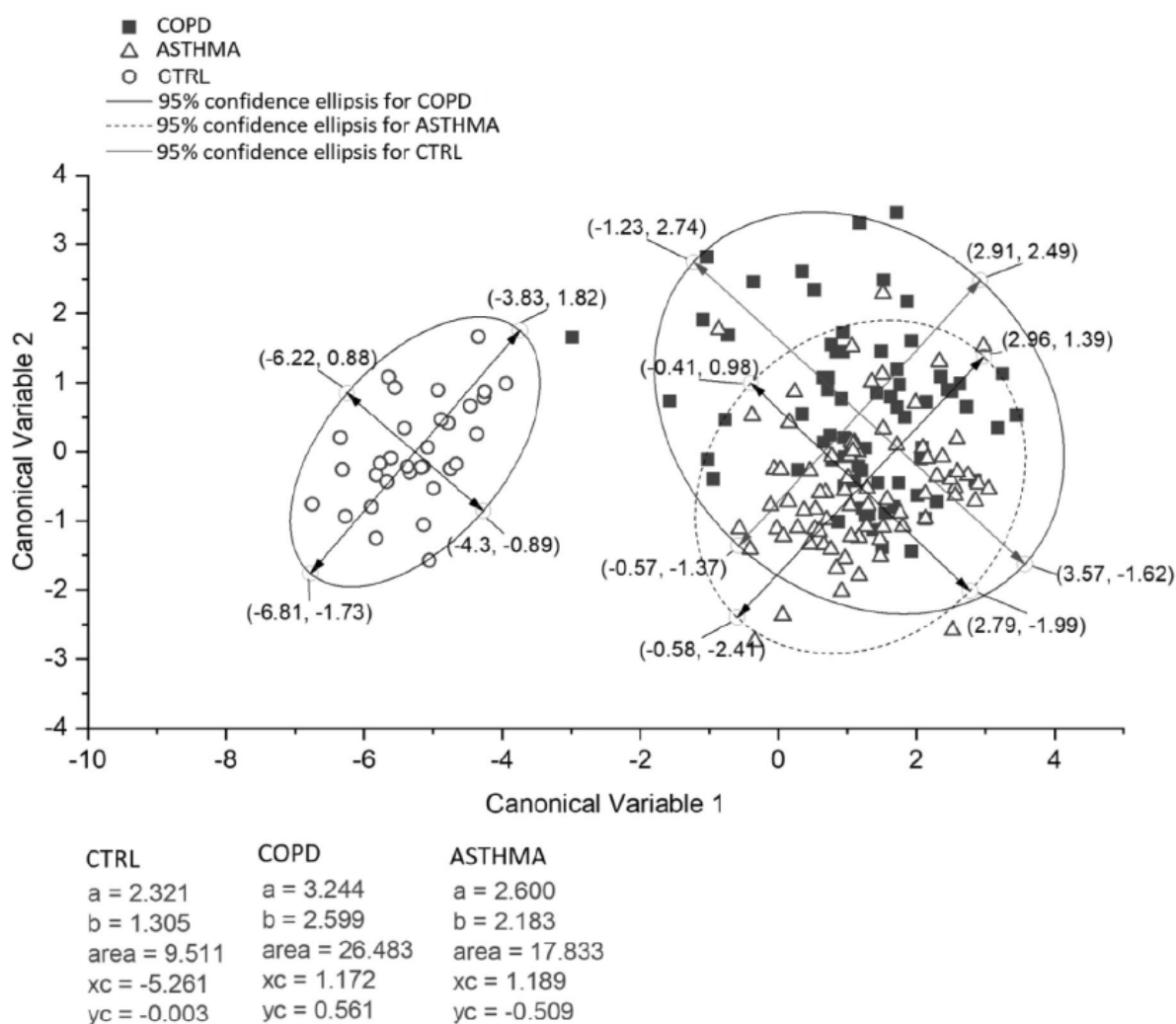
The number of Principal Components (PCs) included in the LDA model was optimized by increasing the number of components until the cross-validation error rate reached a minimum and began to increase, indicating overfitting. LDA was performed with equal prior probability, using a linear discriminant function and leave-one-out cross-validation (LOOCV).

For benchtop Raman, the PCA-LDA model built on salivary Raman spectra showed robust diagnostic capability. Using the first 15 PCs as input variables, the model correctly classified COPD vs asthma vs CTRL with an overall accuracy of 84.4%, a sensitivity of 75.2%, and a specificity of 88.6%.

Class-specific performance was also strong:

- COPD vs ASTHMA and CTRL → accuracy 77%
- ASTHMA vs COPD and CTRL → accuracy 77.6%
- CTRL vs respiratory disease (COPD + asthma): accuracy → >98%

In LDA, the spectral data are transformed into canonical variables, in order to maximize separation between classes. The number of canonical variables is always  $n-1$ , where  $n$  is the number of experimental groups compared. Since Method 1 included three groups (CTRL, COPD, and ASTHMA), the analysis produced two canonical variables, which were used to visualize group separation. Canonical Variable 1 and Canonical Variable 2 separated the three clinical groups into distinct clusters, each surrounded by a 95% confidence ellipse derived from PCA-LDA canonical scores (Fig. 1).



**Figure 1.** Two-dimensional PCA-LDA scatter plot showing the distribution of salivary Raman spectra from CTRL, COPD, and ASTHMA subjects along Canonical Variable 1 (x-axis) and Canonical Variable 2 (y-axis). For each clinical group, the 95% confidence ellipse is displayed together with the coordinates of its center and the vertices of the major and minor axes. CTRL: center  $(-5.261, -0.003)$ ; major-axis vertices  $(-3.83, 1.82)$  and  $(-6.81, -1.73)$ ; minor-axis vertices  $(-6.22, 0.88)$  and  $(-4.3, -0.89)$ . COPD: center  $(1.172, 0.561)$ ; major-axis vertices

*(-1.23, 2.74) and (3.57, -1.62); minor-axis vertices (2.91, 2.49) and (-0.57, -1.37). ASTHMA: center (1.189, -0.509); major-axis vertices (2.96, 1.39) and (-0.58, -2.41); minor-axis vertices (-0.41, 0.98) and (2.79, -1.99).*

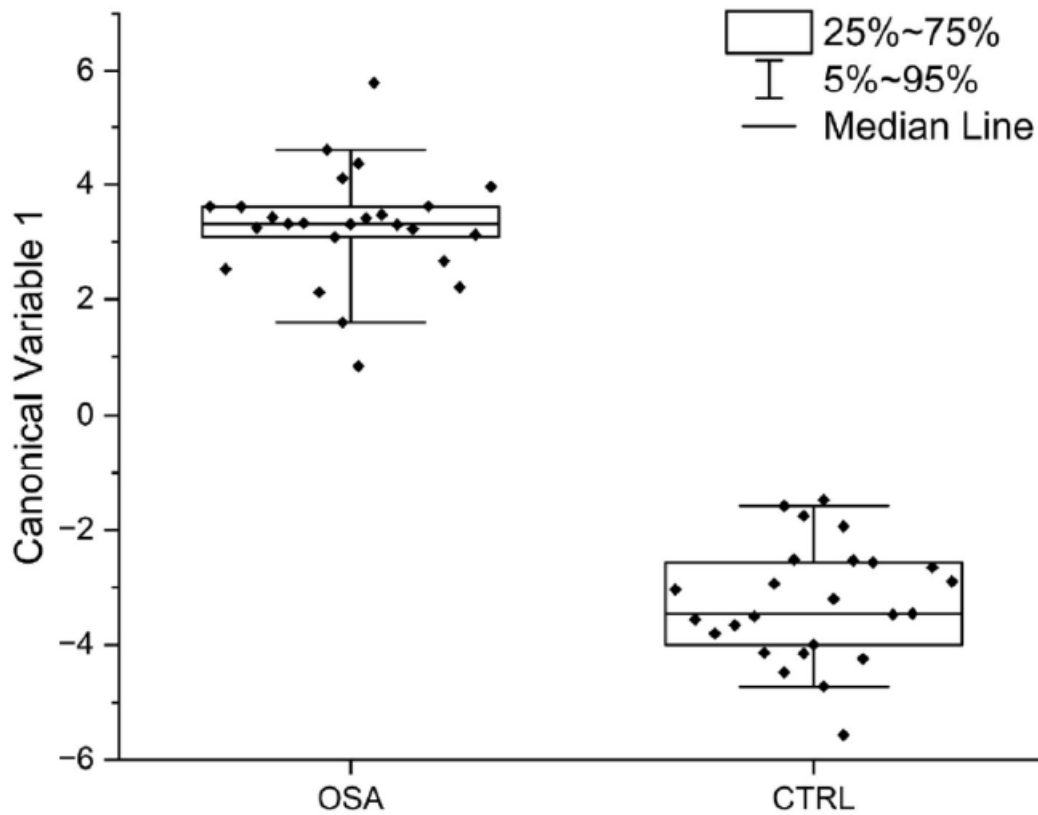
For portable Raman a smaller cohort was also tested using this PCA-LDA model, confirming the robustness of the approach. Indeed, COPD vs ASTHMA were discriminated with 71.9% accuracy, demonstrating feasibility for point-of-care applications.

A second model was developed specifically to discriminate OSA from CTRL. PCA was again performed on normalized spectra, and at least 15 PCs were extracted. After optimization to minimize cross-validation error, LDA was performed with equal priors, linear discriminant functions, and LOOCV.

Again, the PCA-LDA model built using the first 15 PCs achieved excellent diagnostic accuracy:

- Overall accuracy → 96%
- Sensitivity → 96%
- Specificity → 88.6%

In this case, only one canonical variable was required to separate the two groups, and 95% confidence intervals on Canonical Variable 1 confirmed minimal overlap between OSA and CTRL (Fig. 2).



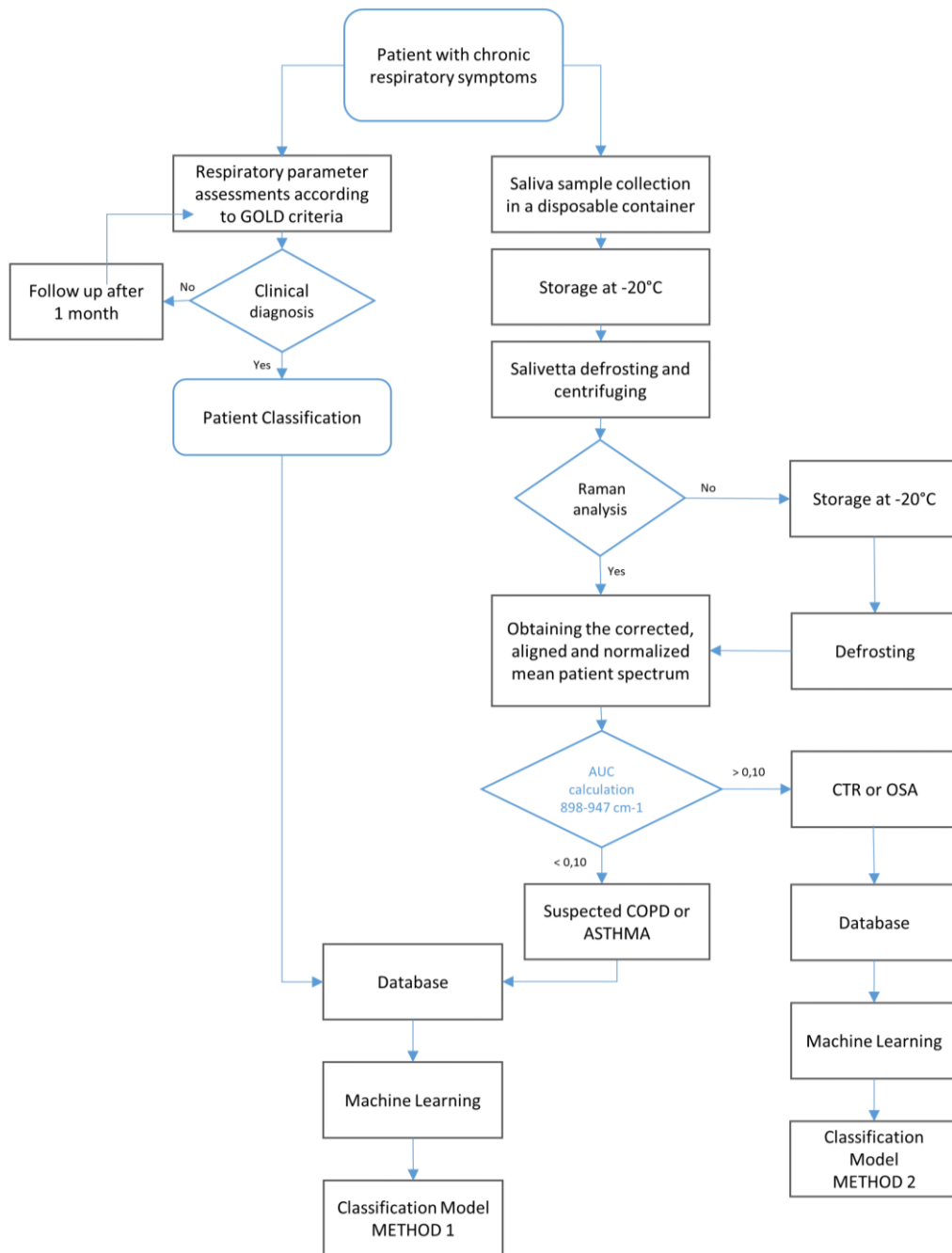
**Figure 2.** Box plot of Canonical Variable 1 (CV1) values obtained from multivariate PCA-LDA analysis of Raman spectra. Each point represents the CV1 value assigned to the average spectrum of an individual subject, either a CTRL or a subject with OSA. The plot illustrates the different distribution of CV1 values between the two groups.

### 3.1.3.2 Integration of the Two Models into a Sequential Diagnostic Workflow

To classify unknown saliva samples, the area under the curve (AUC) of the lactate region is evaluated. This area differs between OSA, ASTHMA, and COPD, as there is a known relationship between nocturnal hypoxia and lactate levels in sleep-related breathing disorders<sup>22</sup>. If the AUC is less than 0.1, the sample is considered suspected of having COPD or ASTHMA and is therefore directed towards method 1. If, on the other hand, the AUC is less than 0.1, the biochemical profile is compatible with the OSA spectra, and analysis is carried out using method 2 (Fig. 3).

The Method 1 provides differential diagnosis between COPD, ASTHMA, and CTRL, while method 2 confirms or excludes OSA from CTRL.

Together, they form a comprehensive system able to distinguish three major respiratory states (ASTHMA, COPD, and OSA) from CTRL subjects, using a single biofluid and a rapid, label-free vibrational spectroscopy approach.



**Figure 3.** Flow chart illustrating the decision process for classifying saliva samples based on the AUC lactate region.

### 3.1.4 Conclusion and Future Perspective

In this patented work, we demonstrated that RS applied to saliva represents a rapid, non-invasive, and label-free method able to capture disease-related biochemical signatures between main respiratory disorders. By integrating Raman spectral profiling with PCA-LDA analysis, we developed two diagnostic models able to discriminate between CTRL, ASTHMA, COPD, and OSA. Both models achieved high accuracy and reproducibility, demonstrating that salivary Raman fingerprints capture biochemical alterations related to metabolic changes in respiratory diseases. Therefore, the combined workflow presents a foundation for an accessible, point-of-care diagnostic tool.

As a future perspective, translating portable Raman devices into the clinical field could transform the clinical management of chronic respiratory diseases. Indeed, bringing a portable Raman directly to the bedside of patients would enable continuous monitoring, early recognition of exacerbations, and a more accurate phenotypic stratification. Furthermore, this approach will provide indicators of treatment adherence, offering clinicians timely feedback on therapeutic effectiveness, improving personalized disease management.

### 3.1.5 References

- (1) AL Wachami, N.; Guennouni, M.; Iderdar, Y.; Boumendil, K.; Arraji, M.; Mourajid, Y.; Bouchachi, F. Z.; Barkaoui, M.; Louerdi, M. L.; Hilali, A.; Chahboune, M. Estimating the Global Prevalence of Chronic Obstructive Pulmonary Disease (COPD): A Systematic Review and Meta-Analysis. *BMC Public Health* **2024**, *24* (1), 297. <https://doi.org/10.1186/s12889-024-17686-9>.
- (2) Mathers, C. D.; Loncar, D. Projections of Global Mortality and Burden of Disease from 2002 to 2030. *PLOS Medicine* **2006**, *3* (11), e442. <https://doi.org/10.1371/journal.pmed.0030442>.
- (3) Rodrigues, S. de O.; Cunha, C. M. C. da; Soares, G. M. V.; Silva, P. L.; Silva, A. R.; Gonçalves-de-Albuquerque, C. F. Mechanisms, Pathophysiology and Currently Proposed Treatments of Chronic Obstructive Pulmonary Disease. *Pharmaceuticals* **2021**, *14* (10), 979. <https://doi.org/10.3390/ph14100979>.
- (4) Simoni, M.; Carrozzi, L.; Baldacci, S.; Borbotti, M.; Pistelli, F.; Pede, F. D.; Maio, S.; Angino, A.; Viegi, G. Respiratory Symptoms/Diseases, Impaired Lung Function, and Drug Use in Two Italian General Population Samples. *Respiratory Medicine* **2008**, *102* (1), 82–91. <https://doi.org/10.1016/j.rmed.2007.08.002>.
- (5) Mirza, S.; Benzo, R. Chronic Obstructive Pulmonary Disease Phenotypes: Implications for Care. *Mayo Clinic Proceedings* **2017**, *92* (7), 1104–1112. <https://doi.org/10.1016/j.mayocp.2017.03.020>.
- (6) Finch, D.; Lange, P.; Halpin, D.; O'Donnell, D.; MacNee, W. Diagnosis, Assessment, and Phenotyping of COPD: Beyond FEV1. *COPD* **2016**, *3*. <https://doi.org/10.2147/COPD.S85976>.
- (7) Çolak, Y.; Afzal, S.; Marott, J. L.; Nordestgaard, B. G.; Vestbo, J.; Ingebrigtsen, T. S.; Lange, P. Prognosis of COPD Depends on Severity of Exacerbation History: A

- Population-Based Analysis. *Respiratory Medicine* **2019**, *155*, 141–147. <https://doi.org/10.1016/j.rmed.2019.07.021>.
- (8) Singh, D.; Agustí, A.; Volgelmeier, C.; Halpin, D. Reply: Spirometry Remains the GOLD Standard Test for COPD Diagnosis. *European Respiratory Journal* **2025**, *65* (6). <https://doi.org/10.1183/13993003.00794-2025>.
  - (9) Agustí, A.; Celli, B. R.; Criner, G. J.; Halpin, D.; Anzueto, A.; Barnes, P.; Bourbeau, J.; Han, M. K.; Martinez, F. J.; Montes de Oca, M.; Mortimer, K.; Papi, A.; Pavord, I.; Roche, N.; Salvi, S.; Sin, D. D.; Singh, D.; Stockley, R.; López Varela, M. V.; Wedzicha, J. A.; Vogelmeier, C. F. Global Initiative for Chronic Obstructive Lung Disease 2023 Report: GOLD Executive Summary. *Am J Respir Crit Care Med* **2023**, *207* (7), 819–837. <https://doi.org/10.1164/rccm.202301-0106PP>.
  - (10) Bourbeau, J.; Bartlett, S. J. Patient Adherence in COPD. **2008**. <https://doi.org/10.1136/thx.2007.086041>.
  - (11) Fermont, J. M.; Masconi, K. L.; Jensen, M. T.; Ferrari, R.; Lorenzo, V. A. P. D.; Marott, J. M.; Schuetz, P.; Watz, H.; Waschki, B.; Müllerova, H.; Polkey, M. I.; Wilkinson, I. B.; Wood, A. M. Biomarkers and Clinical Outcomes in COPD: A Systematic Review and Meta-Analysis. **2019**. <https://doi.org/10.1136/thoraxjnl-2018-211855>.
  - (12) Yuan, L.; Tao, J.; Wang, J.; She, W.; Zou, Y.; Li, R.; Ma, Y.; Sun, C.; Bi, S.; Wei, S.; Chen, H.; Guo, X.; Tian, H.; Xu, J.; Dong, Y.; Ma, Y.; Sun, H.; Lv, W.; Shang, Z.; Jiang, Y.; Lv, H.; Zhang, M. Global, Regional, National Burden of Asthma from 1990 to 2021, with Projections of Incidence to 2050: A Systematic Analysis of the Global Burden of Disease Study 2021. *eClinicalMedicine* **2025**, *80*. <https://doi.org/10.1016/j.eclinm.2024.103051>.
  - (13) Iannella, G.; Pace, A.; Bellizzi, M. G.; Magliulo, G.; Greco, A.; De Virgilio, A.; Croce, E.; Gioacchini, F. M.; Re, M.; Costantino, A.; Casale, M.; Moffa, A.; Lechien, J. R.; Cocuzza, S.; Vicini, C.; Caranti, A.; Marchese Aragona, R.; Lentini, M.; Maniaci, A. The Global Burden of Obstructive Sleep Apnea. *Diagnostics* **2025**, *15* (9), 1088. <https://doi.org/10.3390/diagnostics15091088>.
  - (14) Bouloukaki, I.; Fanaridis, M.; Testelmans, D.; Pataka, A.; Schiza, S. Overlaps between Obstructive Sleep Apnoea and Other Respiratory Diseases, Including COPD, Asthma and Interstitial Lung Disease. *Breathe (Sheff)* **2022**, *18* (3), 220073. <https://doi.org/10.1183/20734735.0073-2022>.
  - (15) Qi, Y.; Chen, E. X.; Hu, D.; Yang, Y.; Wu, Z.; Zheng, M.; Sadi, M. A.; Jiang, Y.; Zhang, K.; Chen, Z.; Chen, Y. P. Applications of Raman Spectroscopy in Clinical Medicine. *Food Frontiers* **2024**, *5* (2), 392–419. <https://doi.org/10.1002/fft2.335>.
  - (16) Jones, R. R.; Hooper, D. C.; Zhang, L.; Wolverson, D.; Valev, V. K. Raman Techniques: Fundamentals and Frontiers. *Nanoscale Res Lett* **2019**, *14* (1), 231. <https://doi.org/10.1186/s11671-019-3039-2>.
  - (17) Laxton, C. S.; Peno, C.; Hahn, A. M.; Allicock, O. M.; Perniciaro, S.; Wyllie, A. L. The Potential of Saliva as an Accessible and Sensitive Sample Type for the Detection of Respiratory Pathogens and Host Immunity. *The Lancet Microbe* **2023**, *4* (10), e837–e850. [https://doi.org/10.1016/S2666-5247\(23\)00135-0](https://doi.org/10.1016/S2666-5247(23)00135-0).
  - (18) Koster, H. J.; Guillen-Perez, A.; Gomez-Diaz, J. S.; Navas-Moreno, M.; Birkeland, A. C.; Carney, R. P. Fused Raman Spectroscopic Analysis of Blood and Saliva Delivers High Accuracy for Head and Neck Cancer Diagnostics. *Sci Rep* **2022**, *12* (1), 18464. <https://doi.org/10.1038/s41598-022-22197-x>.
  - (19) Carlomagno, C.; Banfi, P. I.; Gualerzi, A.; Picciolini, S.; Volpato, E.; Meloni, M.; Lax, A.; Colombo, E.; Ticozzi, N.; Verde, F.; Silani, V.; Bedoni, M. Human Salivary Raman Fingerprint as Biomarker for the Diagnosis of Amyotrophic Lateral Sclerosis. *Sci Rep* **2020**, *10* (1), 10175. <https://doi.org/10.1038/s41598-020-67138-8>.

- (20) Dzelve, P.; Legzdiņa, A.; Krūmiņa, A.; Tirzīte, M. Utility of Raman Spectroscopy in Pulmonary Medicine. *Advances in Respiratory Medicine* **2024**, *92* (5), 421–428. <https://doi.org/10.3390/arm92050038>.
- (21) Movasaghi, Z.; Rehman, S.; Rehman, I. U. Raman Spectroscopy of Biological Tissues. *Applied Spectroscopy Reviews* **2007**, *42* (5), 493–541. <https://doi.org/10.1080/05704920701551530>.
- (22) *Nocturnal Hypoxia and Arterial Lactate Levels in Sleep-Related Breathing Disorders*. <https://sma.org/southern-medical-journal/article/nocturnal-hypoxia-and-arterial-lactate-levels-in-sleep-related-breathing-disorders/> (accessed 2025-11-27).

### 3.2 Molecular Understanding of the Surface-Enhanced Raman Spectroscopy Salivary Fingerprint in People after Sars-COV-2 Infection and in Vaccinated Subjects

This sub-chapter is related to the published study carried out at the Laboratory of Nanomedicine and Clinical Biophotonics (LABION): Rodà Francesca, Gualerzi Alice, Picciolini Silvia, Forleo Luana, **Mangolini Valentina**, Mancuso Roberta, Agostini Simone, Rossetto Rudy Alexander, Pierucci Paola, Banfi Paolo Innocente, Bedoni Marzia (2024). The title of this work is “*Molecular understanding of the salivary Raman fingerprint in people after Sars-CoV-2 infection and in vaccinated subjects*” published on Chemosensors with open access.

DOI: <https://doi.org/10.3390/chemosensors12070136>

The present work focuses on a study investigating the application of surface-enhanced Raman spectroscopy (SERS) to saliva for the detection and monitoring of COVID-19.

To understand whether viral components and/or immune response contribute to the spectral variations induced by the infection, salivary SERS spectra from SARS-CoV-2 infected and vaccinated subjects were retrospectively analysed.

Saliva was collected every week for one month after COVID-19 diagnosis and, in vaccinated subjects, before initiating the COVID-19 vaccination cycle (Tv0) and one month after receiving the second dose of the Pfizer vaccine (Tv1).

These longitudinal SERS analyses showed that the salivary biochemical profile present progressive modification over the course of infection and recovery, with detectable spectral changes persisting up to one month after the first positive swab. Furthermore, multivariate analyses were performed between SARS-CoV-2 current (COV+) infection, SARS-CoV-2 past (COV-) infection, and healthy controls (CTRL). In this case, Principal Component Analysis and Linear Discriminant Analysis demonstrated that COV- remained largely indistinguishable from COV+, suggesting that the biochemical signature of COVID-19 remains partially retained in saliva after negativization. Conversely, both groups showed clear separation from CTRL.

To further investigate the reasons behind the spectral overlap observed between COV+ and COV- saliva, the study explored whether immunoglobulins produced during the adaptive immune response to SARS-CoV-2 might contribute to the SERS fingerprint. In particular, immunoglobulin A (IgA) and immunoglobulin G (IgG) were considered, as both increase following infection and vaccination. The average SERS spectra recorded at Tv0 and Tv1 were compared, showing similar peak patterns. This observation suggested that the vaccine-induced antibodies did not produce easily detectable modifications in the salivary SERS signature.

Finally, these results indicate that SERS can identify a biochemical fingerprint associated with mild COVID-19 and track its gradual normalization over time. Although the molecular origin of the spectral changes cannot be attributed to specific viral proteins or immune molecules, the persistent alterations in the salivary profile highlight the sensitivity of SERS to subtle infection-related biochemical processes.

For a detailed description of the study design, methods, results, and discussion, the full published article is provided as a PDF at the end of this thesis.

### 3.3 Biochemical Characterization of Human Salivary Extracellular Vesicles as A Valuable Source of Biomarkers

This sub-chapter is related to the published study carried out at the Laboratory of Nanomedicine and Clinical Biophotonics (LABION): **Valentina Mangolini**, Alice Gualerzi, Silvia Picciolini, Francesca Rodà, Angela Del Prete, Luana Forleo, Rudy Alexander Rossetto, and Marzia Bedoni (2023). The title of this work is: “*Biochemical characterization of human salivary extracellular vesicles as a valuable source of biomarkers*” published on Biology with open access.

DOI: <https://doi.org/10.3390/biology12020227>

The present work focuses on the study of Salivary Extracellular Vesicles (SEV) as potential biomarkers through biochemical characterization using Raman spectroscopy (RS). As previously discussed, EVs have emerged as important carriers of bioactive molecules, with applications ranging from disease diagnostics to monitoring pathological processes. However, most studies have focused on blood-derived EVs that often present the problem related to the co-isolated particles, such as lipoproteins. In contrast, saliva represents a valid alternative but its potential for EV-based diagnostics remains largely underexplored, due to the lower EVs concentration compared with EVs from blood.

In this study, SEV were isolated and compared to EVs from serum in terms of concentration, purity, and biochemical composition. In particular, colorimetric nanoplasmonic assay revealed that SEV exhibited higher purity than EVs from serum, with reduced co-isolated molecules. Indeed, analysis by nanoparticles tracking analysis revealed that EVs from serum present a higher concentration respect to SEV. Probably, this is due to the presence of co-isolated lipoproteins, a situation that does not occur in saliva which is naturally free of lipoproteins, providing thereby a cleaner EV preparation, but lower concentration.

Despite the lower SEV concentration compared to EVs from serum, RS was able to capture a reproducible EV biochemical signature, confirming their typical spectral profile. Notably, differences in Raman peaks were found between SEV and EVs from serum, such as in the Amide I and Amide III regions, which reflected differences in both protein cargo and their biomolecular corona. Furthermore, bands related to carbohydrates in SEV spectra suggested the presence of glycosylated molecules, such as mucins, consistent with their expected origin from ciliated epithelium of upper airways. Moreover, differences in spectroscopic ratios (Nucleic Acids/Proteins and Proteins/Lipids) supported the hypothesis that SEV may carry a

higher nucleic acid load than serum-EVs, reinforcing their potential as carriers of RNA biomarkers.

Finally, despite the lower concentration of SEV compared to serum-EVs, RS was able to capture a reproducible EV biochemical fingerprint, highlighting SEV Raman signature as a valuable diagnostic biomarker, particularly in contexts where plasma lipoproteins may interfere or where blood withdrawal is not feasible.

Therefore, RS represents a turning point in the application of SEV in clinics, taking advantage of the simple method of collection of the liquid biopsy and the quick, sensitive and label-free biophotonics-based approach.

For a detailed description of the study design, methods, results, and discussion, the full published article is provided as a PDF at the end of this thesis.

## CHAPTER 4 – BLOOD

While the previous chapter highlighted the potential of saliva as a non-invasive and clinically attractive biofluid, its use as a routine diagnostic matrix is still emerging. To date, blood remains the gold standard matrix in clinical diagnostics. Indeed, as previously discussed, blood contains a wide spectrum of circulating molecules that are already used for the detection of several biomarkers in oncology.

For this reason, in this chapter, I present works employing blood-derived biofluids, in particular serum, plasma, and their EV fractions, as potential sources of biomarkers for different pathologies.

Within this context, Raman spectroscopy, surface-enhanced Raman spectroscopy (SERS), Fourier-transform infrared (FTIR) spectroscopy, and nuclear magnetic resonance (NMR) were employed as label-free analytical tools to characterize global molecular signatures in plasma or serum and in their EV fractions, enabling a multiscale understanding of the biochemical changes associated with the diseases under investigation.

In detail:

- Sub-chapter 4.1 presents a combined Raman–NMR analysis of whole plasma from patients with sepsis-associated Intensive Care Unit-Acquired Weakness (ICU AW+S). This study evidences the potential of the Raman and NMR integration, supporting the molecular understanding of this poorly characterized condition;
- Sub-chapter 4.2 describes the use of FTIR and RS for the analysis of plasma-derived EVs from patients with ICU AW+S. Exploring these two complementary techniques, the study highlights the capability to establish distinctive mesoscale spectral signatures that could act as early biomarkers and support clinicians with bedside management of patients;
- Sub-chapter 4.3 focuses on the use of FTIR spectroscopy to analyze plasma-derived EVs from Chronic Myeloid Leukemia patients under different tyrosine kinase inhibitors treatments or in treatment-free remission. By profiling therapy-associated EV fingerprints, the study highlights the potential of FTIR as a label-free and non-invasive tool for disease monitoring and treatment stratification;
- Sub-chapter 4.4 describes the use of Raman spectroscopy to highlight the impact of an intensive treadmill-based rehabilitation in patients with Parkinson’s disease, using EV-like particles Raman signature.

## **4.1 Combined Raman and NMR Spectroscopy of human plasma reveals glutamate, alanine, and dimethylamine variation in ICU- acquired weakness patients**

This sub-chapter presents a study resulting from an international collaboration established during my research visit at the NMR Research Unit led by Prof. Ville-Veikko Telkki of the University of Oulu in Finland.

The work described herein is based on ongoing research and has not yet been published.

### **4.1.1 Introduction**

Advances in spectroscopy have opened new avenues for probing the molecular composition of biological samples with high specificity and minimal sample preparation. Among these, vibrational spectroscopies, particularly Raman spectroscopy (RS), have attracted significant attention in clinical research<sup>1,2</sup>. RS is a label-free technique capable of providing a molecular fingerprint of biofluids, tissues, and cells with small sample requirements. The global spectral information obtained through RS enables a broad range of applications, including disease diagnostics, patient stratification, and monitoring of biochemical alterations<sup>3</sup>.

RS exploits the inelastic scattering of light to reveal the vibrational modes of molecular bonds within a sample. However, when applied to complex biological matrices, the Raman spectrum represents an overlap of signals from numerous molecular species. This spectral overlap complicates the precise assignment of peaks to specific biomolecules, limiting the interpretability of the data<sup>4,5</sup>. Given the intrinsic complexity of Raman spectra derived from biological matrices advanced computational and analytical strategies are often required to achieve reliable interpretation<sup>6</sup>. Recent studies have comprehensively discussed how approaches such as Principal Component Analysis (PCA), Self-Organizing Maps (SOM), and Support Vector Machines (SVM), when integrated with RS, can significantly enhance spectral deconvolution and classification accuracy<sup>7</sup>. In line with these advancements, RS has also demonstrated strong potential in forensic and clinical diagnostics, where subtle biochemical differences must be detected in complex biological matrices. For instance, recent work has applied RS combined with machine learning approaches to differentiate between asphyxia and Sudden Cardiac Death (SCD) in experimental rat models<sup>8</sup>.

Similar applications of RS have been demonstrated in plasma samples from patients with colorectal cancer (CRC). In this context, RS identified characteristic spectral signatures associated with proteins, lipids, nucleic acids, and aromatic amino acids, differentiating CRC patients from individuals with inflammatory or diverticular conditions. Longitudinal analyses

before and after surgical resection further showed that these spectral alterations reverted postoperatively, linking the Raman profile directly to tumor presence and highlighting its potential for both diagnostic and disease-monitoring purposes<sup>9</sup>. Beyond oncology, RS has also shown promise in infectious complications such as sepsis following burn trauma. Analysis of plasma-derived extracellular vesicles revealed spectral features associated with bacterial glycoconjugates, enabling discrimination between septic and non-septic patients with high sensitivity and specificity. This further demonstrates the versatility of RS in detecting disease-specific molecular signatures across diverse clinical contexts<sup>10</sup>.

Furthermore, RS is often complemented by other standard techniques, such as ELISA, to support peak assignment and enhance molecular interpretation. However, the sensitivity of conventional biochemical assays such as ELISA is often limited, meaning that low-abundance metabolites may remain undetected. In contrast, RS can capture subtle molecular variations even at lower concentrations, providing a more comprehensive biochemical profile<sup>11</sup>.

Complementary to RS, NMR spectroscopy provides a powerful tool for metabolomic profiling. NMR exploits the magnetic properties of atomic nuclei, such as  $^1\text{H}$  and  $^{13}\text{C}$ , which align in characteristic spin states under a strong magnetic field. Application of a radiofrequency pulse induces transitions between these states, and the resulting relaxation signals are detected and translated into spectra. These spectra provide detailed information on chemical environments, molecular connectivity, and dynamics, enabling precise elucidation of molecular structures and composition<sup>12</sup>. High-resolution NMR has been applied in metabolomics studies of biofluids. For example, Anupam Guleria and colleagues employed high-field NMR to analyze serum and muscle samples from patients with idiopathic inflammatory myopathies, identifying metabolic signatures indicative of increased anaerobic activity, disrupted oxidative pathways, decreased muscle mass, and lipid imbalances. This approach enabled distinction between active and inactive disease independently of clinical subtype<sup>13</sup>. Similarly,  $^1\text{H}$  NMR has been used to characterize serum metabolite alterations in sepsis patients, revealing shifts in energy and inflammation-related metabolites correlated with disease severity and prognosis<sup>14</sup>.

While both NMR and RS provide complementary molecular information, their analytical perspectives differ substantially. NMR offers molecule-specific and quantitative insights, allowing precise identification and absolute quantification of metabolites in complex biological matrices. In contrast, RS provides a biochemical fingerprint based on vibrational modes corresponding to specific chemical bonds, reflecting the overall molecular composition rather than individual species<sup>15</sup>. Conversely, RS, being rapid, label-free, and minimally invasive, is more suitable for high-throughput or point-of-care analysis<sup>16</sup>. Therefore, integrating both

techniques leverages the strengths of each: NMR's molecular specificity complements Raman's sensitivity to subtle biochemical changes, offering a more comprehensive understanding of disease-related metabolic alterations.

Indeed, integration of RS and NMR has been demonstrated to enhance molecular interpretation in complex biological samples. In one study, Yan Chen et al. combined  $^1\text{H}$  NMR and RS to investigate metabolomic signatures associated with different differentiation states of nasopharyngeal carcinoma cell lines. NMR provided quantitative molecular information on amino acid, lipid, TCA cycle, and energy metabolism, while RS not only confirmed these findings but also revealed additional spectral variations at the single-cell level<sup>17</sup>.

Building on these methodological advances, the present study aims to establish a proof-of-concept framework for the integration of RS and NMR in the analysis of plasma samples from patients with ICU AW+S and from HC. ICU AW is defined as clinically evident weakness in critically ill patients with no cause other than the underlying critical illness. It is the most common neuromuscular complication in the ICU, affecting 30–50% of patients and up to 67% of those with sepsis. ICU-AW is linked to difficult ventilator weaning, prolonged ICU stay, increased healthcare costs, and greater long-term morbidity and mortality. Diagnosis is mainly clinical, based on the Medical Research Council (MRC) sum score and handgrip dynamometry for limb weakness, and recognition of ventilator dependency for diaphragmatic weakness. Currently, no validated biomarkers are available for ICU-AW<sup>18,19</sup>.

Given the limited sample size, the aim of this study is not to identify disease-specific biomarkers, but rather to illustrate how NMR metabolite profiling can support the interpretation of complex Raman spectra. Indeed, by integrating NMR data, it becomes possible to more accurately associate specific Raman features with their corresponding molecular species. This combined approach not only facilitates a more precise interpretation of the spectral data but also offers insights into the underlying metabolic pathways, potentially improving our understanding of pathophysiological mechanisms in diseases that are currently difficult to diagnose in the clinical setting. To our knowledge, this is the first application of combined RS and NMR to human clinical samples, highlighting the potential of this integrative approach for the future help to clinicians in early diagnosis and bedside management of ICU AW+S patients.

## **4.1.2 Experimental Section**

### *4.1.2.1 Subject Recruitment and Sample Collection*

Plasma samples were obtained from patients diagnosed with ICU AW+S, (n=9; age range: 50–84 years, mean age: 71.9; 3 females and 6 males) and from matched HC, (n=5; age range: 43–

82 years, mean age: 65.6; 3 females and 2 males) enrolled from the Intensive Care Unit and Orthopedic ward at Spedali Civili University Hospital in Brescia, Italy respectively.

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (Protocol number 34 68). It was approved by the Ethics Committee of Brescia. Written informed consent was obtained from all participants prior to blood collection.

Whole blood was collected into EDTA-containing tubes and processed using a sequential centrifugation protocol: initially at  $800 \times g$  for 10 minutes, followed by two additional centrifugation steps at  $2,500 \times g$  for 15 minutes each. All centrifugations were performed at room temperature with low acceleration and brake. The resulting plasma was aliquoted and stored at  $-80^{\circ}\text{C}$  until analysis<sup>20</sup>.

#### *4.1.2.2 Raman Spectroscopy*

Raman analyses were performed using a LabRAM Aramis system (Horiba Jobin Yvon S.A.S., Lille, France) equipped with a 785 nm laser source. Three  $\mu\text{L}$  of plasma were spotted onto an aluminum slide (Platypus Technologies, LLC) to optimize signal collection and minimize background interference and left to dry at room temperature for approximately 20 minutes under a biological safety cabinet.

Spectral acquisitions were conducted using a  $50\times$  objective lens (Olympus, Japan), using a 600 grooves/mm diffraction grating, a  $50\text{ }\mu\text{m}$  entrance slit, a  $400\text{ }\mu\text{m}$  confocal hole, and an integration time of 20 seconds for 10 accumulation covering a spectral range of  $400\text{--}1800\text{ cm}^{-1}$ . Ten spectra were collected per subject.

Raman shift calibration was performed daily using the zero-order line and the characteristic silicon band at  $520.7\text{ cm}^{-1}$  as reference.

Spectral processing was carried out using LabSpec6 and OriginPro2024b software, including polynomial baseline correction, unit vector normalization, and post-acquisition wavenumber calibration to account for autofluorescence, background interference, and potential thermal drift. Subsequently, the Area Under the Curve (AUC) of selected Raman peaks was calculated. To this end, all individual spectra from HC and ICU AW+S respectively were overlaid, and the peaks of interest were identified. The mean AUC for each peak was then determined for each experimental group using the Peak Analyzer tool in OriginPro2024b.

#### *4.1.2.3 $^1\text{H}$ -NMR Spectroscopy*

$^1\text{H}$ -NMR spectroscopy was performed using a Bruker Avance 600 MHz spectrometer equipped with a diffusion probe. For each sample,  $300\text{ }\mu\text{L}$  of plasma was mixed with  $300\text{ }\mu\text{L}$  of deuterium

oxide (D<sub>2</sub>O) containing 2.5 mM formate as a quantification standard. The mixture was transferred into a 5 mm NMR tube.

Spectra were acquired at 282 K (4 °C) using the standard zg30 pulse program. For each sample, 512 scans were collected, and eight replicate spectra were acquired, with a total acquisition time of approximately 17 hours per sample. The spectral width was set to 10 ppm.

Data were processed using Bruker TopSpin 3.7.0, including calibration and manual phase. Quantification of metabolites was performed by integrating the area under selected peaks and correcting for the number of contributing protons. The data were subjected to Fourier transformation and manual baseline correction in MATLAB, and further statistical analysis was carried out in OriginPro2024b (OriginLab, Northampton, MA, USA).

#### *4.1.2.4 Statistical analysis*

RS data and NMR-derived metabolite concentrations were analysed using both descriptive and multivariate statistical approaches in OriginPro2024. Principal Component Analysis (PCA) was applied separately to the normalized RS spectra and to the concentration matrix obtained from the integration of selected NMR peaks. PCA was used to reduce dimensionality and to identify the main sources of variance across experimental groups.

To enhance group discrimination and assess class separability, Linear Discriminant Analysis (LDA) was performed on the PCA scores of Raman spectra. To avoid model overfitting, only the minimal number of PCs explaining relevant variance were selected as input for LDA.

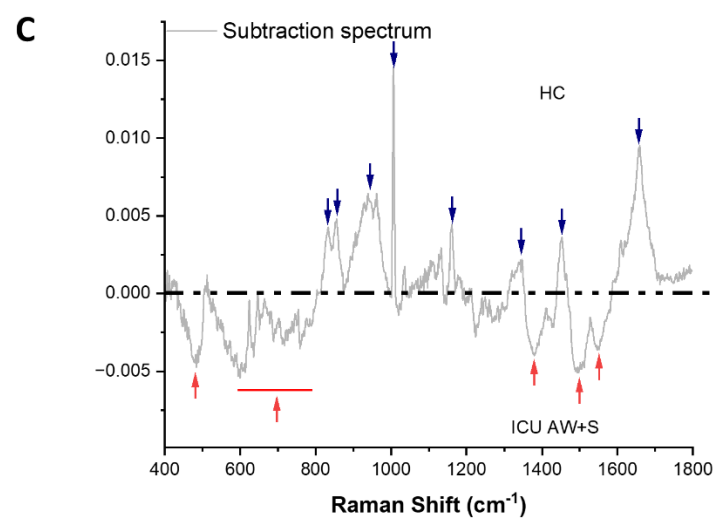
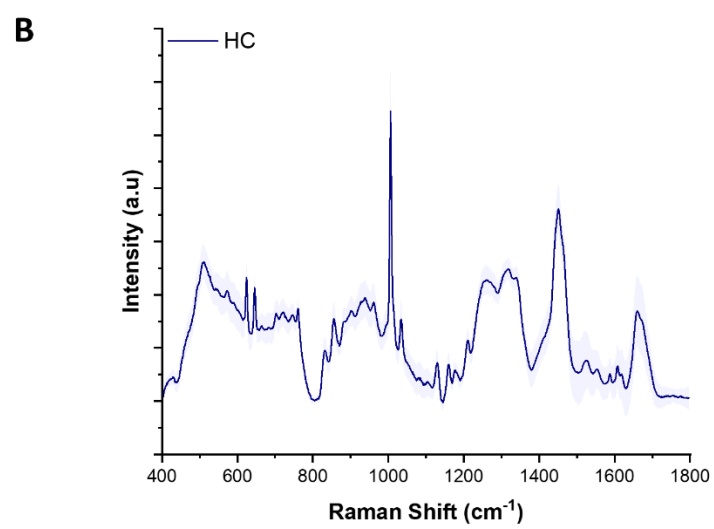
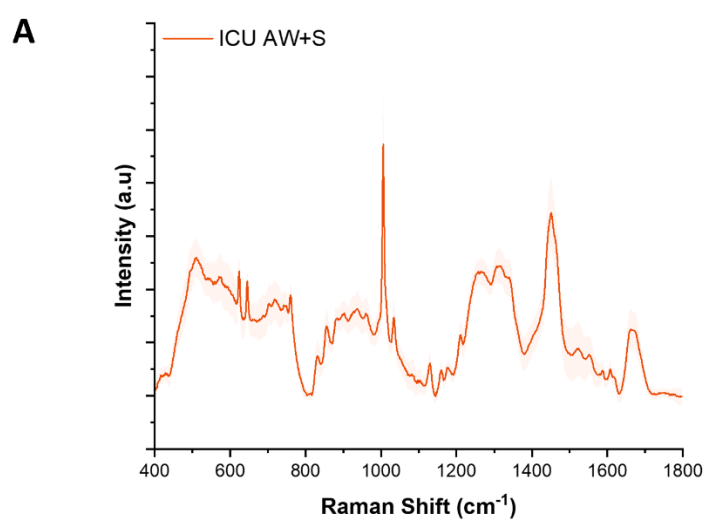
The performance of the PCA-LDA model was evaluated using leave-one-out cross-validation, assessing sensitivity, specificity, and overall classification accuracy.

### **4.1.3 Results and Discussion**

#### *4.1.3.1 Raman Spectral Analysis of Total Plasma*

Starting from a previously optimised protocol developed for saliva samples<sup>21,22</sup>, we acquired Raman spectra representative of the overall biochemical composition of whole plasma from ICU AW+S patients and HC using the spectral range 400-1800 cm<sup>-1</sup>. For each sample, 10 spectra were collected from randomly selected points along the edge of the dried plasma droplet, taking advantage of the natural concentration of molecules in the so-called “coffee ring” region. Figure 1A-B displays the average Raman spectra ( $\pm 1$  standard deviation) obtained from whole plasma samples of ICU AW+S patients and HC, respectively. Spectra show a good signal-to-noise ratio and highlight the methodological reproducibility across samples. To better highlight the spectral differences between the two experimental groups, the average spectra were overlaid, with selected bands color-coded according to their molecular origin (e.g.,

proteins, lipids, nucleic acids, saccharides and carotenoids). See Supplementary Information for more details (Figure 1SI and Table 1SI). Furthermore, a subtraction spectrum was generated by subtracting the mean spectrum of the HC group from that of the ICU AW+S patients (Fig. 1C). The resulting spectrum provides a clear and intuitive visualization of the most prominent spectral variations between the two groups, allowing the identification of specific regions and peaks that contribute to their biochemical distinction (highlighted by arrows in figure 1C). As summarized in Table 1<sup>23</sup>, the difference spectrum reveals distinct peak variations between the two experimental groups. HC showed higher intensities in several bands assigned to amino acids (833, 856, 928-940, 1006  $\text{cm}^{-1}$ ), as well as in characteristic carotenoid peaks (1161  $\text{cm}^{-1}$ ), and glutamate peaks<sup>24</sup> (938  $\text{cm}^{-1}$ ) suggesting a more robust antioxidant status and a greater capacity to neutralize free radicals respect to ICU AW+S patients<sup>25</sup>. Additional differences were also observed at 1342  $\text{cm}^{-1}$ , corresponding to CH deformation modes primarily arising from proteins and carbohydrates, and at 1450  $\text{cm}^{-1}$ , assigned to C–H deformation bands attributable to lipid groups, protein side-chain vibrations, and carbohydrate components. A pronounced band at 1660  $\text{cm}^{-1}$ , associated with amide I vibrations, was also more intense in HC. This region reflects contributions from protein secondary structures ( $\alpha$ -helix,  $\beta$ -sheet)<sup>23</sup>. The decreased intensity observed in ICU AW+S patients could suggest altered protein conformation, or reduced protein turnover. Conversely, ICU AW+S patients exhibited stronger signals in regions attributed to nucleotides (600-800  $\text{cm}^{-1}$ ), particularly DNA, raising the possibility of genomic instability or increased cellular turnover<sup>26</sup>. They also showed enhanced bands associated with glycogen (484  $\text{cm}^{-1}$ ), with CH bending (1380  $\text{cm}^{-1}$ ), and amide II (1480 – 1575  $\text{cm}^{-1}$ ), which could reflect elevated plasma protein levels and a reduced glycogen mobilization due to prolonged inactivity. Such metabolic adaptations are consistent with the catabolic and inflammatory state underlying ICU AW<sup>18,19</sup>.



**Figure 1.** (A-B) Average Raman spectrum of whole plasma from ICU AW+S and HC respectively obtained using 785 nm laser and 10 accumulation. Shaded areas represent  $\pm 1$  standard deviation. (C) The subtraction spectrum generated by subtracting the mean spectrum of two experimental groups (HC - ICU AW+S).

**Table 1.** The table presents the assignments of the Raman peaks identified in the subtraction spectrum obtaining by subtracting the mean spectrum of total plasma from HC and ICU AW+S<sup>23</sup>.

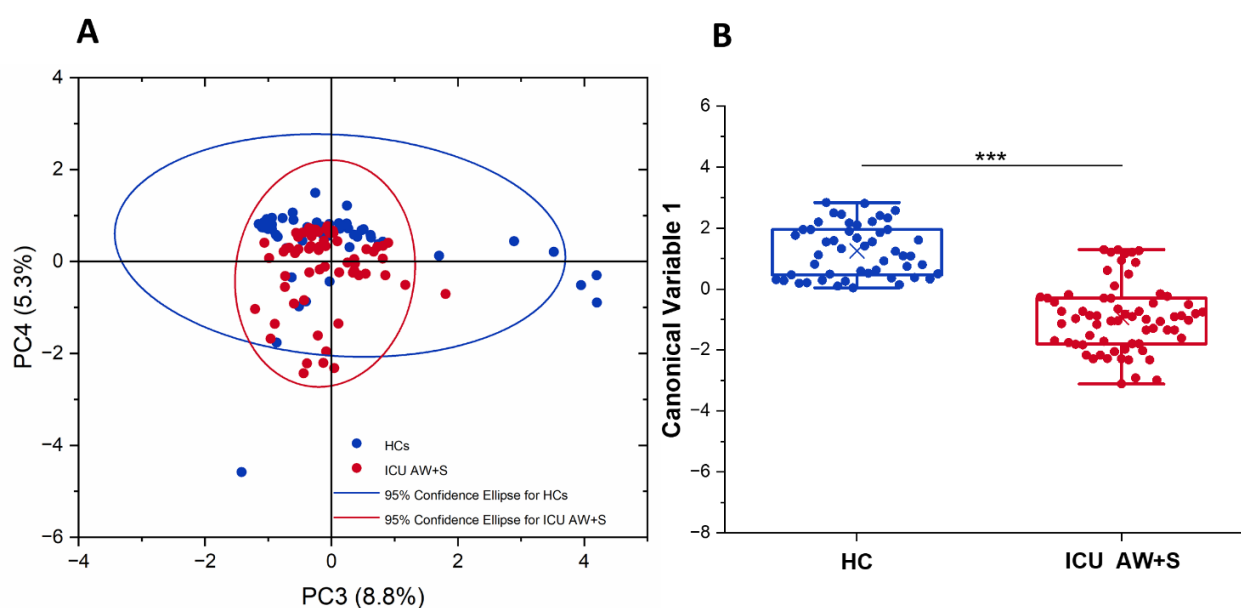
| cm-1        | HC<br>Assignment                                                                                                  | ICU AW+S<br>Assignment  |
|-------------|-------------------------------------------------------------------------------------------------------------------|-------------------------|
| 481         | /                                                                                                                 | DNA                     |
| 484         | /                                                                                                                 | Glycogen                |
| 600-800     | /                                                                                                                 | Nucleotide conformation |
| 833         | Asymmetric O-P-O stretching, tyrosine                                                                             | /                       |
| 856         | Proline, hydroxyproline, tyrosine, C-C stretching                                                                 | /                       |
| 928-940     | n(C-C), stretching in amino acids                                                                                 | /                       |
| 1006        | Phenylalanine (proteins)                                                                                          | /                       |
| 1161        | Carotenoids                                                                                                       | /                       |
| 1290 - 1400 | /                                                                                                                 | CH bending              |
| 1342        | CH deformation (proteins and carbohydrates)                                                                       | /                       |
| 1450        | C-H deformation bands (CH functional groups in lipids, amino acids side chains of the proteins and carbohydrates) | /                       |
| 1480 - 1575 | /                                                                                                                 | Amide II                |
| 1660        | Amide I / Lipids                                                                                                  | /                       |

#### 4.1.3.2 Multivariate Analysis

Although specific peaks could be identified for each experimental group, plasma is a highly complex biological fluid, and many vibrational modes tend to overlap, making spectral interpretation challenging. Therefore, to better discriminate between the two groups, multivariate analysis such as Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) were applied. These techniques allow the reduction of spectral dimensionality while enhancing class separation. Firstly, we obtained the Principal Components (PCs) that describe the differences among spectra, with the PCs representing variable percentage of total data variance. As shown in figure 2A, PC3 and PC4 capture emerging trends between ICU AW+S patients and HC. However, the two groups are not clearly separated in the corresponding score plot. This difficulty in achieving separation through PCA is likely due to the high intra-

group variability inherent in biological samples. Such heterogeneity can mask discriminant features in unsupervised models<sup>27</sup>. To address this, a supervised approach (LDA), was subsequently employed. The first eight principal components were used as input variables for LDA, which generated the Canonical Variable (CV) scores shown in Figure 2B. These scores condense the variance observed between the Raman spectra of the two groups into a single discriminant dimension, facilitating both group comparison and the development of the classification model.

To evaluate the performance of the model, a leave-one-out cross-validation approach was applied. The model achieved a mean classification error rate of 14,22%, reflecting the complexity of the biological samples. Statistical comparison of CV scores between ICU AW+S patients and HC using the Mann–Whitney test revealed a significant difference ( $p$  value  $< 0.0001$ ), supporting the ability of RS combined with LDA to detect differences in plasma composition between the two experimental groups.



**Figure 2.** (A) PCA with the 95% confidence ellipse of the Principal Component 3 (8.8%), and Principal Components 4 (5.3%) cumulatively representing the 14.1% of variance; (B) Box plot illustrating the distribution of the canonical variable obtained from the PCA-LDA classification model. The analysis was performed using the first seven principal component scores as input for LDA. Each point corresponds to the mean spectrum of an individual subject. A non-parametric Mann–Whitney test confirmed a significant difference between the HC and ICU AW+S groups (\*\*\*)  $p < 0.0001$ .

#### 4.1.3.3 Quantitative Metabolomic Profiling by $^1\text{H}$ -NMR

Plasma samples from ICU AW+S patients and HC were analyzed using  $^1\text{H}$  NMR spectroscopy as described in the Materials and Methods section. Each spectrum was acquired with a spectral width of 10 ppm, ensuring comprehensive coverage of the low-molecular-weight metabolite region. For absolute quantification, a known concentration of formate (2.5 mM) was added to each sample prior to acquisition. The formate resonance, located in a region devoid of overlapping plasma signals, served as an internal standard and did not interfere with metabolite identification (Figure 3A).

Figure 3A presents a representative  $^1\text{H}$  NMR spectrum from a plasma sample after baseline correction, showing the main resonances corresponding to circulating metabolites, including amino acids (e.g., valine, leucine, isoleucine, glutamate, glutamine), organic acids (e.g., lactate, citrate), energy-related metabolites (e.g., glucose), and other endogenous molecules such as dimethylamine. A full list of annotated metabolites is provided in Table 2<sup>28,29</sup>.

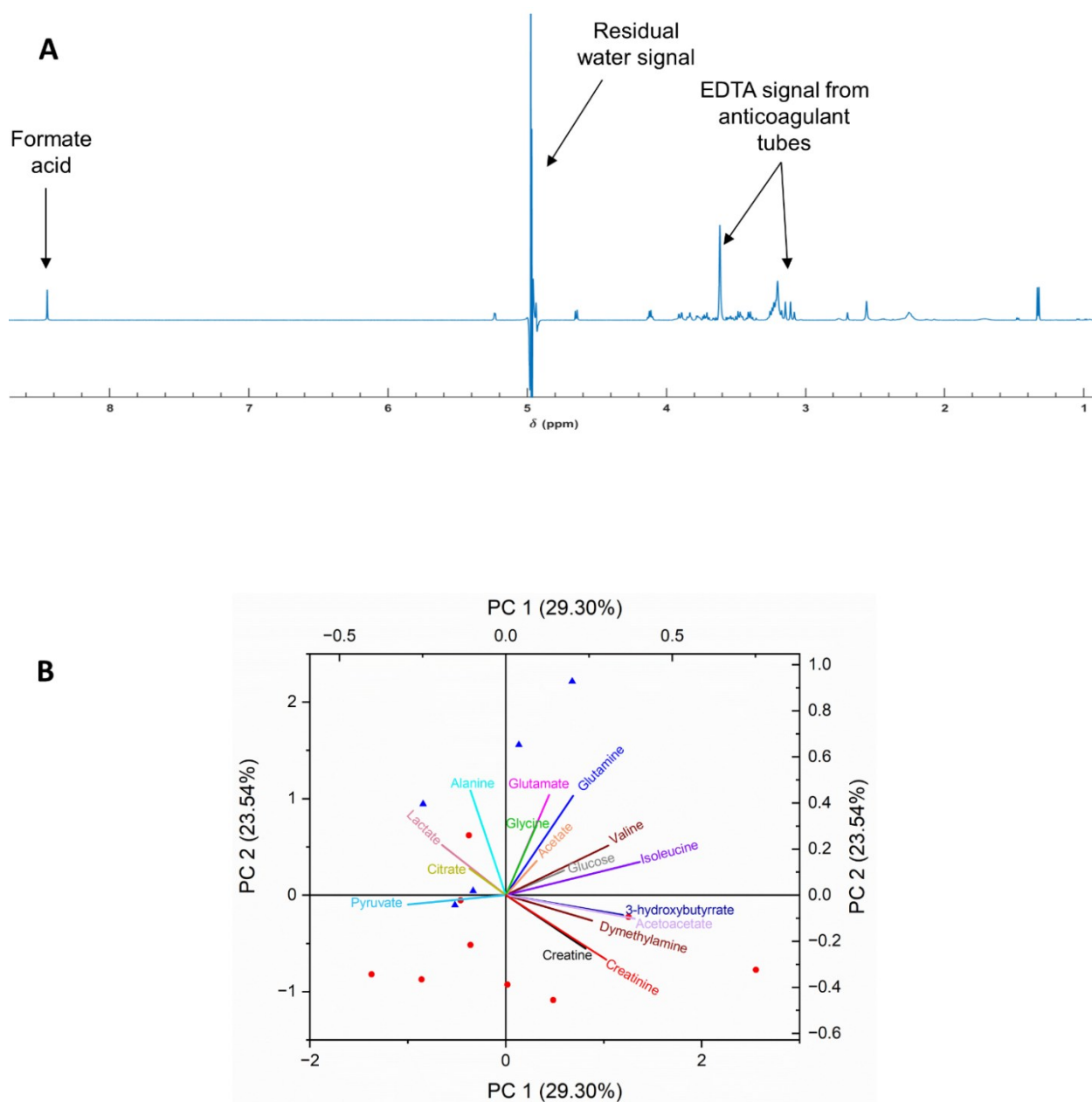
Peaks corresponding to EDTA, originating from anticoagulant tubes, were also detected but excluded from further analysis due to their exogenous origin<sup>30</sup>.

PCA was performed on quantified metabolite concentrations to obtain an overview of group-dependent metabolic variations. The resulting score plot (Fig. 3B) revealed a clear separation between HC and ICU AW+S patients, indicating distinct metabolic profiles. The corresponding loading plot showed that glutamate, glycine, glutamine, valine, alanine, and lactate were more concentrated in HC, whereas dimethylamine, creatinine, and creatine were elevated in ICU AW+S samples. Metabolites positioned near the origin exhibited similar concentrations between groups, suggesting no significant variation.

From a biological point of view, data suggest that ICU AW+S patients display a metabolic phenotype characterized by disrupted amino acid homeostasis, potentially reflecting enhanced muscle protein catabolism and reduced protein turnover. The higher glutamate levels quantified in HC respect to ICU +S may indicate a more efficient antioxidant defense, given glutamate's pivotal role<sup>25</sup> as a precursor in glutathione biosynthesis, which is compromised in ICU AW+S patients<sup>18,19</sup>. In contrast, elevated dimethylamine levels in ICU AW+S patients likely reflect intensified protein degradation or altered methylamine metabolism, both consistent with a catabolic and stress-associated metabolic state<sup>31</sup>. Collectively, these observations support the presence of metabolic reprogramming in ICU AW+S, with implications for energy balance, oxidative stress, and amino acid utilization<sup>18,19</sup>.

**Table 2.** The table presents the metabolites individuated by NMR<sup>28,29</sup>

| Metabolite        | Assignement               | Peaks (ppm) |
|-------------------|---------------------------|-------------|
| $\alpha$ -Glucose | H <sub>1</sub>            | 5.23        |
| $\beta$ -Glucose  | H <sub>1</sub>            | 4.65        |
| Lactate           | CH – CH <sub>3</sub>      | 4.12 – 1.33 |
| Creatinine        | CH <sub>2</sub>           | 4.05        |
| Glycerole         | CH <sub>2</sub>           | 3.65        |
| Valine            | $\alpha$ -CH              | 3.57        |
| Glycine           | CH <sub>2</sub>           | 3.55        |
| Creatinine        | CH <sub>3</sub>           | 3.04        |
| Creatine          | CH <sub>3</sub>           | 3.03        |
| Dymethylamine     | CH <sub>3</sub>           | 2.72        |
| Citrate           | CH <sub>2</sub>           | 2.66        |
| Glutamine         | $\gamma$ -CH <sub>2</sub> | 2.44        |
| Pyruvate          | CH <sub>2</sub>           | 2.37        |
| Acetoacetate      | CH <sub>3</sub>           | 2.23        |
| Glutamate         | $\beta$ -CH <sub>2</sub>  | 2.13        |
| Acetate           | CH <sub>3</sub>           | 1.92        |
| Alanine           | CH <sub>3</sub>           | 1.47        |
| 3-hydroxybutyrate | $\gamma$ -CH <sub>3</sub> | 1.2         |
| Valine            | CH <sub>3</sub>           | 1.04        |
| Isoleucine        | $\beta$ -CH <sub>3</sub>  | 1.01        |



**Figure 3.** (A) Representative  $^1\text{H}$ -NMR spectrum of total plasma sample. The manual baseline correction was performed. (B) PCA of quantified NMR metabolite concentrations. PC1 and PC2 account for 29.30% and 23.54% of the total variance, respectively (cumulative variance = 52.84%). Longer vectors indicate metabolites with a stronger contribution to group discrimination respect to the other closer to the origin.

#### 4.1.3.4 Correlating RS with NMR Results

Building on the findings analyses, we next focused on integrating NMR and RS data to highlight their ability to provide orthogonal converging information.

Notably, we found a strong correspondence between some NMR-quantified metabolites levels (glutamine, glutamate, alanine, and dimethylamide) and Raman spectral features in the amino acid region (833, 856, 928-940  $\text{cm}^{-1}$ ), particularly in proximity to the phenylalanine peak (1006

cm<sup>-1</sup>), glutamate peak (938 cm<sup>-1</sup>), amide I peak (1660 cm<sup>-1</sup>) and peaks related to N-H stretching vibrations<sup>23,24</sup>. In particular, alanine and glutamate were found to be decreased in ICU AW+S patients based PCA analysis of metabolites quantify by NMR. The same trend was also observed for glutamine, which showed higher concentrations in HC compared to patients. This finding is consistent with literature describing reduced glutamine levels in critically ill patients<sup>32</sup>. Accordingly, the Raman spectra of ICU AW+S patients exhibited lower intensity in the amino acid region, further supporting the correspondence between the metabolic alterations detected by NMR and the spectral features observed by RS (Fig. 2SI).

Moreover, the amide I Raman peak at 1660 cm<sup>-1</sup> further contributes to explaining the elevated concentrations of certain amino acids observed in both NMR and Raman analyses. As described in the section of the RS results, the amide I band reflects the C=O stretching vibrations in the protein backbone and it is indicative of secondary protein structures such as  $\alpha$ -helices and  $\beta$ -sheets<sup>26</sup>. Notably, the amide I peak not only exhibited increased intensity in HC patients but also displayed a distinct conformational profile (Fig. 3SI). The amide I peaks from ICU AW+S and HC were analyzed by integrating the AUC, revealing a statistically significant difference (Fig. 4SI). Changes observed in the amide I band likely reflect modifications in the structural integrity or dynamics of protein backbones, which could in turn affect the balance of amino acid pools. Accordingly, the specific evaluation of glutamate provided further insight into these molecular alterations. The mean Raman intensity of the glutamate peak at 938<sup>24</sup> cm<sup>-1</sup> appeared lower in ICU AW+S patients compared to HC, consistent with NMR results (Fig.4A). In addition, the AUC of the corresponding Raman peak was calculated for each individual spectrum, revealing a statistically significant decrease in ICU AW+S patients (Fig.4B). The qualitative reduction observed in the Raman spectra is consistent with the NMR findings and supports the overall trend of altered amino acid metabolism. Glutamate reduction may reflect broader metabolic disturbances<sup>18,19</sup>. Indeed, it serves as a key precursor for glutathione, which is essential for reactive oxygen species (ROS) regulation and maintenance of redox homeostasis. Glutamate is also involved in GABA metabolism<sup>33</sup>, supporting neuronal transmission, and can be converted to  $\alpha$ -ketoglutarate to enter the Krebs cycle. Therefore, decreased glutamate levels may indicate impaired oxidative metabolism and reduced energy production<sup>25</sup>.

Consistently, alanine concentrations were also lower in ICU AW+S patients compared to controls (Fig. 5SI). Alanine is a key amino acid for protein synthesis, and its reduction may reflect impaired protein metabolism, potentially contributing to the muscle atrophy observed in ICU-AW. The combined decrease in glutamate and alanine suggests that the cells may

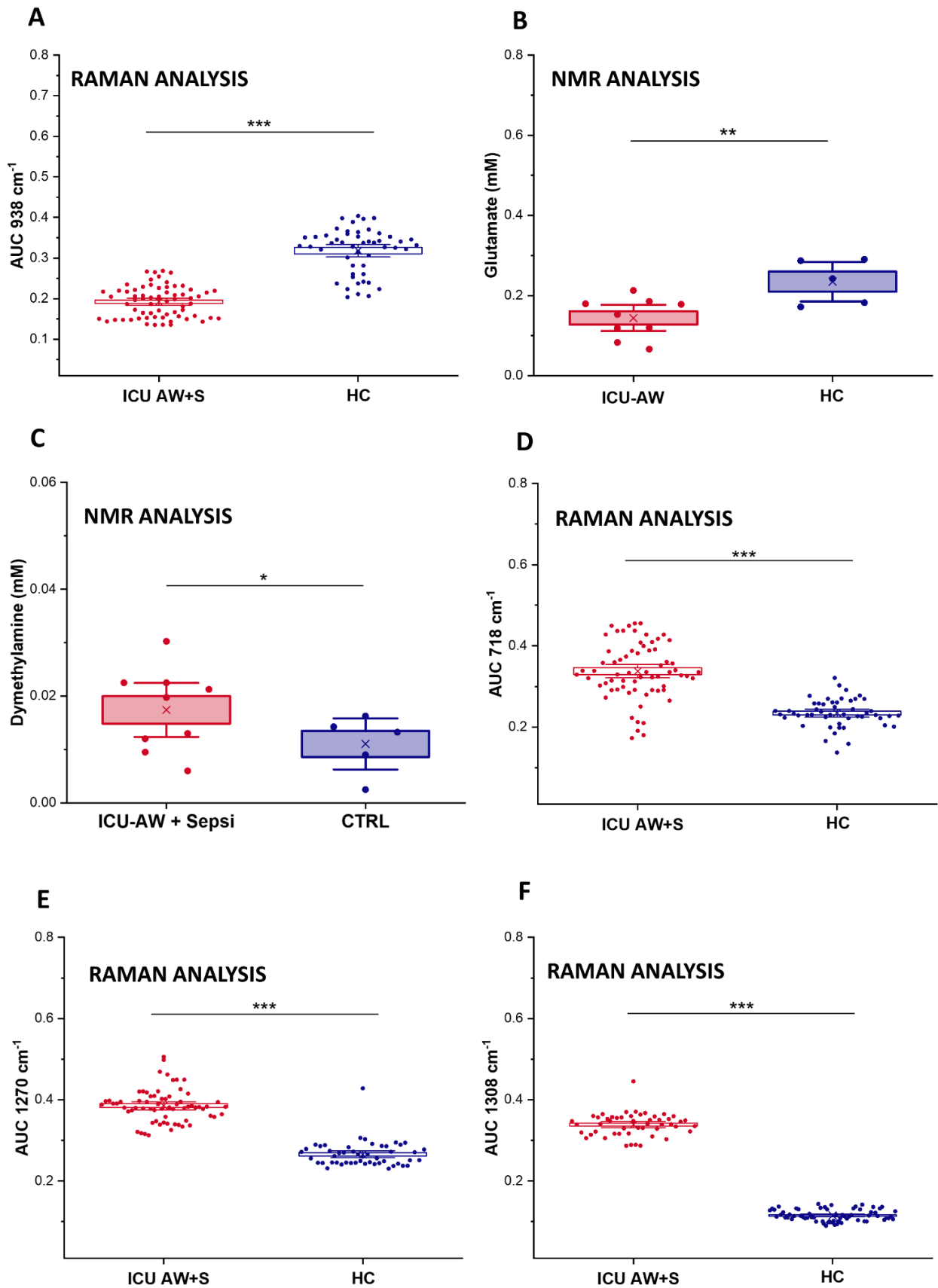
experience both diminished antioxidant capacity and limited substrate availability for energy production via the Krebs cycle, highlighting a link between oxidative stress, disrupted energy metabolism, and muscle wasting in these patients<sup>34</sup>.

Importantly, the observed changes in amino acid and protein-related signals might not solely reflect the disease as these types of patients were septic. Consequently, bacterial metabolism could also contribute to these biochemical signatures influencing both the Raman spectral profile and NMR-detected metabolite concentrations.

Furthermore, dimethylamine was found to be more abundant in ICU AW+S patients compared to HC, as quantified by NMR (Fig. 4C). The presence of the additional methyl group is often associated with metabolic dysregulation, as dimethylamine is typically considered a metabolic waste product<sup>35</sup>. Indeed, critically ill patients, often exhibit elevated ADMA and DMA, which are linked to organ failure and increased mortality<sup>31</sup>. Consistently, several Raman bands corresponding to N–H bonds (718, 1270, and 1308  $\text{cm}^{-1}$ ) displayed increased intensity and different shape in ICU AW+S spectra relative to HC. Evaluation based on AUC further confirmed a statistically significant increase, reinforcing the concordance between Raman and NMR findings as shown in figure 4D, E, F. These spectral changes likely reflect the higher concentration of dimethylamine found on NMR, as the Raman signal corresponds to the increased prevalence of its characteristic chemical bonds.

This concordance further illustrates how NMR and RS provide complementary insights, linking molecular concentration to structural features detectable by vibrational spectroscopy, thereby aiding the interpretation of the underlying metabolic context.

Finally, taken together, these results demonstrate how the combination of NMR and RS enhances the interpretability of complex Raman spectra, enabling more accurate biochemical insights.



**Figure 4.** (A) Box plot showing the AUC of the Raman peak at  $938\text{ cm}^{-1}$ , attributed to glutamate<sup>24</sup>. A non-parametric Mann–Whitney test confirmed a significant difference between HC and ICU AW+S patients ( $***p < 0.0001$ ); (B) Box plot of NMR-derived glutamate concentrations, where a Student’s *t*-test indicated a significant difference between ICU AW+S and HC groups ( $**p = 0.00885$ ); (C) Box plot of NMR-derived dimethylamine concentrations, with a Student’s *t*-test confirming a significant difference between ICU AW+S and HC groups ( $*p = 0.01251$ ). (D–F) Box plots of Raman peaks at  $718$ ,  $1270$ , and  $1308\text{ cm}^{-1}$  corresponding to C–H bond vibrations representative of dimethylamine. A non-parametric Mann–Whitney test confirmed a significant difference between HC and ICU-AW+S groups ( $***p < 0.0001$ ).

#### 4.1.4 Conclusion

This study demonstrates that the integrated use of RS and NMR provides a robust and complementary analytical strategy for the biochemical characterization of human plasma. By combining bond-level vibrational information from RS with molecule-specific quantitative data from NMR, this multimodal approach enabled a comprehensive assessment of metabolic alterations associated with ICU AW+S<sup>18,19</sup>.

RS revealed significant spectral differences between ICU AW+S patients and HC, particularly in regions related to amino acids, proteins, and nucleotides. Correspondingly, NMR metabolomic profiling confirmed decreased concentrations of glutamate, glutamine, and alanine, alongside increased levels of dimethylamine, creatine, and creatinine in ICU AW+S plasma<sup>29,30</sup>. The cross-validation between Raman and NMR findings demonstrated consistent molecular patterns across both techniques. Indeed, the association of the  $938\text{ cm}^{-1}$  (glutamate)<sup>24</sup> and  $1660\text{ cm}^{-1}$  (amide I)<sup>23</sup> Raman peaks correlate, in a specific way with the elevated concentrations of glutamate, but also with other amino acids observed in NMR.

Likewise, the elevated dimethylamine levels detected by NMR agreed with the increased Raman N–H vibrational bands ( $718$ ,  $1270$ , and  $1308\text{ cm}^{-1}$ ), whose AUC was significantly higher in ICU AW+S samples, further confirming the molecular origin of these spectral features.

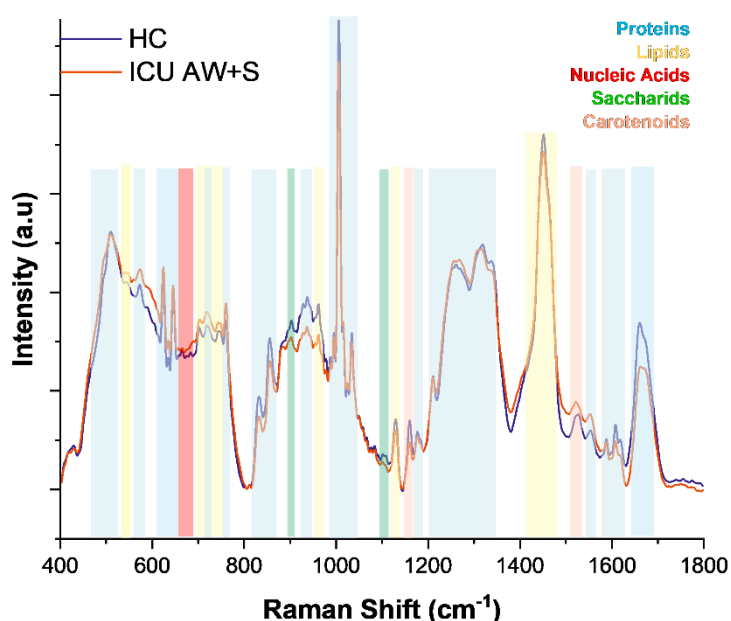
These findings highlight the complementary strengths of the two spectroscopic methods: NMR provides quantitative molecular specificity, while RS captures biochemical and structural variations. Although the current sample size limits the identification of definitive biomarkers, the strong agreement between the two datasets reinforces the analytical and interpretative value of this integrated approach. Notably, the combined RS-NMR analysis enabled the identification of three metabolites that were altered in ICU AW+S patients: glutamate, alanine, and dimethylamine.

Future studies should expand the sample cohort and include an additional experimental group of patients with sepsis only, in order to exclude metabolic differences attributable solely to bacterial infection. This will help to identify biomarkers specifically associated with ICU-AW pathophysiology. Moreover, advanced NMR approaches such as diffusion-ordered spectroscopy (DOSY)<sup>36</sup> should be incorporated to investigate lipid-related contributions. Overall, the synergistic use of RS and NMR represents a promising framework to understand the pathophysiological mechanisms of diseases with a great potential for biomarker discovery in complex clinical conditions as ICU AW+S.

#### 4.1.5 Supplementary Materials

##### *Raman Spectral Analysis of Total Plasma*

Raman spectra were acquired and processed as described in the main manuscript. The overlaid mean Raman spectra of ICU AW+S patients and HC, with selected bands color-coded according to their molecular origin, are reported in this section together with the corresponding table summarizing peak assignments (Figure 1SI and Table 1SI).



**Figure 1SI.** Overlaid mean Raman Spectra of ICU AW+S (red line) and HC (blue line) with selected band color: blue for proteins, yellow for lipids, red for nucleic acids, green for saccharides, and orange for carotenoids.

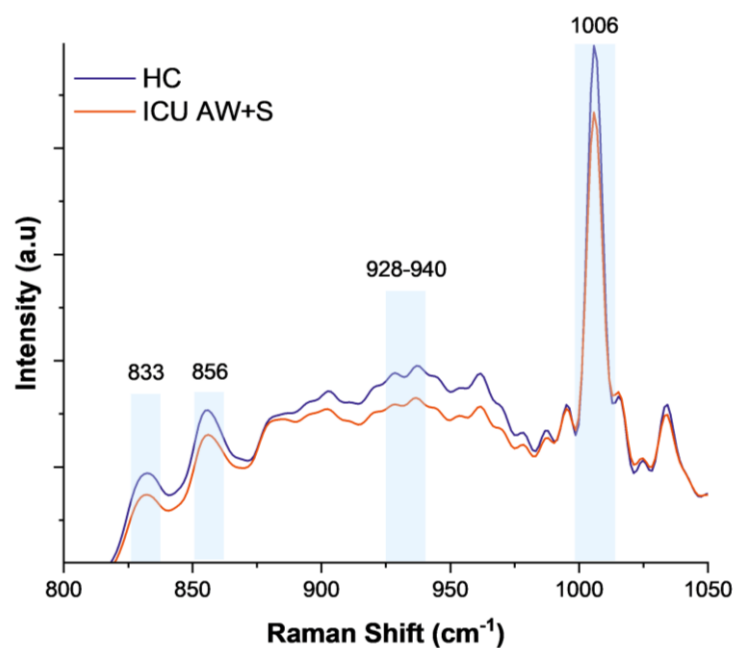
**Table 1SI.** The table reports the assignments of the Raman peaks identified in the mean spectrum of ICU AW+S and HC.

| cm-1    | Assignment                                                                                                                                                                                                                   |
|---------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 509     | S-S disulfide stretching band of cysteine                                                                                                                                                                                    |
| 545     | Cholesterol                                                                                                                                                                                                                  |
| 573     | Tryptophan, Cystoine, Guanine                                                                                                                                                                                                |
| 600-800 | Nucleic Acid                                                                                                                                                                                                                 |
| 624     | C-C twisting mode of phenylalanine (proteins)                                                                                                                                                                                |
| 634     | Glycerol                                                                                                                                                                                                                     |
| 645     | C-C twisting mode of phenylalanine (proteins)                                                                                                                                                                                |
| 666     | G, T (ring breathing modes in the DNA bases), tyrosine-G backbone in RNA                                                                                                                                                     |
| 683     | Ring breathing modes in the DNA bases                                                                                                                                                                                        |
| 703     | Cholesterol, cholesterol ester                                                                                                                                                                                               |
| 718     | Symmetric stretch vibration of choline group, N+(CH <sub>3</sub> ) <sub>3</sub> , characteristic for phospholipids, phosphatidylcholine, sphengomyelin, C-C-N symmetric stretching in phosphatidylcholine (lipid assignment) |
| 743     | Cholesterol ester (as part of the hydrophobic core of the LPs)                                                                                                                                                               |
| 760     | Tryptophan                                                                                                                                                                                                                   |
| 833     | Asymmetric O-P-O stretching, tyrosine                                                                                                                                                                                        |
| 856     | Proline, hydroxyproline, tyrosine, C-C stretching                                                                                                                                                                            |
| 901     | Monosaccharides (b-glucose), (C-O-C) skeletal mode, disaccharide (maltose), (C-O-C) skeletal mode                                                                                                                            |
| 928-940 | n(C-C), stretching in amino acids                                                                                                                                                                                            |
| 938     | Glutamate                                                                                                                                                                                                                    |
| 953     | CH <sub>3</sub> of proteins (a-helix)                                                                                                                                                                                        |
| 961     | Symmetric stretching vibration of phosphate, lipid                                                                                                                                                                           |
| 1006    | Phenylalanine (proteins)                                                                                                                                                                                                     |
| 1105    | Carbohydrates peak                                                                                                                                                                                                           |
| 1129    | n(C-C) skeletal of acyl backbone in lipid                                                                                                                                                                                    |
| 1161    | Carotenoids                                                                                                                                                                                                                  |
| 1176    | C-H bending tyrosine (proteins)                                                                                                                                                                                              |

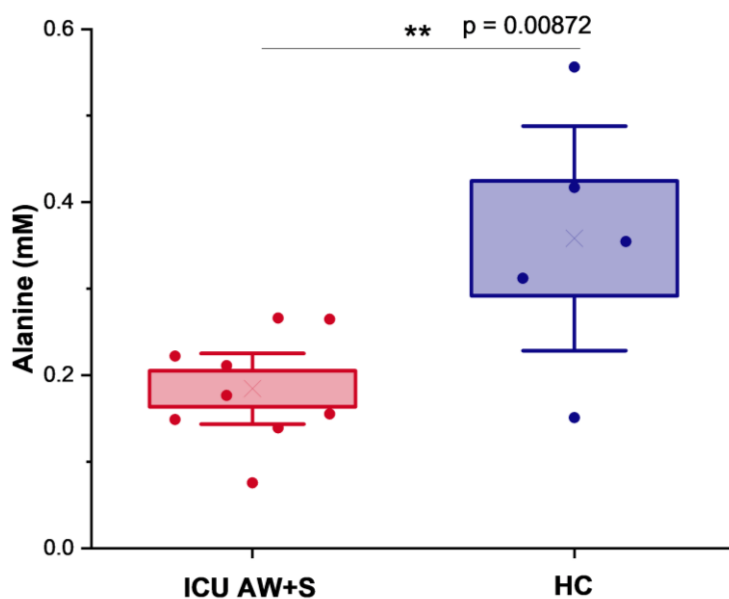
|           |                                                                                                                   |
|-----------|-------------------------------------------------------------------------------------------------------------------|
| 1340      | Nucleic acid mode                                                                                                 |
| 1200-1350 | Amide III                                                                                                         |
| 1270      | Amide III, a C-N stretch from $\alpha$ -helix proteins                                                            |
| 1308      | C-N asymmetric stretching in asymmetric aromatic amines                                                           |
| 1319      | CH <sub>2</sub> twisting mode of the hydrophobic chains of fatty acids                                            |
| 1450      | C-H deformation bands (CH functional groups in lipids, amino acids side chains of the proteins and carbohydrates) |
| 1521-1527 | C=C carotenoid                                                                                                    |
| 1553      | Amide II                                                                                                          |
| 1558      | C-N and N-H amide II (protein assignment)                                                                         |
| 1588      | Phenylalanine, hydroxyproline                                                                                     |
| 1607      | Tyrosine, phenylalanine ring vibration, C=C phenylalanine, tyrosine                                               |
| 1618      | C=C Tryptophan                                                                                                    |
| 1660      | Amide I                                                                                                           |

### ***Correlating RS with NMR results***

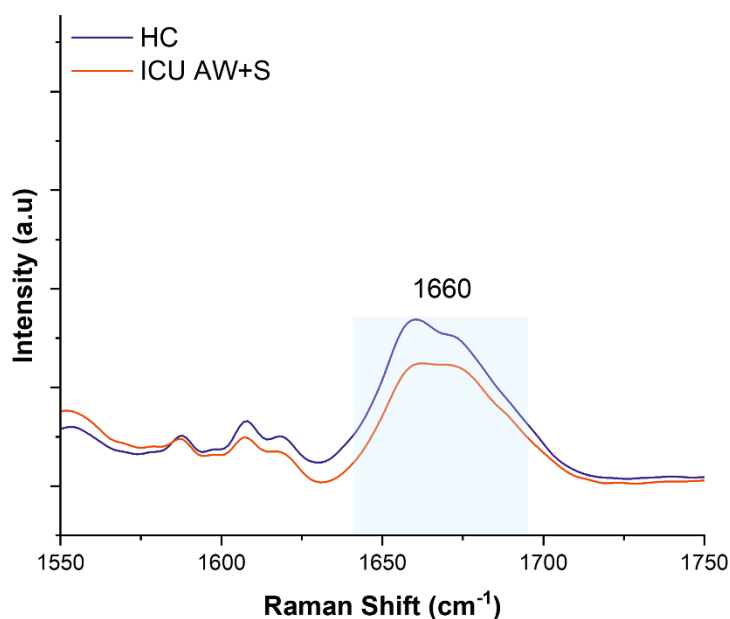
NMR and Raman data provided convergent information, facilitating a more accurate interpretation of the results. Both datasets showed similar trends in amino acid profiles, with Raman peaks (Fig. 2SI) and NMR-quantified amino acids, including glutamate, glycine, glutamine, valine, and notably alanine, higher in HC than in ICU AW+S patients. This decrease in alanine (Fig. 3SI), as reported in the main manuscript, may reflect altered protein metabolism in patients. Additional support comes from the Raman peak at 1660 cm<sup>-1</sup> (amide I), which is more intense and conformationally distinct in controls, with its AUC showing statistically significant differences indicative of modifications in protein backbone structure (Fig. 4SI–5SI).



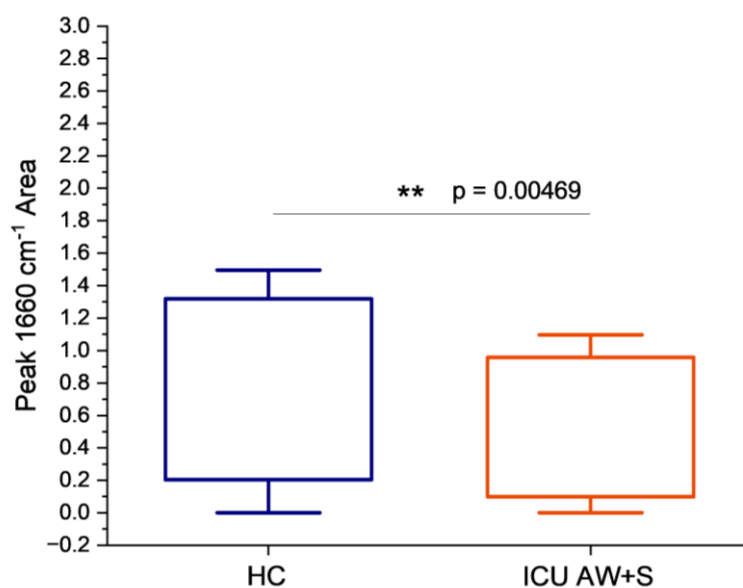
**Figure 2SI.** Mean Raman spectra of HC (blue), and ICU AW+S (red) with an inset highlighting amino acid-related peaks. Higher intensities are observed in HC compared to ICU AW+S.



**Figure 3SI.** Box plots showing alanine concentrations in healthy controls (HC, blue) and ICU-AW+S patients (red) quantified by NMR spectroscopy. Alanine levels were significantly higher in HC compared to ICU-AW+S patients ( $p$  value = 0.00872), as determined by Student's  $t$ -test.



**Figure 4SI.** Focus on the Raman peak at  $1660\text{ cm}^{-1}$  corresponding to the amide I band. Overlapped spectra of HC (blue) and ICU AW+S (red) show greater intensity in HC as well as a distinct conformational difference of the peak compared to ICU AW+S.



**Figure 5SI.** Area under the curve (AUC) of the  $1660\text{ cm}^{-1}$  amide I peak calculated for HC and ICU AW+S patients. Statistical comparison using Student's *t*-test reveals a significant difference ( $p = 0.00469$ ).

#### 4.1.5 References

- (1) A Molecular Typing Method for Invasive Breast Cancer by Serum Raman Spectroscopy - Clinical Breast Cancer. [https://www.clinical-breast-cancer.com/article/S1526-8209\(24\)00048-X/abstract](https://www.clinical-breast-cancer.com/article/S1526-8209(24)00048-X/abstract) (accessed 2025-11-02).
- (2) Sharma, M.; Li, Y.-C.; Manjunatha, S. N.; Tsai, C.-L.; Lin, R.-M.; Huang, S.-F.; Chang, L.-B. Identification of Healthy Tissue from Malignant Tissue in Surgical Margin Using Raman

- Spectroscopy in Oral Cancer Surgeries. *Biomedicines* **2023**, *11* (7), 1984. <https://doi.org/10.3390/biomedicines11071984>.
- (3) Wang, Y.; Fang, L.; Wang, Y.; Xiong, Z. Current Trends of Raman Spectroscopy in Clinic Settings: Opportunities and Challenges. *Adv Sci (Weinh)* **2023**, *11* (7), 2300668. <https://doi.org/10.1002/advs.202300668>.
- (4) Schulze, H. G.; Rangan, S.; Vardaki, M. Z.; Blades, M. W.; Turner, R. F. B.; Piret, J. M. Demixing and Analysis of Complex Biological Raman Hyperspectra Based on Peak Fitting, Amplitude Trend Clustering, and Spectrum Reconstruction. *Appl Spectrosc* **2025**, *79* (9), 1303–1312. <https://doi.org/10.1177/00037028241311296>.
- (5) Kuhar, N.; Sil, S.; Verma, T.; Umapathy, S. Challenges in Application of Raman Spectroscopy to Biology and Materials. *RSC Adv.* **2018**, *8* (46), 25888–25908. <https://doi.org/10.1039/C8RA04491K>.
- (6) Bertazioli, D.; Piazza, M.; Carlomagno, C.; Gualerzi, A.; Bedoni, M.; Messina, E. An Integrated Computational Pipeline for Machine Learning-Driven Diagnosis Based on Raman Spectra of Saliva Samples. *Computers in Biology and Medicine* **2024**, *171*, 108028. <https://doi.org/10.1016/j.compbiomed.2024.108028>.
- (7) Pan, L.; Zhang, P.; Daengngam, C.; Peng, S.; Chongcheawchamnan, M. A Review of Artificial Intelligence Methods Combined with Raman Spectroscopy to Identify the Composition of Substances. *J Raman Spectroscopy* **2022**, *53* (1), 6–19. <https://doi.org/10.1002/jrs.6225>.
- (8) Zhang, K.; Liu, R.; Wei, X.; Wang, Z.; Huang, P. Use of Raman Spectroscopy to Study Rat Lung Tissues for Distinguishing Asphyxia from Sudden Cardiac Death. *RSC Adv.* **2024**, *14* (8), 5665–5674. <https://doi.org/10.1039/D3RA07684A>.
- (9) Morasso, C.; Daveri, E.; Bonizzi, A.; Truffi, M.; Colombo, F.; Danelli, P.; Albasini, S.; Rivoltini, L.; Mazzucchelli, S.; Sorrentino, L.; Corsi, F. Raman Spectroscopy on Dried Blood Plasma Allows Diagnosis and Monitoring of Colorectal Cancer. *MedComm* **2024**, *5* (11), e774. <https://doi.org/10.1002/mco2.774>.
- (10) O'Toole, H. J.; Lowe, N. M.; Arun, V.; Kolesov, A. V.; Palmieri, T. L.; Tran, N. K.; Carney, R. P. Plasma-Derived Extracellular Vesicles (EVs) as Biomarkers of Sepsis in Burn Patients via Label-Free Raman Spectroscopy. *Journal of Extracellular Vesicles* **2024**, *13* (9), e12506. <https://doi.org/10.1002/jev2.12506>.
- (11) Forleo, L.; Picciolini, S.; Gualerzi, A.; Battaglia, E.; Compalati, E.; Banfi, P. I.; Bedoni, M. Development of a Raman-Based Method for the Diagnosis of People with Obstructive Sleep Apnea Syndrome: The Role of Lactic Acid. *International Journal of Molecular Sciences* **2025**, *26* (18), 9095. <https://doi.org/10.3390/ijms26189095>.
- (12) Tognarelli, J. M.; Dawood, M.; Shariff, M. I. F.; Grover, V. P. B.; Crossey, M. M. E.; Cox, I. J.; Taylor-Robinson, S. D.; McPhail, M. J. W. Magnetic Resonance Spectroscopy: Principles and Techniques: Lessons for Clinicians. *Journal of Clinical and Experimental Hepatology* **2015**, *5* (4), 320–328. <https://doi.org/10.1016/j.jceh.2015.10.006>.
- (13) Guleria, A.; Kumar, U.; Kumar, D.; R, N.; Anuja, A. K.; Singh, M. K.; Sharma, P.; Agarwal, V.; Misra, R.; Gupta, L. NMR-Based Serum and Muscle Metabolomics for Diagnosis and Activity Assessment in Idiopathic Inflammatory Myopathies. *Analytical Science Advances* **2021**, *2* (11–12), 515–526. <https://doi.org/10.1002/ansa.202000171>.
- (14) Jaurila, H.; Koivukangas, V.; Koskela, M.; Gäddnäs, F.; Myllymaa, S.; Kullaa, A.; Salo, T.; Ala-Kokko, T. I. <sup>1</sup>H NMR Based Metabolomics in Human Sepsis and Healthy Serum. *Metabolites* **2020**, *10* (2), 70. <https://doi.org/10.3390/metabo10020070>.

- (15) Moco, S. Studying Metabolism by NMR-Based Metabolomics. *Front. Mol. Biosci.* **2022**, *9*. <https://doi.org/10.3389/fmolb.2022.882487>.
- (16) Bratchenko, I. A.; Bratchenko, L. A.; Moryatov, A. A.; Khristoforova, Y. A.; Artemyev, D. N.; Myakinin, O. O.; Orlov, A. E.; Kozlov, S. V.; Zakharov, V. P. In Vivo Diagnosis of Skin Cancer with a Portable Raman Spectroscopic Device. *Experimental Dermatology* **2021**, *30* (5), 652–663. <https://doi.org/10.1111/exd.14301>.
- (17) Chen, Y.; Chen, Z.; Su, Y.; Lin, D.; Chen, M.; Feng, S.; Zou, C. Metabolic Characteristics Revealing Cell Differentiation of Nasopharyngeal Carcinoma by Combining NMR Spectroscopy with Raman Spectroscopy. *Cancer Cell Int* **2019**, *19*, 37. <https://doi.org/10.1186/s12935-019-0759-4>.
- (18) Piva, S.; Fagoni, N.; Latronico, N. Intensive Care Unit–Acquired Weakness: Unanswered Questions and Targets for Future Research. *F1000Research* April 17, 2019. <https://doi.org/10.12688/f1000research.17376.1>.
- (19) Chen, J.; Huang, M. Intensive Care Unit-Acquired Weakness: Recent Insights. *J Intensive Med* **2024**, *4* (1), 73–80. <https://doi.org/10.1016/j.jointm.2023.07.002>.
- (20) Grossi, I.; Radeghieri, A.; Paolini, L.; Porrini, V.; Pilotto, A.; Padovani, A.; Marengoni, A.; Barbon, A.; Bellucci, A.; Pizzi, M.; Salvi, A.; De Petro, G. MicroRNA-34a-5p Expression in the Plasma and in Its Extracellular Vesicle Fractions in Subjects with Parkinson’s Disease: An Exploratory Study. *Int J Mol Med* **2021**, *47* (2), 533–546. <https://doi.org/10.3892/ijmm.2020.4806>.
- (21) Carlomagno, C.; Bertazioli, D.; Gualerzi, A.; Picciolini, S.; Banfi, P. I.; Lax, A.; Messina, E.; Navarro, J.; Bianchi, L.; Caronni, A.; Marengo, F.; Monteleone, S.; Arienti, C.; Bedoni, M. COVID-19 Salivary Raman Fingerprint: Innovative Approach for the Detection of Current and Past SARS-CoV-2 Infections. *Sci Rep* **2021**, *11* (1), 4943. <https://doi.org/10.1038/s41598-021-84565-3>.
- (22) Rodà, F.; Gualerzi, A.; Picciolini, S.; Forleo, L.; Mangolini, V.; Mancuso, R.; Agostini, S.; Rossetto, R. A.; Pierucci, P.; Banfi, P. I.; Bedoni, M. Molecular Understanding of the Surface-Enhanced Raman Spectroscopy Salivary Fingerprint in People after Sars-COV-2 Infection and in Vaccinated Subjects. *Chemosensors* **2024**, *12* (7), 136. <https://doi.org/10.3390/chemosensors12070136>.
- (23) Movasaghi, Z.; Rehman, S.; Rehman, I. U. Raman Spectroscopy of Biological Tissues. *Applied Spectroscopy Reviews* **2007**, *42* (5), 493–541. <https://doi.org/10.1080/05704920701551530>.
- (24) Masui, K.; Nawa, Y.; Tokumitsu, S.; Nagano, T.; Kawarai, M.; Tanaka, H.; Hamamoto, T.; Minoshima, W.; Tani, T.; Fujita, S.; Ishitobi, H.; Hosokawa, C.; Inouye, Y. Detection of Glutamate Encapsulated in Liposomes by Optical Trapping Raman Spectroscopy. *ACS Omega* **2022**, *7* (11), 9701–9709. <https://doi.org/10.1021/acsomega.1c07206>.
- (25) Wu, G.; Lupton, J. R.; Turner, N. D.; Fang, Y.-Z.; Yang, S. Glutathione Metabolism and Its Implications for Health. *The Journal of Nutrition* **2004**, *134* (3), 489–492. <https://doi.org/10.1093/jn/134.3.489>.
- (26) Haberecht-Müller, S.; Krüger, E.; Fielitz, J. Out of Control: The Role of the Ubiquitin Proteasome System in Skeletal Muscle during Inflammation. *Biomolecules* **2021**, *11* (9), 1327. <https://doi.org/10.3390/biom11091327>.
- (27) Guo, S.; Rösch, P.; Popp, J.; Bocklitz, T. Modified PCA and PLS: Towards a Better Classification in Raman Spectroscopy–Based Biological Applications. *Journal of Chemometrics* **2020**, *34* (4), e3202. <https://doi.org/10.1002/cem.3202>.

- (28) Jeremy K. Nicholson, Peta J. D. Foxall, Manfred. Spraul, R. Duncan. Farrant, John C. Lindon. *750 MHz <sup>1</sup>H and <sup>1</sup>H-<sup>13</sup>C NMR Spectroscopy of Human Blood Plasma*. *Anal. Chem.* **1995**, 67, 5, 793–811. <https://doi.org/10.1021/ac00101a004>
- (29) Psychogios, N.; Hau, D. D.; Peng, J.; Guo, A. C.; Mandal, R.; Bouatra, S.; Sinelnikov, I.; Krishnamurthy, R.; Eisner, R.; Gautam, B.; Young, N.; Xia, J.; Knox, C.; Dong, E.; Huang, P.; Hollander, Z.; Pedersen, T. L.; Smith, S. R.; Bamforth, F.; Greiner, R.; McManus, B.; Newman, J. W.; Goodfriend, T.; Wishart, D. S. The Human Serum Metabolome. *PLOS ONE* **2011**, 6 (2), e16957. <https://doi.org/10.1371/journal.pone.0016957>.
- (30) Barton, R. H.; Waterman, D.; Bonner, F. W.; Holmes, E.; Clarke, R.; Consortium, the P.; Nicholson, J. K.; Lindon, J. C. The Influence of EDTA and Citrate anticoagulant Addition to Human Plasma on Information Recovery from NMR-Based Metabolic Profiling Studies. *Mol. BioSyst.* **2009**, 6 (1), 215–224. <https://doi.org/10.1039/B907021D>.
- (31) Nijveldt, R. J.; Teerlink, T.; Van Der Hoven, B.; Siroen, M. P. C.; Kuik, D. J.; Rauwerda, J. A.; van Leeuwen, P. a. M. Asymmetrical Dimethylarginine (ADMA) in Critically Ill Patients: High Plasma ADMA Concentration Is an Independent Risk Factor of ICU Mortality. *Clin Nutr* **2003**, 22 (1), 23–30. <https://doi.org/10.1054/clnu.2002.0613>.
- (32) Smedberg, M.; Rooyackers, O.; Norberg, Å.; Tjäder, I.; Wernerman, J. Endogenous Production of Glutamine and Plasma Glutamine Concentration in Critically Ill Patients. *Clinical Nutrition ESPEN* **2020**, 40, 226–230. <https://doi.org/10.1016/j.clnesp.2020.09.015>.
- (33) Sedlak TW, Paul BD, Parker GM, Hester LD, Snowman AM, Taniguchi Y, Kamiya A, Snyder SH, Sawa A. The glutathione cycle shapes synaptic glutamate activity. *Proc Natl Acad Sci U S A.* **2019**, 12;116(7):2701-2706. doi: 10.1073/pnas.1817885116.
- (34) Yiew, N. K. H.; Vazquez, J. H.; Martino, M. R.; Kennon-McGill, S.; Price, J. R.; Allard, F. D.; Yee, E. U.; Layman, A. J.; James, L. P.; McCommis, K. S.; Finck, B. N.; McGill, M. R. Hepatic Pyruvate and Alanine Metabolism Are Critical and Complementary for Maintenance of Antioxidant Capacity and Resistance to Oxidative Insult. *Molecular Metabolism* **2023**, 77, 101808. <https://doi.org/10.1016/j.molmet.2023.101808>.
- (35) Tsikas, D. Urinary Dimethylamine (DMA) and Its Precursor Asymmetric Dimethylarginine (ADMA) in Clinical Medicine, in the Context of Nitric Oxide (NO) and Beyond. *J Clin Med* **2020**, 9 (6), 1843. <https://doi.org/10.3390/jcm9061843>.
- (36) Harvey, N.; Takis, P. G.; Lindon, J. C.; Li, J. V.; Jiménez, B. Optimization of Diffusion-Ordered NMR Spectroscopy Experiments for High-Throughput Automation in Human Metabolic Phenotyping. *Anal. Chem.* **2023**, 95 (6), 3147–3152. <https://doi.org/10.1021/acs.analchem.2c04066>.

## **4.2 Spectroscopic Profiling of Extracellular Vesicles as Emerging Biomarkers for Sepsis-Associated ICU-Acquired Weakness**

This sub-chapter presents preliminary unpublished data obtained from samples collected as part of the Ricerca Finalizzata 2021 project (RF-2021-12375279), in collaboration with the Intensive Care Unit (Prof. Nicola Latronico and Dr. Simone Piva) and the Orthopedics Ward (Dr. Stefano Cattaneo) of the Spedali Civili in Brescia.

### **4.2.1 Introduction**

Intensive Care Unit–Acquired Weakness (ICU-AW) is a frequent neuromuscular impairment that affects critically ill patients, particularly those with sepsis, where the incidence is up to 67%<sup>[1]</sup>.

ICU-AW manifests as diffuse, symmetrical muscle weakness that prolongs mechanical ventilation, delays rehabilitation, worsens overall prognosis, and consequently leads prolonged ICU stay, higher hospitalization charges, and increased long-term morbidity and mortality<sup>[2]</sup>. Despite its high prevalence and clinical impact, the pathophysiology of ICU-AW remains only partially understood, and no reliable biomarkers currently exist for its early detection or monitoring.

To date, the diagnosis of ICU-AW is based on the assessment of muscle strength using Medical Research Council Score (MRC-SS) where a total score of <48 and <36 are diagnosed with ICU-AW and severe ICU-AW, respectively<sup>[3]</sup>. However, these manual methods require patients awake and cooperative, which is challenge in case of coma or sedation. Indeed, when manual testing is not possible, imaging techniques such as neuromuscular ultrasound<sup>[1]</sup> and computed tomography<sup>[4]</sup> are employed. Furthermore, electromyography and nerve conduction measurements can also help with the diagnosis of ICU-AW in uncooperative patients<sup>[5]</sup>, but they usually require professional equipment, trained personnel, and time-consuming.

For all these reasons, the identification of sensitive, non-invasive biomarkers able to capture the systemic and muscular alterations associated with ICU-AW, especially when sepsis is present, represents an urgent clinical need.

In this context, Extracellular Vesicles (EVs) have emerged as potential biomarkers in clinical field due to their ability to reflect the physiological and pathological state of their cells of origin<sup>[6]</sup>. Indeed, EVs are released by all cell types and circulate in all biofluids, transporting bioactive molecules, including proteins, lipids, and nucleic acids, both inside them and as biomolecular corona<sup>[7]</sup>. EV molecular composition can change dynamically in response to inflammation<sup>[8]</sup>, infection<sup>[9]</sup>, and tissue damage<sup>[10]</sup>, making them attractive tools for disease

diagnosis, prognosis, and therapeutic monitoring. In the context of ICU-AW sepsis associated, EVs could provide valuable insights into muscle injury, immune dysregulation, and metabolic alterations underlying disease progression. However, the EV clinical translation remains challenging due to their nanoscale dimensions, structural heterogeneity, and long-complicated isolation and characterization<sup>[11]</sup>. Moreover, the most available analytical methods for EV analysis, such as nanoparticle tracking analysis and flow cytometry, require expensive instrumentation and often lack of sensitivity for routine clinical use<sup>[12]</sup>. Indeed, standardization of EV isolation and analysis protocols is still an unmet need, limiting reproducibility across studies.

To overcome these limitations, biophotonic techniques have gained attention as powerful analytical tools able to characterize subpopulations of EV in a rapid and non-destructive manner<sup>[13]</sup>.

Among them, Raman spectroscopy (RS) and Fourier Transform InfraRed (FTIR) Spectroscopy are vibrational and label-free technologies that detect specific molecules of a sample irradiated by a laser and IR radiation respectively. Indeed, vibrational spectroscopies provide a fingerprint of the sample, individuating modifications concerning the levels, and presence of proteins, lipids, metabolites, and nucleic acids allowing a precise biochemical characterization and differences associated with disease states<sup>[14]</sup>.

For example, Hannah J, O'Toole and collaborators investigated the use of RS for the analysis of plasma-derived EVs from burn patients to identify sepsis-related biochemical signatures. Using quadratic discriminant analysis, the Raman spectra of plasma-derived EVs successfully distinguished septic from non-septic burn patients with 97.5% sensitivity and 90.0% specificity, outperforming current biomarker-based approaches for sepsis diagnosis<sup>[15]</sup>.

In a similar way, another work studied plasma-derived EVs from subjects with Duchenne Muscular Dystrophy (DMD) at different stages and Healthy Controls (HC) using FTIR spectroscopy. Researcher focused on protein (Amide I and II) and lipid region, using machine learning approaches such as Principal Component Analysis (PCA) and Random Forest to classify DMD and HC based on spectral features. Distinct alterations in protein and lipid configurations were observed in DMD-derived EVs, demonstrating the potential of FTIR-based analysis as a rapid, automated, and minimally invasive tool for early diagnosis and disease monitoring<sup>[16]</sup>.

Due to the lack of early biomarkers for ICU AW+S and the critical need for bedside patient management, this study aims to analyze EVs separated by Size Exclusion Chromatography (SEC) using Raman and FTIR spectroscopy, for obtaining a molecular fingerprint to serve as a

potential disease biomarker. Given their complementary analytical strengths, Raman and FTIR spectroscopy were together employed, as each technique probes distinct vibrational modes. By integrating both approaches, it is possible to capture a more complete EV molecular profile, improving the detection of subtle alterations related to ICU AW.

This work further seeks to evaluate the potential of EV-based Raman and FTIR profiling as an early, non-invasive diagnostic tool for ICU-AW, ultimately paving the way for the integration of vibrational spectroscopy into clinical workflows and offering new perspectives for precision diagnostics and patient monitoring in critical care.

## **4.2.2 Method and Materials**

### *4.2.2.1 Subject Recruitment and Sample Collection*

Plasma samples were collected from three groups: patients with ICU AW+S (n = 9; age range 58 – 84 mean age 70; 2 females and 7 males), patients with SEPSIS only (SEPSIS; n = 5; age range 59 – 83, mean age 71; 3 females and 2 males), and Healthy Controls (HC; n = 7; age range 43 – 82, mean age 62; 4 females and 3 males). Participants were recruited from the Intensive Care Unit and the Orthopedic Ward at Spedali Civili University Hospital in Brescia, Italy. The study adhered to the principles of the Declaration of Helsinki and Good Clinical Practice (Protocol number 34 68) received approval from the Ethics Committee of Brescia. Written informed consent was obtained from all participants prior to sample collection.

Whole blood was collected into EDTA-containing tubes and processed through a stepwise centrifugation protocol: first at  $800 \times g$  for 10 minutes, followed by two consecutive spins at  $2,500 \times g$  for 15 minutes each. All centrifugation steps were performed at room temperature with low acceleration and brake settings. The resulting plasma was then aliquoted and stored at  $-80^\circ\text{C}$  until EV separation<sup>[17]</sup>.

### *4.2.2.2 EV Separation*

For EV separation, 600  $\mu\text{L}$  of plasma were first centrifuged at  $1500 \times g$  for 10 min at  $4^\circ\text{C}$ . Then, the supernatant was collected and subjected to a second centrifugation at  $10,000 \times g$  for 10 min at  $4^\circ\text{C}$  to remove residual cellular debris. Subsequently, 500  $\mu\text{L}$  of the centrifuged plasma were loaded onto a SEC column (qEV/70 nm series; Izon Science, Christchurch, New Zealand), and fractions from 1 to 17 were collected sequentially (400  $\mu\text{L}$  each).

Each fraction was ultracentrifuged at  $100,000 \times g$  for 2 h at  $4^\circ\text{C}$  (UC) and the resulting EV pellets were resuspended in 60  $\mu\text{L}$  of buffer for subsequent analyses.

#### 4.2.2.3 Trichloroacetic acid (TCA) for protein precipitation

SEC fractions (1–17) were treated using 10% TCA and incubated on ice for 4 hours in order to promote protein precipitation. Following incubation, fractions were centrifuged at  $13,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$ . Then, supernatant was removed without disturbing the protein pellet.

The resulting pellets were resuspended in sodium acetate trihydrate to neutralize the pH. A second centrifugation step was then performed at  $13,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$ . After removal of the supernatant, the residual sodium acetate was allowed to evaporate completely. Pellets were subsequently resuspended in  $2\times$  sample buffer (80 mM Tris, pH 6.8, 2% SDS, 7.5% glycerol, 0.01% bromophenol blue, 2% 2-mercaptoethanol), sonicated for 20 seconds at 60% amplitude, and boiled at  $95^{\circ}\text{C}$  for 5 minutes. Finally, the prepared samples were stored at  $-20^{\circ}\text{C}$  until further analysis<sup>[18]</sup>.

#### 4.2.2.4 SDS-PAGE and Western Blot (WB)

SDS–PAGE and WB were performed according to standard protocols<sup>[19]</sup>. Total plasma (30  $\mu\text{g}$  of protein, as determined by the Bradford assay) was used as a positive control, and SEC fractions 1-17 were analyzed. An equal volume (30  $\mu\text{L}$ ) of each fraction was loaded onto 12.5% acrylamide-bisacrylamide gels and subjected to electrophoresis under denaturing conditions. Following SDS-PAGE, proteins were transferred to PVDF membranes (Bio-Rad) and probed with specific antibodies. CD63 (Millipore, clone RFAC4, CBL553), a canonical EV marker<sup>[20]</sup>, was used to confirm the presence of EVs, while Apolipoprotein A1 (APOA1; Thermo Fisher Scientific, 701239; dilution 1:500) was employed as a lipoprotein marker. Detection was performed using appropriate HRP-conjugated secondary antibodies and a chemiluminescent substrate.

#### 4.2.2.7 Dot Blot (DB)

SEC pooled fractions 6, 7, 8, and individual fractions 13, 15, and 17 were analyzed by DB following the protocol published by Paolini et al.<sup>[21]</sup>. Before to start with DB analysis, SEC fractions were concentrated by ultracentrifugation at  $100,000 \times g$  for 2 h at  $4^{\circ}\text{C}$ , and the resulting pellets were resuspended in 15  $\mu\text{L}$  of  $\text{H}_2\text{O}$  HPLC. Protein concentrations were determined using a BCA assay. For the Dot Blot analysis, 3  $\mu\text{g}$  of protein from the pooled fractions (6,7,8) and from individual fractions 13, 15, and 17 were spotted onto a nitrocellulose membrane (BioRad) and allowed to dry at room temperature. Then, the membrane was incubated with blocking solution (5% dry milk in PBS with 0,1% of Tween-20) for 1 hour at  $37^{\circ}\text{C}$  under gentle agitation. Following blocking, membrane was incubated overnight at  $4^{\circ}\text{C}$

with primary antibodies for CD63 (EV marker)<sup>[20]</sup> and APOA1 (lipoprotein marker). The next day, membrane was washed three times for 10 minutes each with PBS containing 0.1% Tween-20, and subsequently incubated with the appropriate secondary antibodies. Finally, the membrane was detected in chemiluminescent mode.

#### *4.2.2.6 Bicinchoninic acid (BCA) and Bradford assay*

Total plasma protein concentration was determined using the Bradford assay (BioRad), following the manufacturer's instructions. Given the low protein content typically associated with EV preparations, the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA) was used to quantify protein concentration in SEC fractions, according to the manufacturer's instructions. All measurements were performed in triplicate, and protein concentrations were calculated using a Bovine Serum Albumin (BSA) standard curve.

#### *4.2.2.7 Colorimetric NANoplasmonic Assay (CONAN)*

Selected SEC fractions (7, 10, 15, 17) were assessed for purity<sup>[20]</sup> and potential contaminants using the CONAN assay, a method based on the clustering of gold nanoparticles (AuNPs) onto lipid membranes, as previously described by Maiolo et al<sup>[22]</sup>. The assay was performed according to the published protocol<sup>[23]</sup>. Briefly, 2 µL of each SEC fraction were serially diluted in HPLC water (1:1, 1:5, and 1:50) and added to 23 µL of H<sub>2</sub>O. The mixture was combined with 50 µL of 6 nM AuNPs solution and 25 µL of PBS. The resulting samples were incubated and analyzed using a microplate reader (Ensign Multi-Mode Reader, PerkinElmer). Absorbance measurements were carried out in triplicate for each dilution.

#### *4.2.2.8 Atomic Force Microscopy (AFM)*

AFM analysis was performed following previously established protocols<sup>[24,25]</sup>. Briefly, 3 µL of the pooled SEC fractions 6,7,8 were spotted onto mica sheets (grade V-1, 0.15 mm thickness, 15 × 15 mm) and allowed to dry at room temperature. Imaging was carried out using a Nanosurf NaioAFM instrument (Nanosurf AG) equipped with a Multi75-AI-G probe (Budget Sensors) in tapping mode. The images were acquired at a scan speed of 1 second per line.

#### *4.2.2.9 Nanoparticles Tracking Analysis (NTA)*

Pooled fractions (6, 7, 8, and 9, 10, 11) were characterized using NTA (NanoSight NS300; Malvern Panalytical Ltd., Malvern, UK) to evaluate EV size distribution and concentration. Before sample analysis, filtered PBS (0.22µm filtered) was analysed for particle contamination. For all the NTA analysis, pooled samples were diluted in fresh filtered PBS (1:50) in order to

obtain an optimal particles per frame (20-120 particles/frame). EVs were injected into the sample chamber using a Nanosight syringe pump (Malvern, Panalytical LTD, Malvern, UK) that provided a continuous flow (50  $\mu\text{L}/\text{min}$ ) using a 1 ml syringe at room temperature. Recordings of the movements of particles were collected for 60 seconds, 3 times for each sample with 5 as threshold.

#### *4.2.2.10 Raman and FTIR Analysis*

For Raman analysis, after ultracentrifugation of the single SEC fractions, fractions 6,7, and 8, and 9,10, and 11 were pooled, and 4  $\mu\text{L}$  of each pool (6,7,8 – 9,10,11) was deposited onto a  $\text{CaF}_2$  slide and allowed to dry at room temperature. Raman acquisition was performed using an Aramis Raman microscope (Horiba Jobin-Yvon, Palaiseau, France) equipped with a 532 nm laser, following the protocol for bulk EV characterization previously<sup>[26–28]</sup>. Instrument calibration was performed using the silicon reference peak at  $520.7\text{ cm}^{-1}$ . A  $50\times$  objective (Olympus, Tokyo, Japan) was employed for all acquisitions, with an exposure time of 30 seconds, two accumulations, a diffraction grating of 1800 grooves/mm, a  $400\text{ }\mu\text{m}$  entrance slit, and a  $600\text{ }\mu\text{m}$  hole. Spectra were collected using LabSpec6 software (Horiba Scientific) over the ranges  $500\text{--}1800\text{ cm}^{-1}$  and  $2700\text{--}3200\text{ cm}^{-1}$ , with spectral resolution of  $0.8\text{ cm}^{-1}/\text{step}$ . For each sample, approximately 15/20 spectra were acquired per drop in a coffee ring. All spectra were baseline-corrected using a polynomial fit aligned to the reference peaks at  $1001\text{ cm}^{-1}$  by LabSpec6. Finally, spectra were normalized using unit vector normalization to reduce the effects of autofluorescence and background signals by OriginPro2024b (OriginLab, Northampton, MA, USA).

FTIR measurements were performed with a Cary 630 spectrometer (Agilent) operating in transmission mode. An Attenuated Total Reflectance (ATR) diamond accessory was used to analyse 3  $\mu\text{L}$  of the pooled fractions 6,7,8 and 9,10,11 dried under nitrogen. All spectra were acquired in triplicate in the range of  $4000\text{--}400\text{ cm}^{-1}$ , with 32 scans per measurement. All spectra were baseline-corrected using a manual tool in OriginPro2024b and normalized by unit vector normalization.

#### *4.2.2.11 Statistical Analysis*

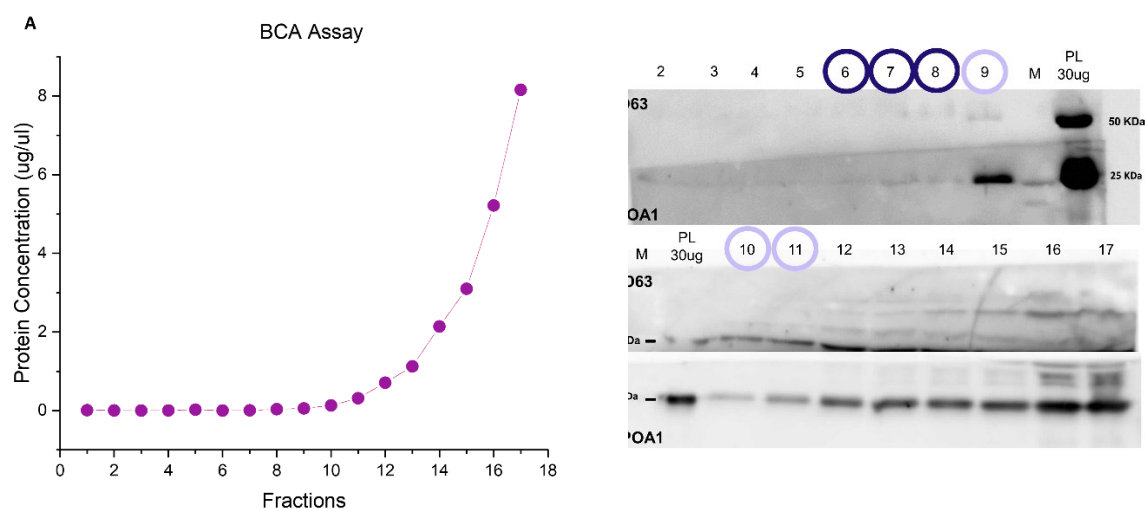
Raman and FTIR spectra analysed by descriptive and multivariate analysis, as Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA), using OriginPro2024b. The Mann-Whitney non-parametric test and the ANOVA One-Way test were used for NTA analysis.

### 4.2.3 Results and Discussion

#### 4.2.3.1 Plasma-derived EV separation and characterization

EVs were successfully separated from human plasma samples from ICU AW+S patients, SEPSIS patients, and HC subjects using the optimized SEC protocol described in the Materials and Methods section. Seventeen fractions, each of 400  $\mu$ l, were collected, and the total protein quantification by BCA assay was subsequently performed. Results confirmed a gradual increase in protein concentration across the fractions, consistent with the expected enrichment of soluble plasma proteins and lipoproteins, leading to higher concentrations in the later fractions (Fig. 1A).

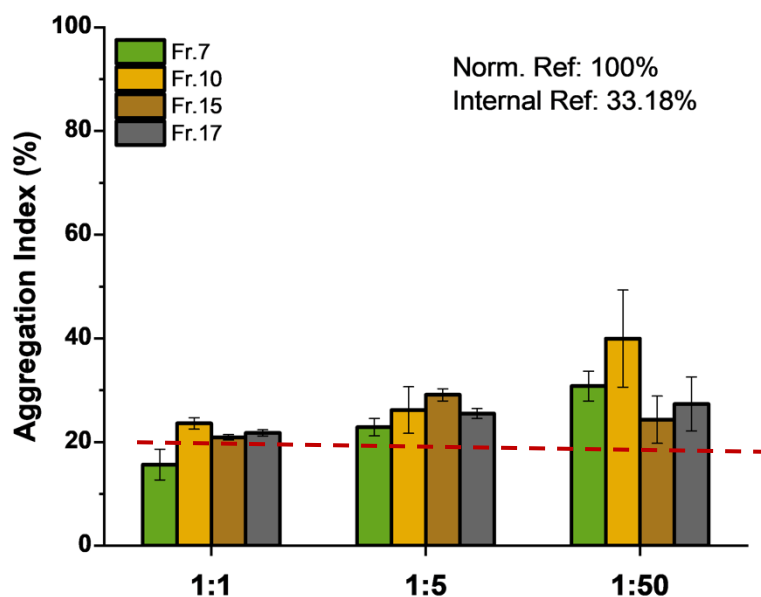
The characterization of the SEC fractions was conducted in accordance with the MISEV2023 guidelines<sup>[20]</sup>. WB analysis was conducted from 1 to 17 fractions, and 30  $\mu$ g of total plasma was used as a positive control. As shown in Figure 1B, WB revealed that the expression of the tetraspanin CD63 started to be expressed from SEC fraction 6. Although the intensity of the CD63 increased in the subsequent fractions, particularly in the later ones, the lipoprotein marker APOA1 showed an even stronger signal, suggesting partial co-isolation of lipoproteins together with EVs. This is due to the size-based method of SEC, in which smaller lipoproteins, particularly high-density lipoproteins (HDL), share a similar size range with EVs and may have been co-isolated during the separation process<sup>[29,30]</sup>. In fractions 6,7,8, despite the weak CD63 signal, the concomitant reduction in APOA1 intensity indicated a higher degree of EV purity. On the other hand, fractions 9,10,11 showed a stronger CD63 band, but also an increase of APOA1 signal, reflecting reduced purity. Later fractions were not further considered, as BCA quantification indicated a high protein content, suggesting an accumulation of soluble contaminants.



**Figure 1.** Protein quantification and biochemical characterization of SEC fractions. A) BCA assay of SEC fractions from 1 to 17. Protein content is negligible across fractions 1-10, indicating minimal co-isolation of soluble proteins. An increase in protein concentration is observed from fraction 11 to 17, consistent with the elution of plasma proteins in later SEC fractions; B) WB analysis of fractions from 1 to 17. Thirty  $\mu\text{g}$  of total plasma (PL) was used as a positive control. Equal volumes of each fraction were loaded and inserted on a 12% acrylamide/bis-acrylamide SDS-PAGE gel. Membranes were probed for CD63 (EV marker) and APOA1 (lipoprotein marker) to assess the distribution of EVs and lipoprotein components across the SEC fractions.

To confirm these data, the CONAN assay<sup>[22,23]</sup> was performed on representative fractions (7, 10, 15, 17) to better assess purity. All analyzed fractions were tested at three dilutions (1:1, 1:5, and 1:50) to achieve an optimal balance between EVs and AuNPs. Indeed, the CONAN assay relies on the optical and colloidal properties of AuNPs: in pure samples (EVs are not contaminated by soluble proteins), AuNPs aggregate on the EV membrane, resulting in a shift in the AuNP plasmon peak and a visible color change from red to blue. Conversely, in the presence of protein contamination, neither color change nor plasmon peak shift is observed. A protein concentration of 0,05  $\mu\text{g}/\mu\text{L}$  (red dashed line in figure 2) was used as a cut-off<sup>[22]</sup>, which is equal to an aggregation index (AI) of 20%. Samples exceeding this threshold were considered impure.

Fraction 7 already had an AI below 20% (AI% = 15.66), indicating it was free of protein contaminants. Furthermore, subsequent dilutions showed an increased aggregation index, likely because the reduced number of EVs in the sample led to a weaker aggregation response of the AuNPs. However, in fractions 10, 15, and 17, a slight increase in the aggregation index was observed even at the 1:1 dilution, indicating a gradual increase in protein contamination, consistent with the BCA assay and Western blot results. Furthermore, the limited increase in the aggregation index can be explained by the presence of lipoproteins, which interact with AuNPs in a manner similar to EVs and are therefore not fully recognized as contaminants by the assay (Fig. 2).

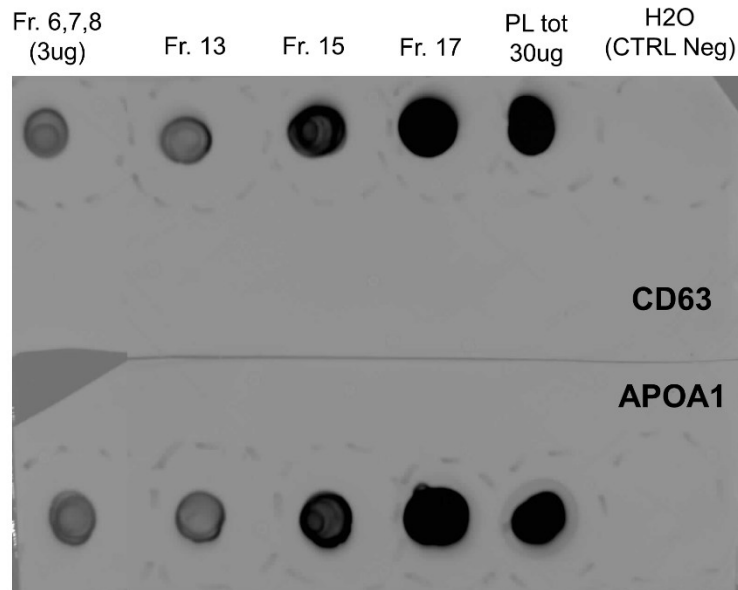


**Figure 2.** CONAN assay to assess the purity of SEC fractions. Aggregation Index (AI%) values obtained from CONAN analysis of selected SEC fractions (7, 10, 15, 17) at serial dilutions (1:1, 1:5, and 1:50). The red dotted line indicates the assay threshold for detecting exogenous protein contaminants (AI% < 20%, corresponding to  $\leq 0.05 \mu\text{g}/\mu\text{L}$  soluble protein). Lower AI% values reflect higher sample purity. Fractions 7 displayed AI% below the threshold, confirming minimal contamination, whereas later fractions showed progressively higher AI%, in line with their increased soluble protein content. Values are reported as mean  $\pm$  standard deviation. The Norm. Ref. corresponds to monodisperse AuNPs in water, while the internal Ref. represents the internal assay control containing no sample.

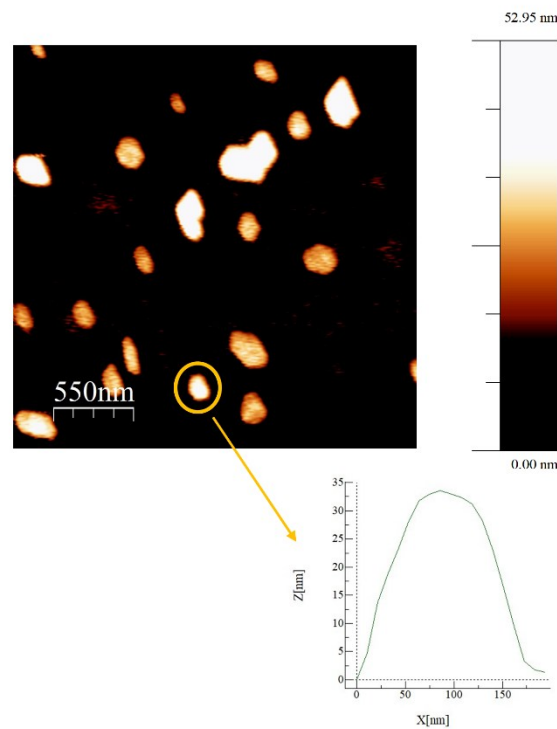
For further analysis, we performed ultracentrifugation at  $100,000 \times g$  for 2 hours to concentrate and then pool the 6, 7, and 8 SEC fractions.

DB analysis was performed on the concentrated pooled fractions 6,7,8 and on the concentrated single fraction 13, 15, and 17 to evaluate the presence of both EVs and lipoproteins. As shown in Figure 3A, CD63 and APOA1 signals in the pooled fractions were approximately equivalent, suggesting a roughly 50:50 distribution between EVs and lipoproteins. In the individual fractions 13, 15, and 17, both signals increased, indicating a gradual enrichment of EVs and co-isolated lipoproteins.

The presence of spherical nanoparticles in the concentrated pooled fractions 6,7,8 was also confirmed by AFM imaging, which revealed nanostructures with characteristic size and morphology (Figure 3B) that are indistinguishable from EVs and lipoproteins. These observations are in line with the previous results, indicating that the pool contained a mixture of EVs and lipoproteins.

**A**

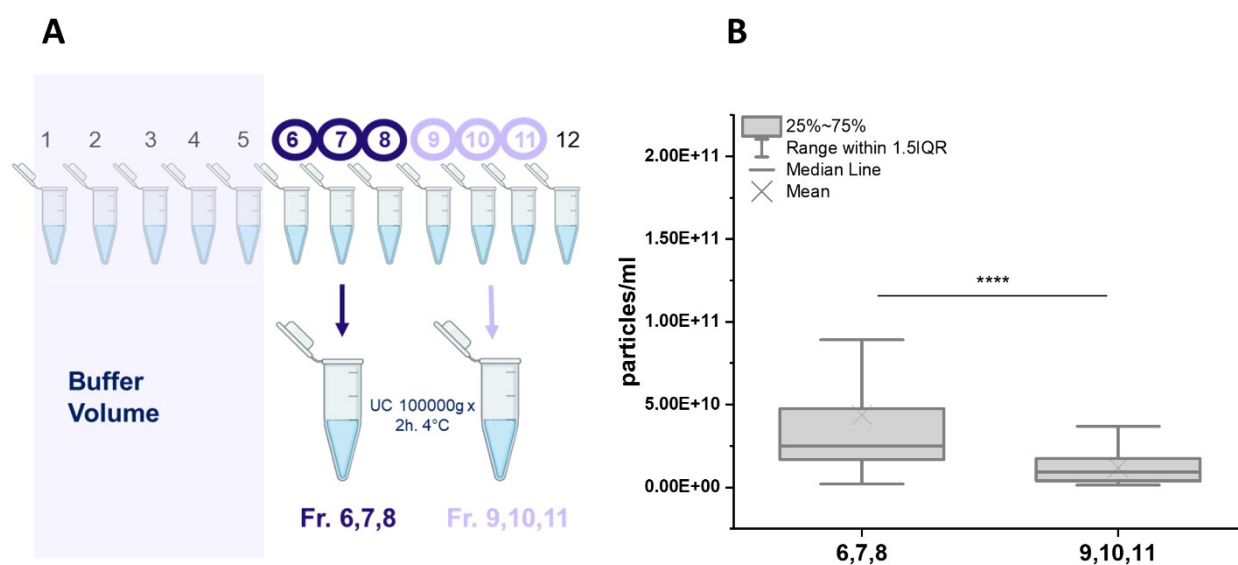
**Figure**  
**3. A)**  
*DB*  
*and*  
*AFM*

**B**

characterization of pooled and single SEC fractions. DB analysis was performed on 3  $\mu$ g of protein from pooled EV-enriched fractions (6,7,8) and from single later fractions (13, 15, 17). Membranes were probed with CD63 antibody for EV marker and APOA1 antibody for lipoprotein marker. Both markers were detectable across fractions, indicating the co-isolation of EVs and lipoproteins.

B) Representative AFM images of pooled fractions 6,7,8. Samples show spherical nanoobjects with size and morphology consistent with EVs, although lipoprotein-like structures cannot be fully excluded.

Given the results obtained from BCA assay, WB, CONAN assay, DB and AFM we decided to continue the analyses by pooling fractions 6,7,8 as purest but less concentrated EV population, and fractions 9,10,11 as higher EVs but also with a high lipoprotein co-isolation (Fig. 4A). However, NTA analysis revealed a higher particle concentration in the 6,7,8 pool compared to 9,10,11 pool (Fig. 4B). This observation may be explained by the presence of smaller lipoproteins in the 9,10,11 fractions, whose signal is probably underestimated due to the instrument's detection limit.



**Figure 4.** A) Schematic representation of the collection fractions. Fractions 1-5 correspond to the void volume containing buffer as indicated on manufacture instruction, while EV-containing fractions start from fraction 6. Fractions 6,7,8 and 9,10,11 were individually concentrated by ultracentrifugation ( $100,000 \times g$ , 2 h,  $4^{\circ}\text{C}$ ) and then pooled as indicated for further analysis.

B) NTA of the pooled fractions shows a higher particle concentration in fractions 6,7,8 compared to 9,10,11. Data are presented as box plots showing the interquartile range (25–75%), median, and mean ( $\times$ ). Statistical significance was determined using the Mann–Whitney test, as the distributions did not meet normality assumptions. Values: \*\*\*\* $p \leq 0.0001$ .

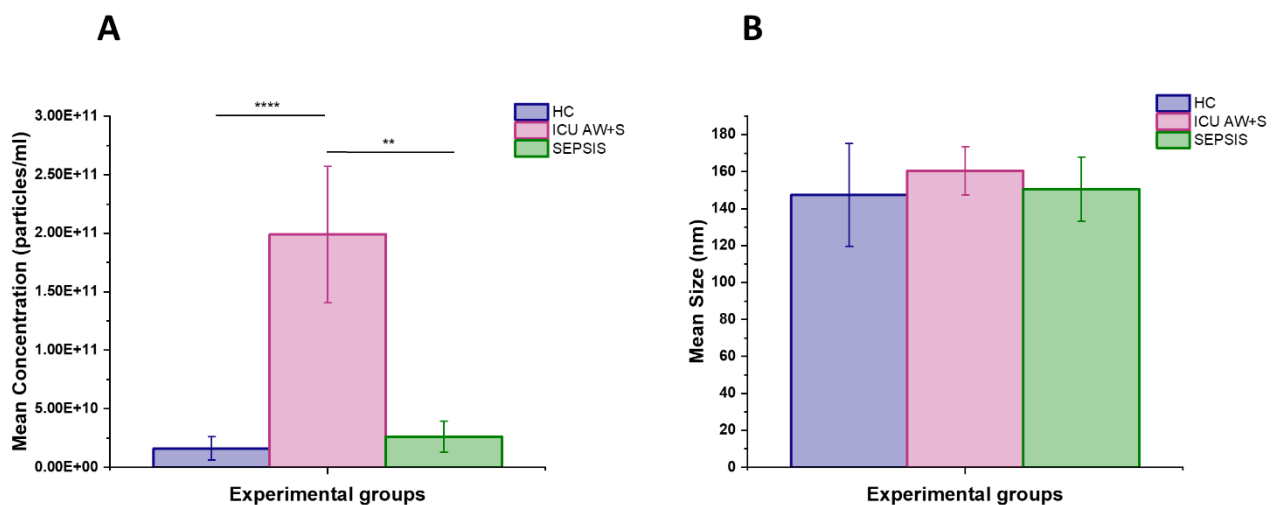
In conclusion, this characterization confirms the presence of EVs in the analyzed fractions, with varying degrees of purity depending on lipoprotein contamination and the specific pool considered. Fractions 6,7,8 represent a purer EV population with lower lipoprotein content with respect to fractions 9,10,11 as demonstrated by WB, BCA, and CONAN assay. These findings highlight the balance between EV purity and yield and provide a basis for selecting the most appropriate fraction pool for the following analyses.

#### 4.2.3.2 NTA Analysis between ICU AW+S, SEPSIS, and HC Groups

Concentrated and pooled EV samples (6,7,8) were analysed by NTA to evaluate EV concentration and size distribution. All samples were diluted 1:50 prior to acquisition to obtain measurements within the instrument's optimal range. For each group, three 60-second captures were acquired, and the resulting values were subjected to One-Way ANOVA to assess potential differences among ICU AW+S, SEPSIS, and HC.

NTA revealed a higher and statistically significant EV concentration in the ICU AW+S group compared with both SEPSIS and HC (Fig. 6A). This enrichment in circulating EVs may reflect the systemic stress and accelerated cellular turnover that characterise ICU AW+S. Critically ill patients with muscle atrophy present an enhanced inflammatory activation, mitochondrial dysfunction, and elevated catabolic signalling, which can increase the release of EVs from different types of cells<sup>[1,2,31]</sup>. This major EV concentration in ICU AW+S patients respect to patients with only sepsis also could represent an intensified cellular stress, tissue remodelling, or intensified metabolic dysregulation linked to muscle atrophy that septic patients do not present.

In contrast, no significant differences were observed in mean EV size across the three experimental groups (Fig.6B). This is consistent with the use of the SEC method to separate EV, which is a technique based on size-separation within a narrow range, minimising variability associated with EV diameter. The stability of size distributions also suggests that the observed differences in concentration are not related to artefacts caused by aggregation or fragmentation during processing.

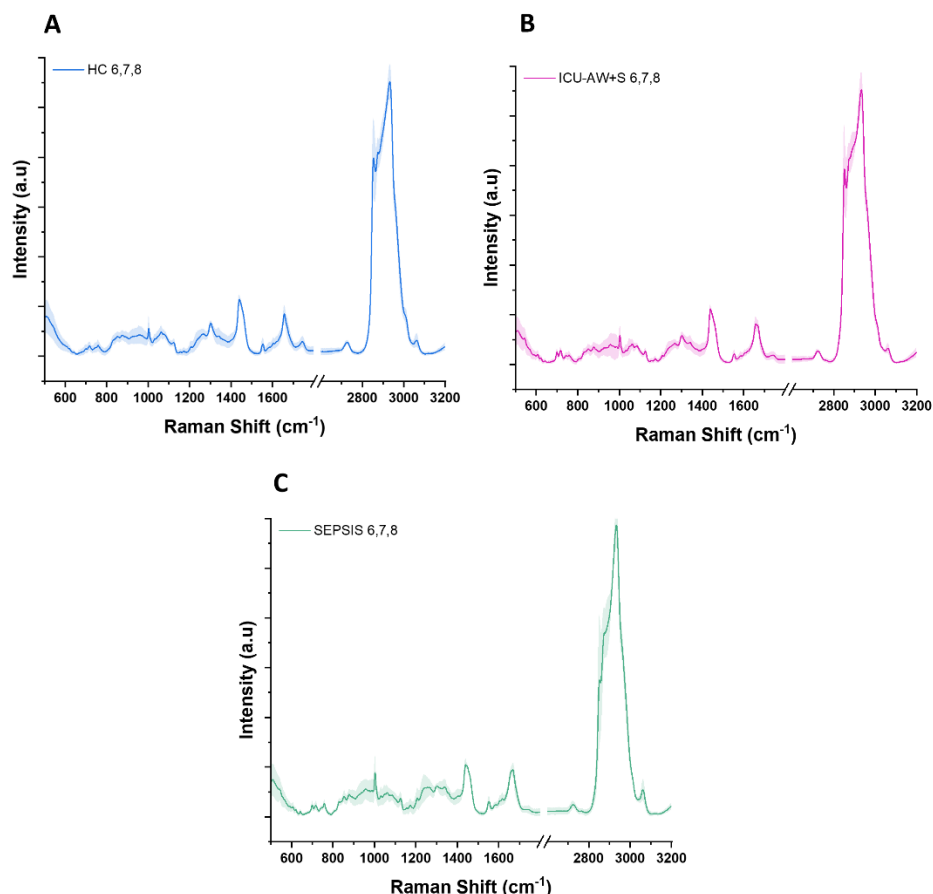


**Figure 5.** Nanoparticle Tracking Analysis (NTA) of pooled EV fractions (6,7,8). A) Concentration measured by NTA shows a significant difference among the three experimental groups. One-way ANOVA revealed a statistically higher EV concentration in ICU AW+S patients compared with both HC ( $p$  value  $< 0,0001$ ) and SEPSIS subjects ( $p$  value  $= 0,00296$ ); B) Mean particle size did not differ significantly between the three experimental groups. Data are reported as mean  $\pm$  SEM. Values: \*\*\*\* $p \leq 0,0001$ ; \*\* $p \leq 0,01$ .

#### 4.2.3.3 Raman Spectroscopy and FTIR Analysis for distinguishing ICU AW+S from SEPSIS and HC subjects

Based on the results of the standard characterization, the RS analyses were performed on EV from ICU AW+S ( $n=9$ ), SEPSIS ( $n=5$ ), and HC ( $n=7$ ) subjects using the pooled fractions 6,7,8, which were identified as the most EV-enriched.

For RS analysis, fractions were concentrated by ultracentrifugation at  $100,000 \times g$  for 2 h and resuspended in 60  $\mu$ L of H<sub>2</sub>O HPLC. For RS, 4  $\mu$ L of freshly EV from each group under analysis were deposited onto CaF<sub>2</sub> slide and allowed to dry at room temperature. Approximately 15-20 spectra were acquired per drop, taking advantage of the concentration of molecules in the coffee ring region. Raman spectra were acquired in the spectral ranges 500-1800  $\text{cm}^{-1}$  and 2700-3200  $\text{cm}^{-1}$  using a 532 nm laser. Spectral pre-processing was performed using LabSpec6 and OriginPro2024b, including despiking to remove cosmic rays. Spectral alignment was performed to the related phenylalanine peak (1006  $\text{cm}^{-1}$ ), which is always present in biological samples, and a univariate vector normalization was applied to correct for intensity spectral variations. Then, the mean spectra ( $\pm 1$  standard deviation) were generated for the three experimental groups. As shown in fig. 7A,B, C, all average spectra present a good signal-to-noise ratio and highlight the methodological reproducibility across samples.

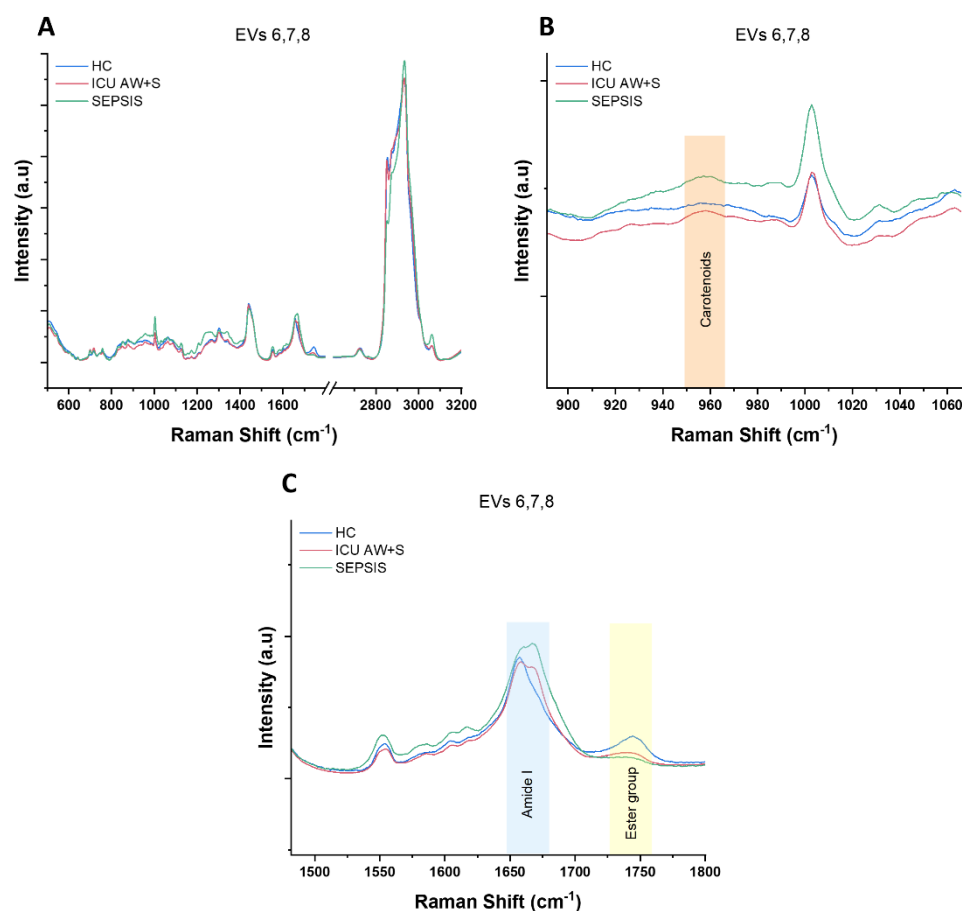


**Figure 6.** Mean Raman spectra of A) HC (blue), B) ICU AW+S (red), and C) SEPSIS (green) samples obtained using 532 nm laser and 30 seconds of exposure for 2 accumulations for each spectrum. The lines indicate the average spectra of each group  $\pm 1$  standard deviation.

To better highlight spectral differences among the three experimental groups, the average Raman spectra of ICU AW+S, SEPSIS, and HC were overlapped (Fig. 8A). Clear differences are evident in the SEPSIS spectrum, likely reflecting the strong influence of bacterial components and associated inflammatory processes. In particular, the  $956\text{ cm}^{-1}$  peak is assigned to carotenoids. As previously reported by Movasaghi and collaborators<sup>[32]</sup>, this peak is typically not present in spectra from healthy tissues. Its presence in SEPSIS and ICU AW+S spectra, but not in HC spectra, may indicate an altered redox state and increased oxidative stress, both known hallmarks of sepsis and systemic inflammation (Fig. 8B).

Moreover, the amide I band (around  $1660\text{ cm}^{-1}$ ) showed differences in shape and intensity across all three groups, suggesting differences in protein composition and folding patterns between HC, SEPSIS, and ICU AW+S. Notably, as shown in Figure 8C, the shape of the amide I peak is much more similar between ICU AW+S and SEPSIS patients than between ICU AW+S and HC patients. Furthermore, changes in the ester group region in the ICU AW+S and SEPSIS spectra further reinforce the idea of modifications in membrane lipid ester bonds,

possibly reflecting oxidative degradation or remodelling of lipid components under septic and catabolic conditions (Fig. 8C).

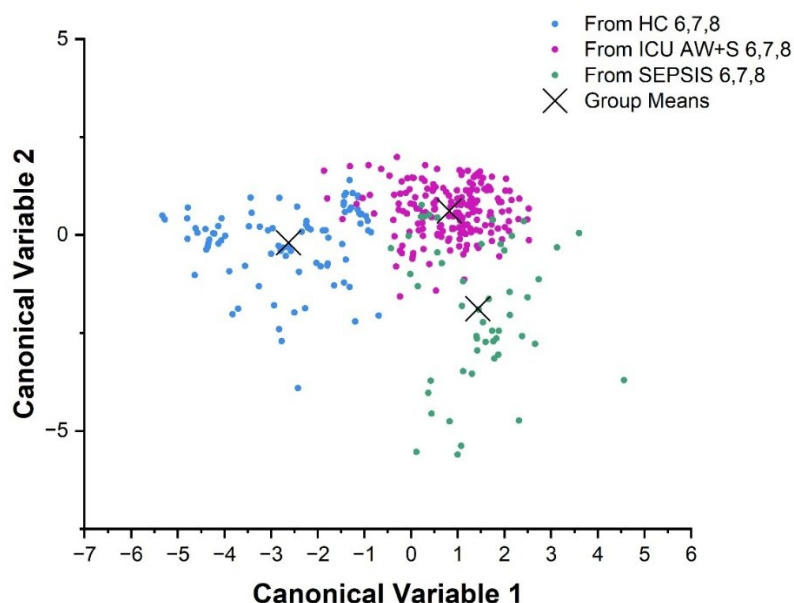


**Figure 7.** A) Overlaid mean Raman spectra of the three experimental groups, showing overall spectral differences; B) Zoom of the carotenoid peak<sup>[32]</sup> (orange) shows an increase of the intensity in SEPSIS and ICU AW+S samples compared with HC; C) Zoom of the Amide I band (blue) displays differences at the peak shape in all of the experimental groups and at the lipid ester peak (yellow)<sup>[32]</sup>, where changes in band intensity and profile reflect distinct lipid compositions among HC, ICU AW+S, and SEPSIS samples.

These results support the potential of RS to reveal disease-specific molecular fingerprints that reflect underlying metabolic and structural changes in EVs, but it is important to note that RS is a vibrational technique in which each peak corresponds to a specific chemical bond. However, the complex molecular composition of biological samples makes it challenging to assign spectral variations to a specific molecule. The overlapping contributions of different bonds result in composite spectra that are difficult to understand. For this reason, multivariate statistical analysis plays a crucial role in the interpretation of Raman data. By reducing data dimensionality and emphasizing the main sources of spectral variance, these approaches allow

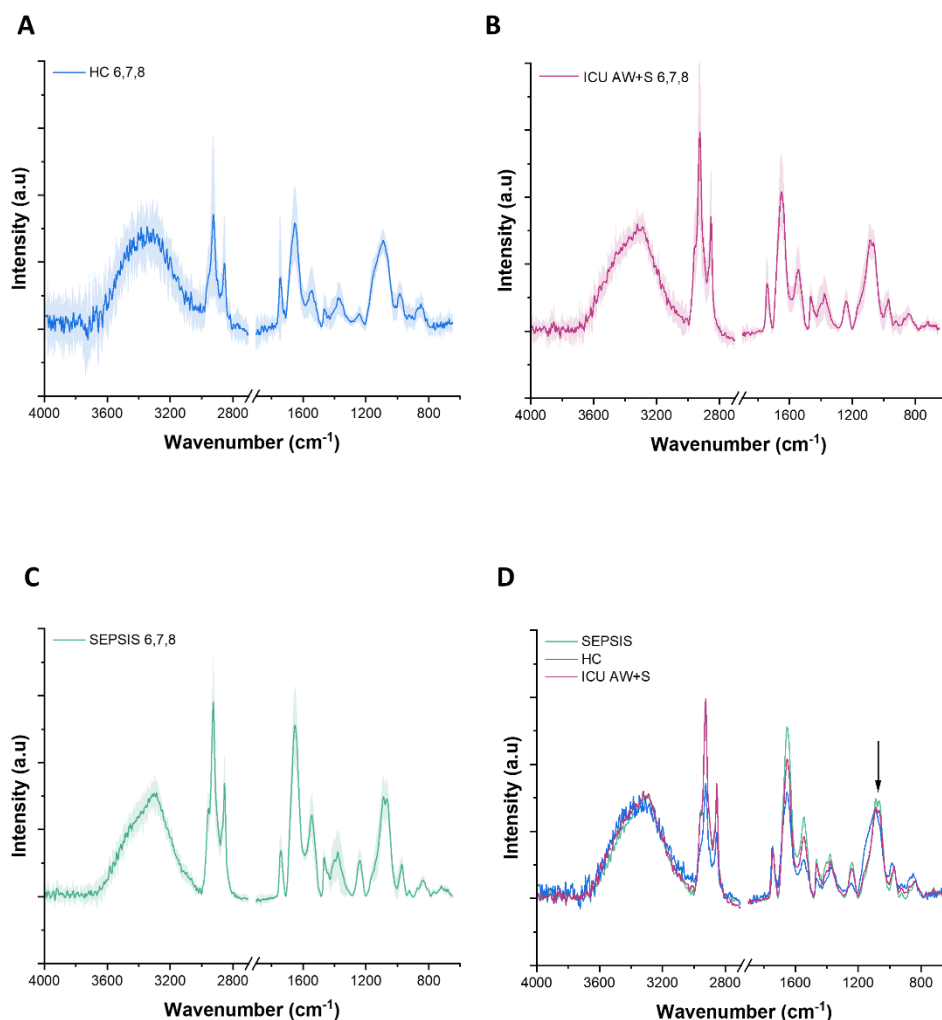
the identification of differences between experimental groups, thereby enhancing the diagnostic and interpretative power of vibrational spectroscopy.

For this reason, multivariate analysis, PCA followed by LDA, was applied to classify ICU AW+S, SEPSIS, and HC subjects based on their Raman fingerprints. The PCA-LDA model demonstrated a statistically significant separation among the three experimental groups with a classification accuracy of 83.47% (Fig. 9).



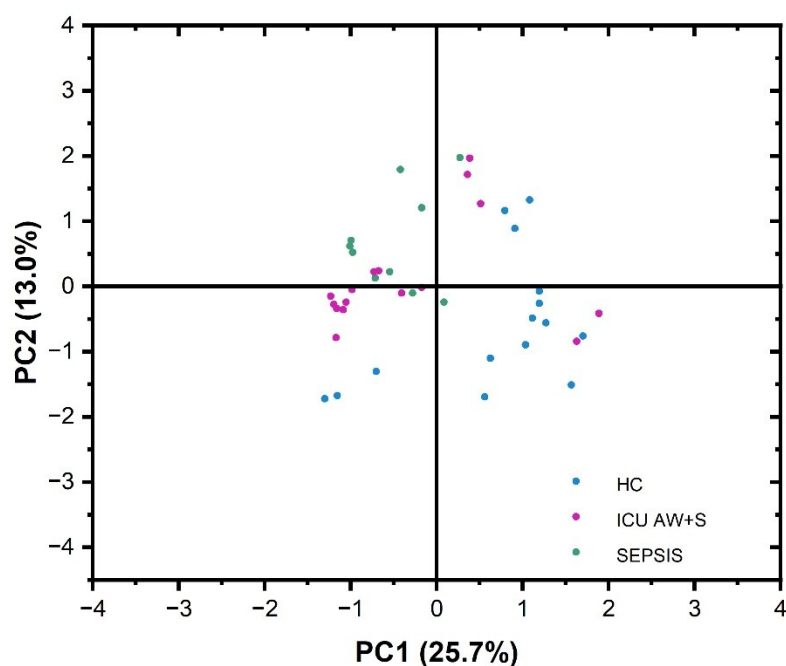
**Figure 8.** Score plot where each point represents an individual Raman spectrum: ICU-AW+S (red), SEPSIS (green), and HC (blue). The graph shows the first two canonical variables (CV1 and CV2), showing a clear separation among the three groups.

For FTIR, 3  $\mu$ l of pooled freshly EVs (6,7,8) were spotted on a diamond ATR crystal and dried under nitrogen. Spectra were acquired in triplicate; the raw data were subsequently normalized using unit vector normalization, and the baseline was determined using a manual tool in OriginPro2024b. The figures 10A, 10B, and 10C show the mean FTIR spectra ( $\pm 1$  standard deviation) of the HC, ICU AW+S, and SEPSIS, respectively, with a good signal-to-noise ratio that suggests the methodological reproducibility of the method. Moreover, the overlaid spectra allowing a direct comparison among the three experimental groups (Fig. 10D). Notably, in the nucleic acid region (approximately 1070–1080  $\text{cm}^{-1}$  band, indicated by the arrow in the figure 10D)<sup>[33]</sup>, a clear difference in spectral shape emerges. Indeed, both SEPSIS and ICU AW+S samples exhibit characteristic peak splitting, suggesting alterations in nucleic acid-related vibrational modes compared to HC.



**Figure 9.** Mean FTIR spectra of A) HC (blue), B) ICU AW+S (red), and C) SEPSIS (green) samples. The lines indicate the average spectra of each group  $\pm 1$  standard deviation; D) Overlaid mean FTIR spectra of the three experimental groups, showing overall spectral differences. The arrow indicates the main visible difference at the nucleic acids band<sup>[33]</sup>.

Moreover, PCA analysis was also performed on the FTIR spectra. As shown in Figure 11, the PCA does not fully discriminate the three experimental groups. Indeed, the clusters exhibit partial overlap, indicating that the variance captured by the first PCs is insufficient for clear separation. Nonetheless, each group follows its own trajectory in multivariate space, suggesting the presence of specific spectral patterns that are not yet strongly expressed due to the limited sample size. With a larger cohort, the separation would become more pronounced, enabling the application of a supervised model, such as PCA-LDA, to FTIR data as well.



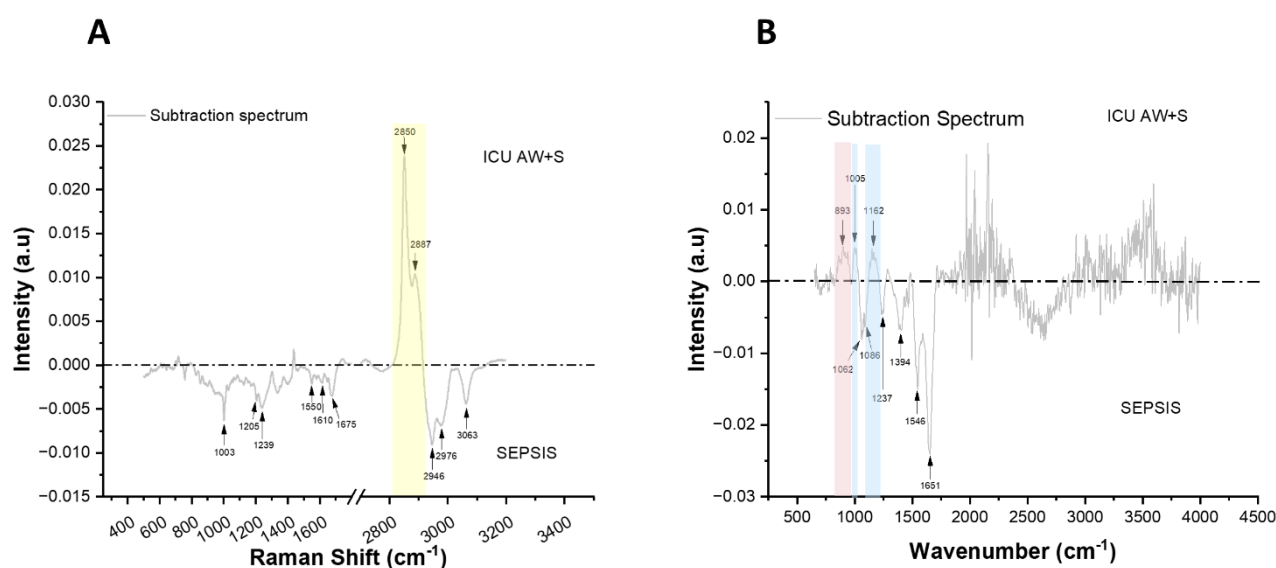
**Figure 10.** PCA of the spectral data from HC, ICU AW+S, and SEPSIS samples. PC1 and PC2 account for 25.7% and 13.0% of the total variance, respectively, highlighting the main patterns of variation among the samples.

#### 4.2.3.4 Combining Results of Raman and FTIR Spectroscopy

Due to different physical principles, Raman and FTIR spectroscopy can detect distinct yet complementary biochemical features. For this reason, subtraction spectra were generated from the mean EV spectrum of ICU AW+S and SEPSIS to enhance the group-specific spectral changes. This approach highlights spectral regions where one group shows higher signal intensity than the other.

As shown in Figure 12A, in the Raman subtraction spectrum (ICU AW+S minus SEPSIS), the most prominent differences are in the lipid-associated region, particularly at  $2850\text{ cm}^{-1}$  ( $\text{CH}_2$  symmetric stretch of fatty acids and membrane lipids) and  $2887\text{ cm}^{-1}$  ( $\text{CH}_2$  asymmetric stretch of lipids, with contributions from protein CH stretching also). Although the  $2887\text{ cm}^{-1}$  vibration can derive from mixed lipid–protein environments, the absence of ICU AW+S-specific signatures in the amide I and amide III regions suggests that these differences most probably originate from lipid alterations rather than significant changes in protein backbone composition<sup>[32]</sup>. Overall, these results demonstrated a lipid-associated biochemical shift in the EV spectrum from ICU AW+S patients compared with SEPSIS patients, supporting an altered lipid metabolism.

Furthermore, the FTIR subtraction (ICU AW+S minus SEPSIS) spectrum was performed (Fig. 12B). In this case, results reveal that the most differences were in regions associated with nucleic acids and protein vibrations. Indeed, the  $893\text{ cm}^{-1}$  peak (C–C and C–O stretching of deoxyribose) and protein-related peaks at  $1005\text{ cm}^{-1}$  (phenylalanine) and  $1162\text{ cm}^{-1}$  (C–OH stretching modes of serine, threonine, and tyrosine residues), were present as positive detection (above the line in the subtraction spectrum), suggesting stress-related or damage-associated biochemical remodelling<sup>[33]</sup>.



**Figure 11.** Raman e FTIR subtraction EV spectra between ICU AW+S and SEPSIS to highlight the main spectral differences. A) In ICU AW+S Raman subtraction EV spectrum, the distinguishing peaks are located in the lipid-associated region (highlighted in yellow); B) In ICU AW+S FTIR subtraction EV spectrum the distinguishing peaks correspond to regions associated with nucleic acids (highlighted in red) and proteins (highlighted in blue).

This complementary vibrational information illustrates that Raman and FTIR spectroscopy capture distinct molecular characteristics of ICU AW+S. Firstly, RS showed differences in lipid composition, whereas FTIR highlighted alterations in nucleic acids and protein moieties. Therefore, integrating information from both techniques provides a more comprehensive biochemical understanding of ICU AW+S, underlining the potential of vibrational spectroscopy in order to use the EV fingerprint as a future biomarker for ICU AW+S.

#### 4.2.4 Conclusion and Future Perspectives

In this part of my PhD thesis, I explored plasma-derived EVs from ICU AW+S, SEPSIS, and HC subjects using SEC as a separation method. Standard characterization and vibrational spectroscopies were employed to study biochemical, morphological, and biophysical properties of EVs. SEC were able to enrich EVs, although the lipoprotein co-isolation, and the pooled and concentrated SEC fractions 6,7,8 were identified as the most suitable for further analyses. Indeed, the method utilized as standard characterization demonstrated a successful EV present. Furthermore, NTA identified differences in EV concentration in ICU AW+S patients compared with SEPSIS and HC subjects. This result is in line with the most recent literature on ICU AW<sup>[1,2,31]</sup>.

Raman and FTIR spectroscopy further demonstrated that ICU AW+S is characterised by distinct spectral alterations in lipids, nucleic acids, carotenoids, and proteins, supporting the hypothesis that EV vibrational fingerprints may serve as a potential new biomarker of ICU AW+S.

Taken together, these results indicate that plasma-derived EVs reflect the systemic metabolic and inflammatory abnormalities in ICU AW+S, and that vibrational spectroscopy is a promising analytical tool for uncovering disease-specific EV signatures. However, the reduced sample size needed validation in larger cohorts to ensure statistical robustness and improve the PCA-LDA model.

Future studies should integrate vibrational spectroscopy with orthogonal molecular techniques to better understand spectral differences. For example, Nuclear Magnetic Resonance (NMR) spectroscopy is a valuable tool for identifying specific metabolites and lipid species that contribute to differences in Raman and FTIR spectra. Once candidate molecules are identified by NMR, their validation can be performed using ultra-sensitive immunoassays such as SiMoA (Single Molecule Array), a technology that enables the detection of proteins at femtomolar concentrations by measuring enzymatic signals at the single-molecule level. This approach would enable linking vibrational signatures to defined biochemical entities.

In summary, expanding the sample size and integrating analytical platforms such as NMR and SiMoA techniques will be essential to better understand the EV molecular fingerprint of ICU AW+S, elucidating the biochemical pathways involved, and ultimately strengthening the translational potential of EV-based vibrational spectroscopy in precision medicine for ICU AW+S diagnosis.

Moreover, the introduction of artificial intelligence and machine learning models could facilitate data interpretation and simplify instrument operation, enabling the potential translation of this technique to portable devices for bedside use.

#### 4.2.5 References

- (1) Piva, S.; Fagoni, N.; Latronico, N. Intensive Care Unit–Acquired Weakness: Unanswered Questions and Targets for Future Research. *F1000Research* April 17, 2019. <https://doi.org/10.12688/f1000research.17376.1>.
- (2) Chen, J.; Huang, M. Intensive Care Unit-Acquired Weakness: Recent Insights. *J Intensive Med* **2024**, *4* (1), 73–80. <https://doi.org/10.1016/j.jointm.2023.07.002>.
- (3) Vanhorebeek, I.; Latronico, N.; Van den Berghe, G. ICU-Acquired Weakness. *Intensive Care Med* **2020**, *46* (4), 637–653. <https://doi.org/10.1007/s00134-020-05944-4>.
- (4) Teixeira, J. P.; Mayer, K. P.; Griffin, B. R.; George, N.; Jenkins, N.; Pal, C. A.; González-Seguel, F.; Neyra, J. A. Intensive Care Unit–Acquired Weakness in Patients With Acute Kidney Injury: A Contemporary Review. *American Journal of Kidney Diseases* **2023**, *81* (3), 336–351. <https://doi.org/10.1053/j.ajkd.2022.08.028>.
- (5) *Clinical review: intensive care unit acquired weakness | Critical Care | Full Text*. <https://ccforum.biomedcentral.com/articles/10.1186/s13054-015-0993-7> (accessed 2025-11-17).
- (6) Hallal, S.; Túzesi, Á.; Grau, G. E.; Buckland, M. E.; Alexander, K. L. Understanding the Extracellular Vesicle Surface for Clinical Molecular Biology. *Journal of Extracellular Vesicles* **2022**, *11* (10), e12260. <https://doi.org/10.1002/jev2.12260>.
- (7) Tassoni, S.; Bergese, P.; Radeghieri, A. The Extracellular Vesicle Biomolecular Corona: Current Insights and Diagnostic Potential. *Nanomedicine* **2025**, *20* (16), 2013–2021. <https://doi.org/10.1080/17435889.2025.2538426>.
- (8) Sanwlani, R.; Gangoda, L. Role of Extracellular Vesicles in Cell Death and Inflammation. *Cells* **2021**, *10* (10), 2663. <https://doi.org/10.3390/cells10102663>.
- (9) You, B.; Yang, Y.; Wei, J.; Zhou, C.; Dong, S. Pathogenic and Therapeutic Roles of Extracellular Vesicles in Sepsis. *Front. Immunol.* **2025**, *16*. <https://doi.org/10.3389/fimmu.2025.1535427>.
- (10) Bruno, S.; Porta, S.; Bussolati, B. Extracellular Vesicles in Renal Tissue Damage and Regeneration. *European Journal of Pharmacology* **2016**, *790*, 83–91. <https://doi.org/10.1016/j.ejphar.2016.06.058>.
- (11) Gandham, S.; Su, X.; Wood, J.; Nocera, A. L.; Alli, S. C.; Milane, L.; Zimmerman, A.; Amiji, M.; Ivanov, A. R. Technologies and Standardization in Research on Extracellular Vesicles. *Trends Biotechnol* **2020**, *38* (10), 1066–1098. <https://doi.org/10.1016/j.tibtech.2020.05.012>.
- (12) George, S. K.; Lauková, L.; Weiss, R.; Semak, V.; Fendl, B.; Weiss, V. U.; Steinberger, S.; Allmaier, G.; Tripisciano, C.; Weber, V. Comparative Analysis of Platelet-Derived Extracellular Vesicles Using Flow Cytometry and Nanoparticle Tracking Analysis. *International Journal of Molecular Sciences* **2021**, *22* (8), 3839. <https://doi.org/10.3390/ijms22083839>.
- (13) Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Rodà, F.; Bedoni, M. Biophotonics for Diagnostic Detection of Extracellular Vesicles. *Advanced Drug Delivery Reviews* **2021**, *174*, 229–249. <https://doi.org/10.1016/j.addr.2021.04.014>.
- (14) Aitekenov, S.; Sultangaziyev, A.; Abdirova, P.; Yussupova, L.; Gaipov, A.; Utegulov, Z.; Bukasov, R. Raman, Infrared and Brillouin Spectroscopies of Biofluids for Medical

- Diagnostics and for Detection of Biomarkers. *Critical Reviews in Analytical Chemistry* **2023**, 53 (7), 1561–1590. <https://doi.org/10.1080/10408347.2022.2036941>.
- (15) O'Toole, H. J.; Lowe, N. M.; Arun, V.; Kolesov, A. V.; Palmieri, T. L.; Tran, N. K.; Carney, R. P. Plasma-Derived Extracellular Vesicles (EVs) as Biomarkers of Sepsis in Burn Patients via Label-Free Raman Spectroscopy. *Journal of Extracellular Vesicles* **2024**, 13 (9), e12506. <https://doi.org/10.1002/jev2.12506>.
  - (16) Rajavel, A.; Essakipillai, N.; Anbazhagan, R.; Ramakrishnan, J.; Venkataraman, V.; Natesan Sella, R. Molecular Profiling of Blood Plasma-Derived Extracellular Vesicles Derived from Duchenne Muscular Dystrophy Patients through Integration of FTIR Spectroscopy and Machine Learning Reveals Disease Signatures. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **2025**, 326, 125236. <https://doi.org/10.1016/j.saa.2024.125236>.
  - (17) Grossi, I.; Radeghieri, A.; Paolini, L.; Porrini, V.; Pilotto, A.; Padovani, A.; Marengoni, A.; Barbon, A.; Bellucci, A.; Pizzi, M.; Salvi, A.; De Petro, G. MicroRNA-34a-5p Expression in the Plasma and in Its Extracellular Vesicle Fractions in Subjects with Parkinson's Disease: An Exploratory Study. *Int J Mol Med* **2021**, 47 (2), 533–546. <https://doi.org/10.3892/ijmm.2020.4806>.
  - (18) Paolini, L.; Radeghieri, A.; Civini, S.; Caimi, L.; Ricotta, D. The Epsilon Hinge-Ear Region Regulates Membrane Localization of the AP-4 Complex. *Traffic* **2011**, 12 (11), 1604–1619. <https://doi.org/10.1111/j.1600-0854.2011.01262.x>.
  - (19) Salvi, A.; Vezzoli, M.; Busatto, S.; Paolini, L.; Faranda, T.; Abeni, E.; Caracausi, M.; Antonaros, F.; Piovesan, A.; Locatelli, C.; Cocchi, G.; Alvisi, G.; De Petro, G.; Ricotta, D.; Bergese, P.; Radeghieri, A. Analysis of a Nanoparticle-enriched Fraction of Plasma Reveals miRNA Candidates for Down Syndrome Pathogenesis. *Int J Mol Med* **2019**, 43 (6), 2303–2318. <https://doi.org/10.3892/ijmm.2019.4158>.
  - (20) Welsh, J. A.; Goberdhan, D. C. I.; O'Driscoll, L.; Buzas, E. I.; Blenkiron, C.; Bussolati, B.; Cai, H.; Di Vizio, D.; Driedonks, T. A. P.; Erdbrügger, U.; Falcon-Perez, J. M.; Fu, Q.-L.; Hill, A. F.; Lenassi, M.; Lim, S. K.; Mahoney, M. G.; Mohanty, S.; Möller, A.; Nieuwland, R.; Ochiya, T.; Sahoo, S.; Torrecilhas, A. C.; Zheng, L.; Zijlstra, A.; Abuelreich, S.; Bagabas, R.; Bergese, P.; Bridges, E. M.; Brucale, M.; Burger, D.; Carney, R. P.; Cocucci, E.; Crescitelli, R.; Hanser, E.; Harris, A. L.; Haughey, N. J.; Hendrix, A.; Ivanov, A. R.; Jovanovic-Talisman, T.; Kruh-Garcia, N. A.; Ku'ulei-Lyn Faustino, V.; Kyburz, D.; Lässer, C.; Lennon, K. M.; Lötval, J.; Maddox, A. L.; Martens-Uzunova, E. S.; Mizenko, R. R.; Newman, L. A.; Ridolfi, A.; Rohde, E.; Rojalin, T.; Rowland, A.; Saftics, A.; Sandau, U. S.; Saugstad, J. A.; Shekari, F.; Swift, S.; Ter-Ovanesyan, D.; Tosar, J. P.; Useckaite, Z.; Valle, F.; Varga, Z.; van der Pol, E.; van Herwijnen, M. J. C.; Wauben, M. H. M.; Wehman, A. M.; Williams, S.; Zembrini, A.; Zimmerman, A. J.; MISEV Consortium; Théry, C.; Witwer, K. W. Minimal Information for Studies of Extracellular Vesicles (MISEV2023): From Basic to Advanced Approaches. *J Extracell Vesicles* **2024**, 13 (2), e12404. <https://doi.org/10.1002/jev2.12404>.
  - (21) Paolini, L.; Orizio, F.; Busatto, S.; Radeghieri, A.; Bresciani, R.; Bergese, P.; Monti, E. Exosomes Secreted by HeLa Cells Shuttle on Their Surface the Plasma Membrane-Associated Sialidase NEU3. *Biochemistry* **2017**, 56 (48), 6401–6408. <https://doi.org/10.1021/acs.biochem.7b00665>.
  - (22) Maiolo, D.; Paolini, L.; Di Noto, G.; Zembrini, A.; Berti, D.; Bergese, P.; Ricotta, D. Colorimetric Nanoplasmonic Assay to Determine Purity and Titrate Extracellular Vesicles. *Anal Chem* **2015**, 87 (8), 4168–4176. <https://doi.org/10.1021/ac504861d>.
  - (23) Zembrini, A.; Paolini, L.; Busatto, S.; Radeghieri, A.; Romano, M.; Wauben, M. H. M.; van Herwijnen, M. J. C.; Nejsun, P.; Borup, A.; Ridolfi, A.; Montis, C.; Bergese, P. Augmented COlorimetric NANoplasmonic (CONAN) Method for Grading Purity and

- Determine Concentration of EV Microliter Volume Solutions. *Frontiers in Bioengineering and Biotechnology* **2020**, 7.
- (24) Paolini, L.; Zendrini, A.; Noto, G. D.; Busatto, S.; Lottini, E.; Radeghieri, A.; Dossi, A.; Caneschi, A.; Ricotta, D.; Bergese, P. Residual Matrix from Different Separation Techniques Impacts Exosome Biological Activity. *Sci Rep* **2016**, 6 (1), 23550. <https://doi.org/10.1038/srep23550>.
  - (25) Radeghieri, A.; Savio, G.; Zendrini, A.; Di Noto, G.; Salvi, A.; Bergese, P.; Piovani, G. Cultured Human Amniocytes Express hTERT, Which Is Distributed between Nucleus and Cytoplasm and Is Secreted in Extracellular Vesicles. *Biochemical and Biophysical Research Communications* **2017**, 483 (1), 706–711. <https://doi.org/10.1016/j.bbrc.2016.12.077>.
  - (26) Gualerzi, A.; Niada, S.; Giannasi, C.; Picciolini, S.; Morasso, C.; Vanna, R.; Rossella, V.; Masserini, M.; Bedoni, M.; Ciceri, F.; Bernardo, M. E.; Brini, A. T.; Gramatica, F. Raman Spectroscopy Uncovers Biochemical Tissue-Related Features of Extracellular Vesicles from Mesenchymal Stromal Cells. *Sci Rep* **2017**, 7 (1), 9820. <https://doi.org/10.1038/s41598-017-10448-1>.
  - (27) Gualerzi, A.; Kooijmans, S. A. A.; Niada, S.; Picciolini, S.; Brini, A. T.; Camussi, G.; Bedoni, M. Raman Spectroscopy as a Quick Tool to Assess Purity of Extracellular Vesicle Preparations and Predict Their Functionality. *Journal of Extracellular Vesicles* **2019**, 8 (1), 1568780. <https://doi.org/10.1080/20013078.2019.1568780>.
  - (28) Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Terenzi, F.; Ramat, S.; Sorbi, S.; Bedoni, M. Raman Profiling of Circulating Extracellular Vesicles for the Stratification of Parkinson's Patients. *Nanomedicine: Nanotechnology, Biology and Medicine* **2019**, 22, 102097. <https://doi.org/10.1016/j.nano.2019.102097>.
  - (29) Liangsupree, T.; Multia, E.; Riekkola, M.-L. Modern Isolation and Separation Techniques for Extracellular Vesicles. *Journal of Chromatography A* **2021**, 1636, 461773. <https://doi.org/10.1016/j.chroma.2020.461773>.
  - (30) Nguyen, P. H. D.; Le, A. H.; Pek, J. S. Q.; Pham, T. T.; Jayasinghe, M. K.; Do, D. V.; Phung, C. D.; Le, M. T. N. Extracellular Vesicles and Lipoproteins – Smart Messengers of Blood Cells in the Circulation. *J Extracell Biol* **2022**, 1 (7), e49. <https://doi.org/10.1002/jex2.49>.
  - (31) Lad, H.; Saumur, T. M.; Herridge, M. S.; Dos Santos, C. C.; Mathur, S.; Batt, J.; Gilbert, P. M. Intensive Care Unit-Acquired Weakness: Not Just Another Muscle Atrophying Condition. *Int J Mol Sci* **2020**, 21 (21), 7840. <https://doi.org/10.3390/ijms21217840>.
  - (32) Movasaghi, Z.; Rehman, S.; Rehman, I. U. Raman Spectroscopy of Biological Tissues. *Applied Spectroscopy Reviews* **2007**, 42 (5), 493–541. <https://doi.org/10.1080/05704920701551530>.
  - (33) Movasaghi, Z.; Rehman, S.; ur Rehman, Dr. I. Fourier Transform Infrared (FTIR) Spectroscopy of Biological Tissues. *Applied Spectroscopy Reviews* **2008**, 43 (2), 134–179. <https://doi.org/10.1080/05704920701829043>.

#### **4.3 Extracellular Vesicles molecular fingerprinting with Fourier Transform Infrared Spectroscopy: novel approach for monitoring Chronic Myeloid Leukemia treatment**

This chapter presents preliminary unpublished data obtained through a collaboration with Dr. Simona Bernardi at the Department of Clinical and Experimental Sciences, University of Brescia, and the CREA Laboratory (Hematological Research AIL Centre), ASST Spedali Civili di Brescia.

### 4.3.1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm that occurs from an acquired genetic alteration in the hematopoietic stem cell compartment, with an incidence of 1–2 cases per 100,000 adults, representing approximately 15% of adult leukemias<sup>1</sup>. The disease is caused by the balanced translocation t(9;22)(q34;q11.2), which generates the BCR::ABL1 fusion gene encoding a constitutively active tyrosine kinase that causes uncontrolled myeloid proliferation<sup>1,2</sup>. Thanks to advances in molecular biology and increasingly refined therapeutic strategies, CML now presents an excellent prognosis, with life expectancy approaching that of the general population<sup>3</sup>.

Indeed, the introduction of tyrosine kinase inhibitors (TKI) revolutionized the management of CML. For example, Imatinib was the first BCR::ABL1-selective inhibitor that transformed CML from a fatal leukemia into a chronic condition<sup>3,4</sup>. However, resistance and intolerance to Imatinib<sup>5</sup> led to the development of second-generation TKI, including Dasatinib<sup>6</sup>, Nilotinib<sup>7</sup>, and Bosutinib<sup>8</sup>, which induce faster and deeper molecular responses<sup>9</sup>. For patients presenting resistant mutations such as T315I, the third-generation inhibitor Ponatinib remains an essential therapeutic resource<sup>10</sup>. Despite the success of TKI, several limitations persist: leukemic stem cells are not fully eradicated, therapy requires long-term adherence, and patients may have chronic toxicities and, consequently, impaired quality of life<sup>11</sup>. Treatment-free remission (TFR) represents the main therapeutic goal, but only a small group of patients achieve molecular responses sufficient for stopping the therapy in a safe way<sup>12,13</sup>. For this reason, a non-invasive, sensitive biomarker is urgently needed to guide patient therapy by identifying molecular changes.

Liquid biopsy approaches have generated increasing attention<sup>14</sup>, in particular with circulating analytes such as Extracellular Vesicles (EVs)<sup>15,16</sup>. EVs are natural nanoparticles released by all cell types in both physiological and pathological conditions, supporting intercellular communication<sup>17</sup>. In hematological malignancies, including CML, EVs can transfer oncogenic proteins, RNA, and other bioactive molecules, contributing to disease progression, modulation of the bone marrow niche, angiogenesis, and immune escape<sup>18–22</sup>. Given their characteristic of mirroring the cell of origin, EVs represent a powerful new candidate as a biomarker for the non-invasive monitoring of disease activity and therapeutic response<sup>17</sup>.

In parallel, vibrational spectroscopies such as Fourier-transform infrared (FTIR) spectroscopy are emerging as powerful analytical tools for biomedical applications<sup>23</sup> for EV characterization also<sup>24,25</sup>. The advantages of FTIR include its rapidity, label-free operation, and minimal

invasiveness as a tool for biochemical characterization of complex biological samples<sup>26</sup>. Indeed, FTIR captures the absorption of infrared radiation associated with specific functional groups. Its sensitivity to identify biochemical variations makes it particularly suitable for capturing disease-related fingerprints and potentially assessing treatment-induced molecular changes<sup>27</sup>. Moreover, integrating spectroscopic data with multivariate statistical approaches has been shown to enhance diagnostic power and classification performance in the context of liquid biopsy<sup>28</sup>. When applied to EVs, these spectroscopic methods offer a unique opportunity to probe their molecular composition without requiring extensive purification or large sample volumes overcoming some of the limitations related to the translation of EVs into the clinical field<sup>29</sup>. One example was reported by Tânia Soares Martins and collaborators, who studied plasma- and serum-derived EVs from patients with Alzheimer's disease (AD) using FTIR spectroscopy. These analyses revealed biochemical alterations across distinct spectral regions, including carbohydrates, nucleic acids, proteins, and lipids. In particular, the discrimination between AD and healthy controls (HC) was found within the 1200-900 cm<sup>-1</sup> interval and the Amide I band by processing the peak through the calculation of the second derivative. Results highlighted the metabolic dysfunction, nucleic acid degradation, and protein aggregation typical of AD pathology, supporting their use as minimally invasive tools for early diagnosis and clinical monitoring<sup>30</sup>.

For this reason, the aim of this study is to investigate the biochemical profiles of plasma-derived EVs from CML patients under different TKI treatments by using FTIR spectroscopy combined with multivariate analysis. By characterizing the therapy-associated EV fingerprint, this work seeks to evaluate FTIR's potential as a complementary tool for disease monitoring and treatment stratification in CML.

#### **4.3.2 Methods and Materials**

##### *4.3.2.1 Subject Recruitment and Sample Collection*

Plasma samples were obtained from patients diagnosed with CML who were undergoing treatment with TKI or were in TFR. In detail, a total of 64 patients were enrolled, with a median age of 57 years (range 30-89), including 40 male patients (62.5%) and 24 female patients (37.5%). Regarding therapy, 14 patients (22%) were undergoing treatment with Imatinib, 11 patients (17%) with Dasatinib, 15 patients (23%) with Nilotinib, 5 patients (8%) with Ponatinib, 7 patients (11%) with Bosutinib, and 12 patients (19%) were in TFR. The study adhered to the principles of the Declaration of Helsinki and received approval from the Ethics Committee.

Written informed consent was obtained from all participants. Finally, all samples were anonymized prior to processing.

Briefly, 10 mL of peripheral blood was collected in EDTA tubes and centrifuged at 2,000 rpm for 10 minutes to obtain plasma. Aliquots of 1.5 mL were transferred into sterile microtubes and stored at  $-20^{\circ}\text{C}$  until EVs analysis.

#### *4.3.2.2 EV Separation*

EVs were separated using the Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Norgen Biotek, #58300), following the manufacturer's protocol and adapting it as needed. Plasma was centrifuged (2,000 rpm, 2 minutes) to remove debris. For each sample, 1.5 mL of plasma was diluted with 3 mL of nuclease-free water, then mixed with 100  $\mu\text{L}$  of ExoC Buffer and 200  $\mu\text{L}$  of Slurry E. The mix was vortexed and incubated at room temperature for 5 minutes. Then, another centrifugation at 2,000 rpm for 2 minutes was performed, and the pellet was resuspended in 200  $\mu\text{L}$  of ExoR Buffer and incubated for 5 minutes. The mix was centrifuged at 500 rpm for 2 minutes, then the supernatant was passed through a Mini Filter Spin Column and centrifuged at 6000 rpm for 1 minute. The final eluates containing 200  $\mu\text{L}$  of EVs were split into two aliquots (each from 100  $\mu\text{L}$ ) and stored at  $-20^{\circ}\text{C}$ .

#### *4.3.2.4 Bradford assay*

Total plasma and EV protein concentration was determined using the Bradford assay (BioRad), following the manufacturer's instructions. All measurements were performed in triplicate, and protein concentrations were calculated using a Bovine Serum Albumin (BSA) standard curve.

#### *4.3.2.5 SDS-PAGE and Western Blot (WB)*

SDS-PAGE and WB were performed according to standard protocols<sup>31</sup>. EVs and total plasma samples were mixed with Sample Buffer 2X (80 mM Tris, pH 6.8, 2% SDS, 7.5% glycerol, 0.01% bromophenol blue, 2% 2-mercaptoethanol), heated at  $95^{\circ}\text{C}$  for 5 min, sonicated for 20 seconds with 60% of power, and subsequently resolved by SDS-PAGE. A total of 30  $\mu\text{g}$  of proteins from both plasma and EVs (measured by Bradford assay) were loaded onto 12.5% acrylamide-bisacrylamide gels. For WB analysis, mouse anti-Flotillin-1 (FLOT-1) and mouse anti-CD63 primary antibodies were used as EV markers<sup>32</sup>, while anti-Apolipoprotein A1 (ApoA1) primary antibody was employed to evaluate potential contamination by high-density lipoproteins (HDL). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Detection was performed using appropriate HRP-conjugated secondary antibodies and a chemiluminescent substrate.

#### *4.3.4.6 Colorimetric NANoplasmonic (CONAN) Assay*

EVs were assessed for purity<sup>32</sup> and potential contaminants using the CONAN assay, a method based on the clustering of gold nanoparticles (AuNPs) onto lipid membranes, as previously described by Maiolo et al<sup>33</sup>. The assay was performed according to the published protocol<sup>34</sup>. Briefly, 2  $\mu\text{L}$  of EVs were added to 23  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , then combined with 50  $\mu\text{L}$  of a 6 nM AuNPs solution and 25  $\mu\text{L}$  of PBS. The resulting samples were incubated and analyzed using a microplate reader (Ensign Multi-Mode Reader, PerkinElmer). Absorbance measurements were carried out in triplicate for each dilution.

#### *4.3.4.7 Atomic Force Microscopy (AFM)*

AFM analysis was performed following previously established protocols<sup>35,36</sup>. Briefly, 3  $\mu\text{L}$  of EVs were spotted onto mica sheets (grade V-1, 0.15 mm thickness, 15  $\times$  15 mm) and allowed to dry at room temperature. Imaging was carried out using a Nanosurf NaioAFM instrument (Nanosurf AG) equipped with a Multi75-AI-G probe (Budget Sensors) in tapping mode. The images were acquired at a scan speed of 1 second per line.

#### *4.3.4.8 FTIR Spectroscopy*

FTIR spectroscopy was used to obtain the EV molecular fingerprints using a Cary 630 spectrometer (Agilent) operating in transmission mode, and an Attenuated Total Reflectance (ATR) diamond accessory was used. EV spectra were acquired in triplicate in the range of 4000–400  $\text{cm}^{-1}$ , and 32 scans per measurement were performed in absorbance mode. Then, post-processing, including normalization of FTIR spectra, was done using OriginPro2024b.

#### *4.3.4.9 Statistical Analysis*

FTIR spectra were analyzed by descriptive and multivariate analysis, as Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA), using OringPro2024b.

### **4.3.5 Results and Discussion**

#### *4.3.5.1 Plasma-derived EV separation and characterization*

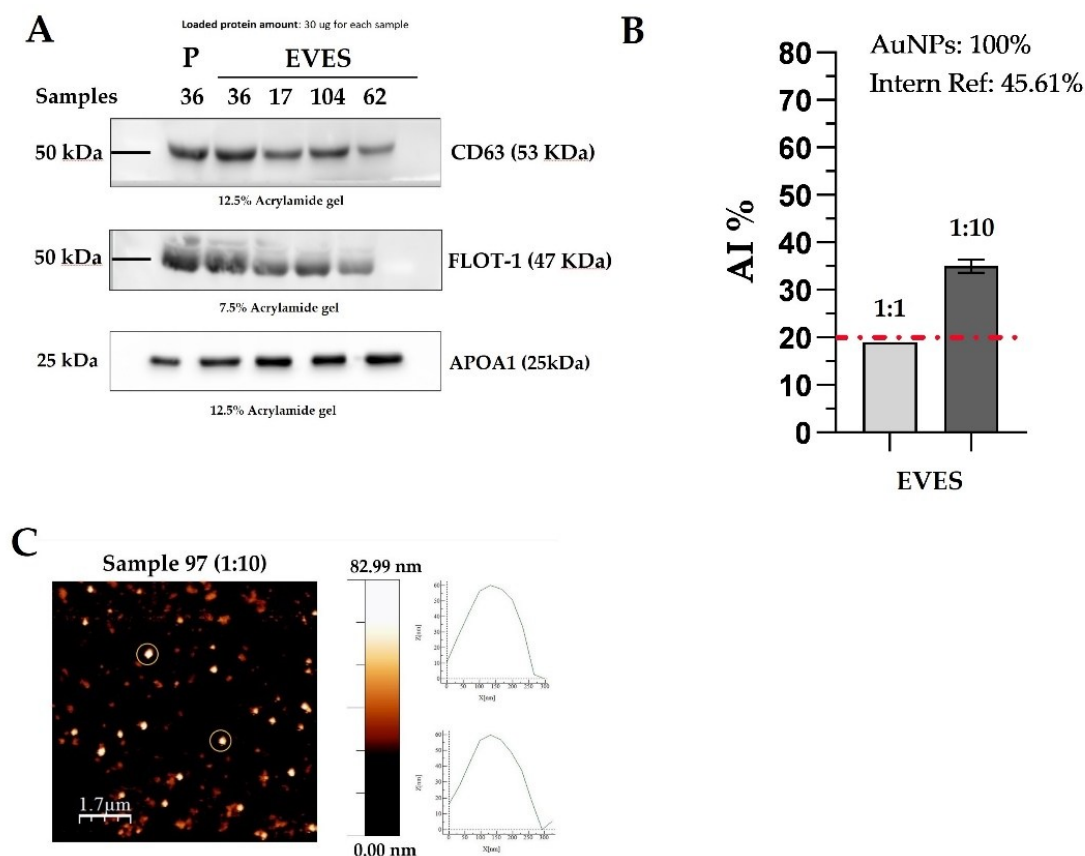
Plasma-derived EV from both patients receiving TKI therapy and patients in TFR were obtained using Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Norgen Biotek, #58300) and subsequently characterized according to MISEV2023 guidelines<sup>32</sup>.

Biochemical analysis by WB confirmed the successful enrichment of EVs, as indicated by the presence of EV markers CD63 and Flotillin-1 in 4 EV samples (36, 17, 104, and 62), as shown

in Figure 1A. Despite that, the presence of APOA1 revealed the co-isolation of HDL particles (Fig. 1A). The co-isolation with lipoprotein was expected since small EVs have a range of dimensions that corresponds to the size of HDL. Furthermore, a recent work has highlighted that EVs do not circulate as isolated entities once released into biological fluids<sup>37</sup>. Instead, they rapidly acquire a biomolecular corona, a dynamic layer of proteins, lipids, and other macromolecules that adsorb onto their surface. This nanoscale phenomenon reflects the interaction between the EV membrane and the molecular composition of the surrounding environment. In plasma, where lipoproteins are abundant and share similar biophysical properties with small EVs, components such as HDL can associate with EV membranes and become part of this corona. As a result, EV preparations may contain lipoprotein-derived signals not only due to co-isolation during size-based separation, but also because lipoproteins bind to the EV surface and contribute to their biological identity<sup>38</sup>.

To assess the EV purity, the CONAN assay was performed. EVs were tested at two dilutions (1:1, 1:10) to achieve an optimal balance between EVs and AuNPs. CONAN assay demonstrated low levels of soluble protein contaminants. Indeed, all preparations displayed Aggregation Index (AI%) values below the 20% threshold (corresponding to  $\leq 0.05 \mu\text{g}/\mu\text{L}$  soluble protein) at 1:1 dilution. These results indicate that EV samples were largely free from exogenous protein aggregates (Fig. 1B).

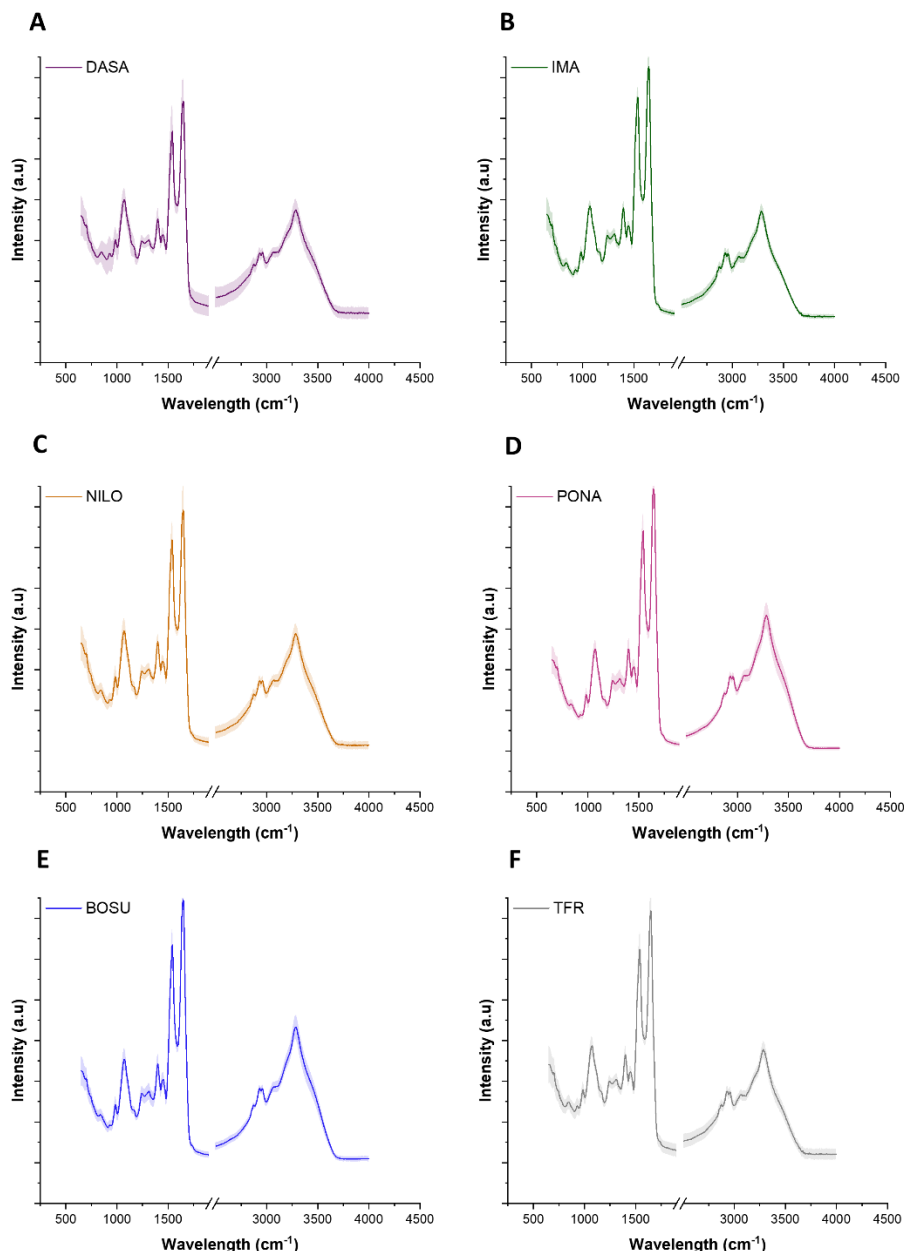
The morphological characterization and size of EVs were performed by AFM. EVs appeared as spherical objects with diameters ranging from tens to hundreds of nanometres, consistent with the expected dimensions of EV populations (Fig. 1C). Collectively, these results confirm that we obtained EV-enriched samples, accompanied by a minor presence of lipoprotein.



**Figure 1.** Characterization of plasma-derived EVs. *A*) Biochemical characterization by WB. Whole plasma and EVs (30  $\mu$ g) were assessed for the presence of EV markers CD63 and FLOT-1, and for APOA1 as a marker of lipoproteins; *B*) Evaluation of soluble protein contaminants using the CONAN assay. The graph represents AI% values for two dilutions (1:1 and 1:10) of a representative sample. The Intern. Ref. represents the internal assay control containing no sample.; *C*) Representative AFM images showing the morphology and topographical profile of EVs isolated from plasma (sample diluted 1:10), highlighting spherical nanoscale structures consistent with EVs.

#### 4.3.5.2 FTIR Analysis

FTIR analyses were performed on EV from patients with different TKI therapies (Dasatinib, Imatinib, Nilotinib, Ponatinib, and Bosutinib) and patients with TFR. 3  $\mu$ L of the EV sample was spotted onto a diamond ATR crystal and dried under nitrogen. Spectra were acquired in triplicate, and the raw data were subsequently normalized using the unit vector normalization by OriginPro2024b. The resulting spectra exhibited clear shapes and minimal background noise as seen from the mean spectrum ( $\pm 1$  standard deviation) in figures 2A,B,C,D,E,F.



**Figure 2.** Mean spectra of the six experimental groups. A) Mean spectrum of patients with Dasatinib (DASA) ( $\pm 1$  standard deviation); B) Mean spectrum of patients with Imatinib (IMA) ( $\pm 1$  standard deviation); C) Mean spectrum of patients with Nilotinib (NILO) ( $\pm 1$  standard deviation); D) Mean spectrum of patients with Ponatinib (PONA) ( $\pm 1$  standard deviation) E) Mean spectrum of patients with Bosutinib (BOSU) ( $\pm 1$  standard deviation); F) Mean spectrum of patients with TFR ( $\pm 1$  standard deviation).

To better highlight differences between the experimental groups, all the mean spectra were overlapped. As shown in Figure 3A, although the variations were not immediately apparent, the spectra encompassed signals from the major EV biomolecular components, including proteins, lipids, nucleic acids, and carbohydrates. Prominent features included the Amide I

(~1650  $\text{cm}^{-1}$ ) and Amide II (~1540  $\text{cm}^{-1}$ ) bands, indicative of protein secondary structures, as well as the  $\text{CH}_2/\text{CH}_3$  stretching region (2850–2950  $\text{cm}^{-1}$ ), associated with membrane lipids. Furthermore, a bond at 1738  $\text{cm}^{-1}$  is related to  $\text{C}=\text{O}$  stretching of lipid content, in particular in biological samples, to indicate certain phospholipids. Additional contributions were detected between 1000 and 1200  $\text{cm}^{-1}$ , a region typically assigned to carbohydrates and phosphate-containing species.

Particularly, subtle variations in peak intensity were detectable in specific spectral regions, for example, within the Amide I and Amide II bands (~1650 and ~1540  $\text{cm}^{-1}$ ) and in the lipid-associated  $\text{CH}_2/\text{CH}_3$  stretching region (2850–2950  $\text{cm}^{-1}$ )<sup>39,40</sup>. These localized changes may reflect treatment-dependent shifts in the protein and lipid composition of EVs. These differences could suggest that the EV biochemical profile may be influenced by the particular TKI administered, serving as an indicator of disease status or therapeutic response.

Because FTIR spectra are derived from different molecules present in the sample under analysis, the relevant differences between samples are not immediately visible in the spectrum. To overcome this problem, the multivariate analysis was applied. In this way, it was possible to reduce the data that captures the main sources of biochemical variance. Indeed, algorithms can isolate combinations of spectral features that best differentiate the experimental groups. Minimal shifts in peak intensity or shape are integrated into discriminative components that allow classification of patients with different types of treatment on their fingerprint. In particular, Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) were used. The PCA-LDA model demonstrated that different patient groups, with distinct TKI or in TFR, presented distinct EV FTIR spectra biochemical fingerprints related to specific treatments, supporting predictive classification (Fig. 3B).

Figure 3B illustrates the performance and limitations of the PCA-LDA model, showing the distribution of spectra from the different experimental groups (DASA, IMA, NILO, TFR, PONA, and BOSU). Each bar corresponds to one predicted group, while the colored band indicates the true group membership of the samples.

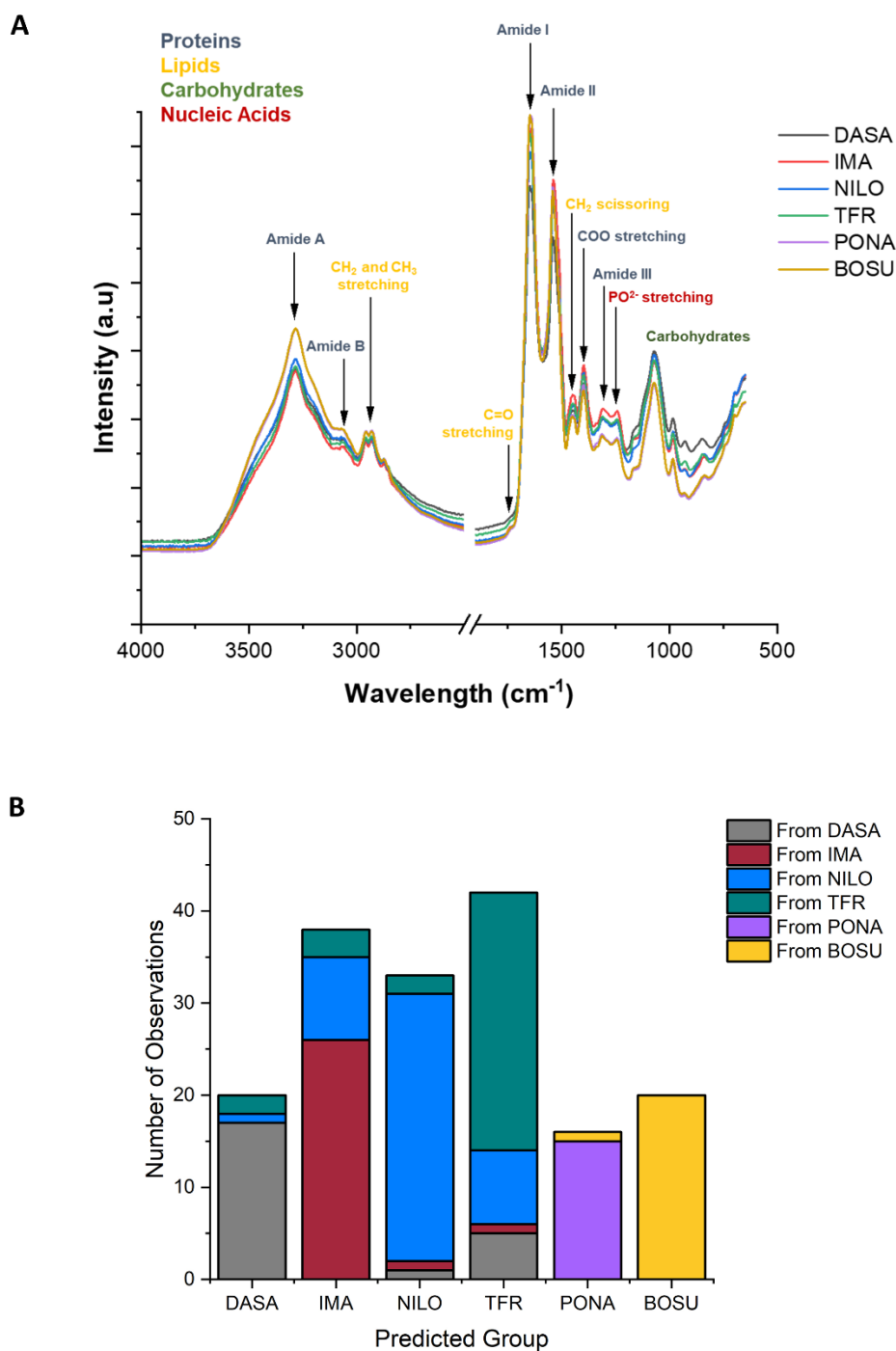
Overall, some therapeutic groups exhibit clearer classification than others, whereas several classes show a degree of overlap that reflects the intrinsic biochemical similarity of their EV FTIR spectra. For example, samples from patients receiving Bosutinib (BOSU) tend to cluster predominantly within the BOSU-predicted bar, suggesting that their EV profiles exhibit distinctive features detectable by the model. In contrast, patients with Nilotinib (NILO) treatment showed substantial cross-assignments, indicating that their FTIR signatures are more

difficult to discriminate and may share overlapping biochemical characteristics that can reflect similarities in drug mechanism or downstream molecular effects.

Taken together, EV FTIR spectra can distinguish biochemical information related to treatment, enabling partial but not complete discrimination between patients on TKI treatment and those in TFR. The observed misclassifications highlight the complexity of the dataset and the need for a larger cohort of patients. Nonetheless, the overall pattern indicates that multivariate chemometric approaches can indeed capture clinically relevant spectral trends associated with different therapeutic regimens, achieving an accuracy of 61% and good sensitivities and specificities in recognizing treatments, as described in Table 1.

**Table 1.** The table reports the sensitivity and specificity (%) for each experimental group, calculated from a confusion matrix generated by the PCA-LDA model.

| <b>Experimental groups</b> | <b>Sensitivity (%)</b> | <b>Specificity (%)</b> |
|----------------------------|------------------------|------------------------|
| Dasatinib                  | 43.5                   | 91.1                   |
| Imatinib                   | 71.4                   | 94.3                   |
| Nilotinib                  | 51.1                   | 86.1                   |
| TFR                        | 57.1                   | 88.8                   |
| Ponatinib                  | 60.0                   | 94.2                   |
| Bosutinib                  | 81.0                   | 98.6                   |



**Figure 3.** A) Overlapped spectra between the six experimental groups with arrows that indicate the specific chemical bond related to lipid, nucleic acids, carbohydrates, and proteins; B) Stacked bar plot illustrating the results of the PCA-LDA model performed on EV FTIR spectra. The x-axis represents the model-predicted groups (Dasatinib, Imatinib, Nilotinib, TFR, Ponatinib, and Bosutinib), while the y-axis shows the number of samples assigned to each group. Each bar is segmented and color-coded by the true group of the samples, supporting classification visualization.

### 4.3.6 Conclusion and Future Perspective

In this study, we successfully separated and characterized plasma-derived EVs from patients with different TKI treatments and patients in TFR. Biochemical, morphological, and purity analysis confirmed the enrichment of EVs, with a contribution from co-isolated lipoproteins, consistent with their similar size in EVs<sup>32</sup> and the formation of a biomolecular corona in plasma<sup>38</sup>. FTIR spectroscopy revealed distinct biochemical signatures associated with EV reflecting their protein, lipid, nucleic acid, and carbohydrate content.

Multivariate analyses using a PCA-LDA classification model demonstrated that EV FTIR spectra can partially discriminate between patients receiving different TKI treatments and those in TFR. The partial misclassification observed underscores the complexity of EV biochemical heterogeneity and the influence of overlapping molecular features among treatments. Despite this, the PCA-LDA model achieved an overall classification accuracy of 61%, with variable sensitivity and specificity across treatment groups.

These findings highlight the potential of EV FTIR profiling, combined with multivariate analysis, as a non-invasive approach to monitor treatment type in patients with CML.

Future studies should expand patient cohorts to improve the robustness of predictive models. Integration of complementary omics approaches, such as proteomics and lipidomics, could enhance the resolution of EV biochemical fingerprints and provide mechanistic insights into treatment-specific effects<sup>41</sup>. Moreover, the implementation of artificial intelligence (AI) and machine learning (ML) tools could significantly optimize both data analysis and interpretation<sup>42</sup>. These approaches are particularly promising for non-expert users, as they can simplify the processing of EV FTIR spectra, automate characteristic extraction, and optimize classification models. By reducing the technical complexity of spectral acquisition and model implementation, AI/ML could facilitate the standardization and scalability of EV FTIR profiling. This advancement would support the future clinical translation of this technology for the non-invasive monitoring of therapeutic response in patients with TKI treatments or in TFR, ultimately enabling more accessible and accurate patient stratification based on EV-derived biochemical fingerprint.

In summary, this work demonstrates the feasibility of using EV FTIR spectra as a minimally invasive tool to capture clinically relevant biochemical information, paving the way for future applications in patient stratification and therapy monitoring.

### 4.3.7 References

- (1) Jabbour, E.; Kantarjian, H. Chronic Myeloid Leukemia: 2025 Update on Diagnosis, Therapy, and Monitoring. *American Journal of Hematology* **2024**, *99* (11), 2191–2212. <https://doi.org/10.1002/ajh.27443>.
- (2) Perrotti, D.; Jamieson, C.; Goldman, J.; Skorski, T. Chronic Myeloid Leukemia: Mechanisms of Blastic Transformation. *J Clin Invest* **2010**, *120* (7), 2254–2264. <https://doi.org/10.1172/JCI41246>.
- (3) Iezza, M.; Cortesi, S.; Ottaviani, E.; Mancini, M.; Venturi, C.; Monaldi, C.; De Santis, S.; Testoni, N.; Soverini, S.; Rosti, G.; Cavo, M.; Castagnetti, F. Prognosis in Chronic Myeloid Leukemia: Baseline Factors, Dynamic Risk Assessment and Novel Insights. *Cells* **2023**, *12* (13), 1703. <https://doi.org/10.3390/cells12131703>.
- (4) Bower, H.; Björkholm, M.; Dickman, P. W.; Höglund, M.; Lambert, P. C.; Andersson, T. M.-L. Life Expectancy of Patients With Chronic Myeloid Leukemia Approaches the Life Expectancy of the General Population. *J Clin Oncol* **2016**, *34* (24), 2851–2857. <https://doi.org/10.1200/JCO.2015.66.2866>.
- (5) Ottmann, O. G.; Druker, B. J.; Sawyers, C. L.; Goldman, J. M.; Reiffers, J.; Silver, R. T.; Tura, S.; Fischer, T.; Deininger, M. W.; Schiffer, C. A.; Baccarani, M.; Gratwohl, A.; Hochhaus, A.; Hoelzer, D.; Fernandes-Reese, S.; Gathmann, I.; Capdeville, R.; O'Brien, S. G. A Phase 2 Study of Imatinib in Patients with Relapsed or Refractory Philadelphia Chromosome-Positive Acute Lymphoid Leukemias. *Blood* **2002**, *100* (6), 1965–1971. <https://doi.org/10.1182/blood-2001-12-0181>.
- (6) Shah, N. P.; Kim, D.-W.; Kantarjian, H.; Rousselot, P.; Llacer, P. E. D.; Enrico, A.; Vela-Ojeda, J.; Silver, R. T.; Khoury, H. J.; Müller, M. C.; Lambert, A.; Matloub, Y.; Hochhaus, A. Potent, Transient Inhibition of BCR-ABL with Dasatinib 100 Mg Daily Achieves Rapid and Durable Cytogenetic Responses and High Transformation-Free Survival Rates in Chronic Phase Chronic Myeloid Leukemia Patients with Resistance, Suboptimal Response or Intolerance to Imatinib. *Haematologica* **2010**, *95* (2), 232–240. <https://doi.org/10.3324/haematol.2009.011452>.
- (7) Kantarjian, H. M.; Giles, F.; Gattermann, N.; Bhalla, K.; Alimena, G.; Palandri, F.; Ossenkoppele, G. J.; Nicolini, F.-E.; O'Brien, S. G.; Litzow, M.; Bhatia, R.; Cervantes, F.; Haque, A.; Shou, Y.; Resta, D. J.; Weitzman, A.; Hochhaus, A.; Le Coutre, P. Nilotinib (Formerly AMN107), a Highly Selective BCR-ABL Tyrosine Kinase Inhibitor, Is Effective in Patients with Philadelphia Chromosome-Positive Chronic Myelogenous Leukemia in Chronic Phase Following Imatinib Resistance and Intolerance. *Blood* **2007**, *110* (10), 3540–3546. <https://doi.org/10.1182/blood-2007-03-080689>.
- (8) Smith, B. D.; Brümmendorf, T. H.; Roboz, G. J.; Gambacorti-Passerini, C.; Charbonnier, A.; Viqueira, A.; Leip, E.; Purcell, S.; Goldman, E. H.; Giles, F.; Ernst, T.; Hochhaus, A.; Rosti, G. Efficacy and Safety of Bosutinib in Patients Treated with Prior Imatinib and/or Dasatinib and/or Nilotinib: Subgroup Analyses from the Phase 4 BYOND Study. *Leukemia Research* **2024**, *139*, 107481. <https://doi.org/10.1016/j.leukres.2024.107481>.
- (9) Hossain, Md. I.; Khalil, I.; Islam, M. R.; Kulsum, U.; Eva, I. I.; Das Joy, A.; Sayed, M. A.; Bilal, E.; Zeima, Z.; Monsur, S.; Rakib, Md. M. A.; Tarannum, S. T.; Emon, Md. Y. A.; Habib, M. A. Comparative Effectiveness of First, Second, and Third-Generation Tyrosine Kinase Inhibitors in Chronic Myeloid Leukemia: A Systematic Review and Frequentist Network Meta-Analysis. *J Clin Oncol* **2025**, *43* (16\_suppl), e18564–e18564. [https://doi.org/10.1200/JCO.2025.43.16\\_suppl.e18564](https://doi.org/10.1200/JCO.2025.43.16_suppl.e18564).
- (10) Kantarjian, H. M.; Jabbour, E.; Deininger, M.; Abruzzese, E.; Apperley, J.; Cortes, J.; Chuah, C.; DeAngelo, D. J.; DiPersio, J.; Hochhaus, A.; Lipton, J.; Nicolini, F. E.; Pinilla-Ibarz, J.; Rea, D.; Rosti, G.; Rousselot, P.; Shah, N. P.; Talpaz, M.; Srivastava, S.; Ren,

- X.; Mauro, M. Ponatinib after Failure of Second-Generation Tyrosine Kinase Inhibitor in Resistant Chronic-Phase Chronic Myeloid Leukemia. *American Journal of Hematology* **2022**, *97* (11), 1419–1426. <https://doi.org/10.1002/ajh.26686>.
- (11) Flynn, K. E.; Atallah, E. Quality of Life and Long-Term Therapy in Patients with Chronic Myeloid Leukemia. *Curr Hematol Malig Rep* **2016**, *11* (2), 80–85. <https://doi.org/10.1007/s11899-016-0306-5>.
  - (12) Laganà, A.; Scalzulli, E.; Carmosino, I.; Bisegna, M. L.; Ielo, C.; Diverio, D.; Martelli, M.; Breccia, M. Treatment Free Remission (TFR) in Chronic Phase-Chronic Myeloid Leukemia (CP-CML): Analysis of Predictive Factors and Novel Baseline Scoring System to Predict Molecular Relapse. *European Journal of Haematology* **2025**, *115* (4), 367–379. <https://doi.org/10.1111/ejh.70006>.
  - (13) Sauße, S.; Richter, J.; Hochhaus, A.; Mahon, F.-X. The Concept of Treatment-Free Remission in Chronic Myeloid Leukemia. *Leukemia* **2016**, *30* (8), 1638–1647. <https://doi.org/10.1038/leu.2016.115>.
  - (14) Hollanda, C. N.; Gualberto, A. C. M.; Motoyama, A. B.; Pittella-Silva, F. Advancing Leukemia Management Through Liquid Biopsy: Insights into Biomarkers and Clinical Utility. *Cancers* **2025**, *17* (9), 1438. <https://doi.org/10.3390/cancers17091438>.
  - (15) Liang, Y.; Leirich, B. M.; Zheng, S.; Lu, M. Emerging Methods in Biomarker Identification for Extracellular Vesicle-Based Liquid Biopsy. *Journal of Extracellular Vesicles* **2021**, *10* (7), e12090. <https://doi.org/10.1002/jev2.12090>.
  - (16) Trino, S.; Lamorte, D.; Caivano, A.; De Luca, L.; Sgambato, A.; Laurenzana, I. Clinical Relevance of Extracellular Vesicles in Hematological Neoplasms: From Liquid Biopsy to Cell Biopsy. *Leukemia* **2021**, *35* (3), 661–678. <https://doi.org/10.1038/s41375-020-01104-1>.
  - (17) Hallal, S.; Túzesi, Á.; Grau, G. E.; Buckland, M. E.; Alexander, K. L. Understanding the Extracellular Vesicle Surface for Clinical Molecular Biology. *Journal of Extracellular Vesicles* **2022**, *11* (10), e12260. <https://doi.org/10.1002/jev2.12260>.
  - (18) Raimondo, S.; Saieva, L.; Corrado, C.; Fontana, S.; Flugey, A.; Rizzo, A.; De Leo, G.; Alessandro, R. Chronic Myeloid Leukemia-Derived Exosomes Promote Tumor Growth through an Autocrine Mechanism. *Cell Communication and Signaling* **2015**, *13* (1), 8. <https://doi.org/10.1186/s12964-015-0086-x>.
  - (19) Taverna, S.; Flugey, A.; Saieva, L.; Kohn, E. C.; Santoro, A.; Meraviglia, S.; De Leo, G.; Alessandro, R. Role of Exosomes Released by Chronic Myelogenous Leukemia Cells in Angiogenesis. *International Journal of Cancer* **2012**, *130* (9), 2033–2043. <https://doi.org/10.1002/ijc.26217>.
  - (20) Gao, X.; Wan, Z.; Wei, M.; Dong, Y.; Zhao, Y.; Chen, X.; Li, Z.; Qin, W.; Yang, G.; Liu, L. Chronic Myelogenous Leukemia Cells Remodel the Bone Marrow Niche via Exosome-Mediated Transfer of miR-320. *Theranostics* **2019**, *9* (19), 5642–5656. <https://doi.org/10.7150/thno.34813>.
  - (21) Jiang, Y.-H.; Liu, J.; Lin, J.; Li, S.-Q.; Xu, Y.-M.; Min, Q.-H.; Zhong, Q.-H.; Sun, F.; Li, J.; You, X.-H.; Liao, K.-L.; Qin, T.-Y.; Zhao, C.; Huang, B.; Wang, X.-Z. K562 Cell-Derived Exosomes Suppress the Adhesive Function of Bone Marrow Mesenchymal Stem Cells via Delivery of miR-711. *Biochem Biophys Res Commun* **2020**, *521* (3), 584–589. <https://doi.org/10.1016/j.bbrc.2019.10.096>.
  - (22) Jafarzadeh, N.; Gholampour, M. A.; Alivand, M.-R.; Kavousi, S.; Arzi, L.; Rad, F.; Sadeghizadeh, M.; Pornour, M. CML Derived Exosomes Promote Tumor Favorable Functional Performance in T Cells. *BMC Cancer* **2021**, *21* (1), 1002. <https://doi.org/10.1186/s12885-021-08734-3>.
  - (23) Fadlilmoula, A.; Pinho, D.; Carvalho, V. H.; Catarino, S. O.; Minas, G. Fourier Transform Infrared (FTIR) Spectroscopy to Analyse Human Blood over the Last 20 Years: A Review

- towards Lab-on-a-Chip Devices. *Micromachines* **2022**, *13* (2), 187. <https://doi.org/10.3390/mi13020187>.
- (24) Di Santo, R.; Vaccaro, M.; Romanò, S.; Di Giacinto, F.; Papi, M.; Rapaccini, G. L.; De Spirito, M.; Miele, L.; Basile, U.; Ciasca, G. Machine Learning-Assisted FTIR Analysis of Circulating Extracellular Vesicles for Cancer Liquid Biopsy. *Journal of Personalized Medicine* **2022**, *12* (6), 949. <https://doi.org/10.3390/jpm12060949>.
  - (25) Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Rodà, F.; Bedoni, M. Biophotonics for Diagnostic Detection of Extracellular Vesicles. *Advanced Drug Delivery Reviews* **2021**, *174*, 229–249. <https://doi.org/10.1016/j.addr.2021.04.014>.
  - (26) Al-Kelani, M.; Buthelezi, N. Advancements in Medical Research: Exploring Fourier Transform Infrared (FTIR) Spectroscopy for Tissue, Cell, and Hair Sample Analysis. *Skin Res Technol* **2024**, *30* (6), e13733. <https://doi.org/10.1111/srt.13733>.
  - (27) Grdadolnik, J. ATR-FTIR SPECTROSCOPY: ITS ADVANTAGES AND LIMITATIONS. *Acta Chim. Slov.* **2002**.
  - (28) Morais, C. L. M.; Lima, K. M. G.; Singh, M.; Martin, F. L. Tutorial: Multivariate Classification for Vibrational Spectroscopy in Biological Samples. *Nat Protoc* **2020**, *15* (7), 2143–2162. <https://doi.org/10.1038/s41596-020-0322-8>.
  - (29) Gandham, S.; Su, X.; Wood, J.; Nocera, A. L.; Alli, S. C.; Milane, L.; Zimmerman, A.; Amiji, M.; Ivanov, A. R. Technologies and Standardization in Research on Extracellular Vesicles. *Trends Biotechnol* **2020**, *38* (10), 1066–1098. <https://doi.org/10.1016/j.tibtech.2020.05.012>.
  - (30) Soares Martins, T.; Ferreira, M.; Magalhães, S.; Leandro, K.; de Almeida, L. P.; Vogelgsang, J.; Breitling, B.; Hansen, N.; Esselmann, H.; Wiltfang, J.; da Cruz e Silva, O. A. B.; Nunes, A.; Henriques, A. G. FTIR Spectroscopy and Blood-Derived Extracellular Vesicles Duo in Alzheimer's Disease. *J Alzheimers Dis* **98** (3), 1157–1167. <https://doi.org/10.3233/JAD-231239>.
  - (31) Salvi, A.; Vezzoli, M.; Busatto, S.; Paolini, L.; Faranda, T.; Abeni, E.; Caracausi, M.; Antonaros, F.; Piovesan, A.; Locatelli, C.; Cocchi, G.; Alvisi, G.; De Petro, G.; Ricotta, D.; Bergese, P.; Radeghieri, A. Analysis of a Nanoparticle-enriched Fraction of Plasma Reveals miRNA Candidates for Down Syndrome Pathogenesis. *Int J Mol Med* **2019**, *43* (6), 2303–2318. <https://doi.org/10.3892/ijmm.2019.4158>.
  - (32) Welsh, J. A.; Goberdhan, D. C. I.; O'Driscoll, L.; Buzas, E. I.; Blenkiron, C.; Bussolati, B.; Cai, H.; Di Vizio, D.; Driedonks, T. A. P.; Erdbrügger, U.; Falcon-Perez, J. M.; Fu, Q.-L.; Hill, A. F.; Lenassi, M.; Lim, S. K.; Mahoney, M. G.; Mohanty, S.; Möller, A.; Nieuwland, R.; Ochiya, T.; Sahoo, S.; Torrecilhas, A. C.; Zheng, L.; Zijlstra, A.; Abuelreich, S.; Bagabas, R.; Bergese, P.; Bridges, E. M.; Brucale, M.; Burger, D.; Carney, R. P.; Cocucci, E.; Crescitelli, R.; Hanser, E.; Harris, A. L.; Haughey, N. J.; Hendrix, A.; Ivanov, A. R.; Jovanovic-Talisman, T.; Kruh-Garcia, N. A.; Ku'ulei-Lyn Faustino, V.; Kyburz, D.; Lässer, C.; Lennon, K. M.; Lötvall, J.; Maddox, A. L.; Martens-Uzunova, E. S.; Mizenko, R. R.; Newman, L. A.; Ridolfi, A.; Rohde, E.; Rojalin, T.; Rowland, A.; Saftics, A.; Sandau, U. S.; Saugstad, J. A.; Shekari, F.; Swift, S.; Ter-Ovanesyan, D.; Tosar, J. P.; Useckaite, Z.; Valle, F.; Varga, Z.; van der Pol, E.; van Herwijnen, M. J. C.; Wauben, M. H. M.; Wehman, A. M.; Williams, S.; Zendrini, A.; Zimmerman, A. J.; MISEV Consortium; Théry, C.; Witwer, K. W. Minimal Information for Studies of Extracellular Vesicles (MISEV2023): From Basic to Advanced Approaches. *J Extracell Vesicles* **2024**, *13* (2), e12404. <https://doi.org/10.1002/jev2.12404>.
  - (33) Maiolo, D.; Paolini, L.; Di Noto, G.; Zendrini, A.; Berti, D.; Bergese, P.; Ricotta, D. Colorimetric Nanoplasmonic Assay to Determine Purity and Titrate Extracellular Vesicles. *Anal Chem* **2015**, *87* (8), 4168–4176. <https://doi.org/10.1021/ac504861d>.

- (34) Zendrini, A.; Paolini, L.; Busatto, S.; Radeghieri, A.; Romano, M.; Wauben, M. H. M.; van Herwijnen, M. J. C.; Nejsum, P.; Borup, A.; Ridolfi, A.; Montis, C.; Bergese, P. Augmented Colorimetric NANoplasmonic (CONAN) Method for Grading Purity and Determine Concentration of EV Microliter Volume Solutions. *Frontiers in Bioengineering and Biotechnology* **2020**, *7*.
- (35) Paolini, L.; Zendrini, A.; Noto, G. D.; Busatto, S.; Lottini, E.; Radeghieri, A.; Dossi, A.; Caneschi, A.; Ricotta, D.; Bergese, P. Residual Matrix from Different Separation Techniques Impacts Exosome Biological Activity. *Sci Rep* **2016**, *6* (1), 23550. <https://doi.org/10.1038/srep23550>.
- (36) Radeghieri, A.; Savio, G.; Zendrini, A.; Di Noto, G.; Salvi, A.; Bergese, P.; Piovani, G. Cultured Human Amniocytes Express hTERT, Which Is Distributed between Nucleus and Cytoplasm and Is Secreted in Extracellular Vesicles. *Biochemical and Biophysical Research Communications* **2017**, *483* (1), 706–711. <https://doi.org/10.1016/j.bbrc.2016.12.077>.
- (37) Musicò, A.; Zendrini, A.; Reyes, S. G.; Mangolini, V.; Paolini, L.; Romano, M.; Papait, A.; Silini, A. R.; Gianvincenzo, P. D.; Neva, A.; Cretich, M.; Parolini, O.; Almici, C.; Moya, S. E.; Radeghieri, A.; Bergese, P. Extracellular Vesicles of Different Cellular Origin Feature Distinct Biomolecular Corona Dynamics. *Nanoscale Horiz.* **2024**, *10* (1), 104–112. <https://doi.org/10.1039/D4NH00320A>.
- (38) Tassoni, S.; Bergese, P.; Radeghieri, A. The Extracellular Vesicle Biomolecular Corona: Current Insights and Diagnostic Potential. *Nanomedicine* **2025**, *20* (16), 2013–2021. <https://doi.org/10.1080/17435889.2025.2538426>.
- (39) Wong, L.-W.; Mak, S.-H.; Goh, B.-H.; Lee, W.-L. The Convergence of FTIR and EVs: Emergence Strategy for Non-Invasive Cancer Markers Discovery. *Diagnostics (Basel)* **2022**, *13* (1), 22. <https://doi.org/10.3390/diagnostics13010022>.
- (40) Movasaghi, Z.; Rehman, S.; ur Rehman, Dr. I. Fourier Transform Infrared (FTIR) Spectroscopy of Biological Tissues. *Applied Spectroscopy Reviews* **2008**, *43* (2), 134–179. <https://doi.org/10.1080/05704920701829043>.
- (41) Kasprzyk-Pochopień, J.; Kamińska, A.; Mielczarek, P.; Porada, R.; Stepień, E.; Piekoszewski, W. The Proteomic Analysis of Platelet Extracellular Vesicles in Diabetic Patients by nanoLC-MALDI-MS/MS and nanoLC-TIMS-MS/MS. *Molecules* **2025**, *30* (6), 1384. <https://doi.org/10.3390/molecules30061384>.
- (42) Di Santo, R.; Vaccaro, M.; Romanò, S.; Di Giacinto, F.; Papi, M.; Rapaccini, G. L.; De Spirito, M.; Miele, L.; Basile, U.; Ciasca, G. Machine Learning-Assisted FTIR Analysis of Circulating Extracellular Vesicles for Cancer Liquid Biopsy. *Journal of Personalized Medicine* **2022**, *12* (6), 949. <https://doi.org/10.3390/jpm12060949>.

#### **4.4 Circulating particles carotenoids are associated with rehabilitation recovery in Parkinson's disease**

This chapter is related to the published study carried out at the Laboratory of Nanomedicine and Clinical Biophotonics (LABION): Alice Gualerzi, Martina Gerli, Aurora Mangolini, Silvia Picciolini, Francesca Rodà, **Valentina Mangolini**, Stefano Doronzio, Diego Longo, Giulio Cherubini, Cristina Polito, Chiara De Santis, Silvia Ramat, Francesca Cecchi, Gemma Lombardi, Marzia Bedoni (2025). The title of this work is: “Circulating particles carotenoids are associated with rehabilitation recovery in Parkinson's disease,” published on Redox Biology with open access.

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The present work describes a study exploring the use of Raman Spectroscopy (RS) to identify biomarkers of neuroplasticity and brain remodelling in people with Parkinson's Disease (pwPD). The focus is on the beneficial effects of an intensive motor rehabilitation program, as detected by changes in EV-like particles. The work was conducted within the VIRTREAD-PD randomized clinical trial (ClinicalTrials.gov ID: NCT05902065), which compares an intensive treadmill training (TT) rehabilitative program with and without augmented virtual reality (AVR) in pwPD with gait disturbances.

Serum samples were collected before (T0) and after treatment (T1), and EV-like particles were isolated using a standardized protocol adapted for Raman analysis.

RS revealed distinct biochemical signatures at T0 and T1, with the most prominent change involving peaks associated with carotenoids (antioxidant molecules linked to redox status). These spectral variations were also strongly correlated with major clinical scales of disability profile. In particular, higher carotenoid Raman peaks were associated with worse motor performance, suggesting that an elevated carotenoid signature in EV-like particles reflects a redox-stress burden that correlates with more severe functional impairment.

Importantly, carotenoid levels at T0 were prognostic. Indeed, patients with higher carotenoids at T0 demonstrated poorer rehabilitation outcomes, indicating that this Raman biomarker can identify responders and non-responders to the rehabilitation program. This highlights the potential of circulating EV-like particles as predictive biomarkers to guide personalized rehabilitation strategies.

Together, these results demonstrate that RS is sensitive to molecular changes in EV-like particles at T0 and T1. Indeed, focusing on the reproducible association between carotenoid signatures, clinical scales of motor disability, and rehabilitation outcomes, this approach

supports the EV-like particles' Raman signature as a minimally invasive tool for patient stratification based on the identification of specific biomarkers (“rehabilomics”). Finally, this approach holds promise for personalized rehabilitation strategies and improving the clinical management of Parkinson’s disease.

For a detailed description of the study design, methods, results, and discussion, the full published article is provided as a PDF at the end of this thesis.

## CHAPTER 5 – CONCLUSIONS AND PERSPECTIVES

This thesis demonstrated how vibrational spectroscopies and NMR can serve as powerful analytical tools for the study of biofluids and their EV fractions. By applying these techniques to saliva, plasma, and serum, the work showed that spectroscopies can capture molecular and mesoscale differences that reflect underlying pathological conditions.

Despite the complexity of these matrices and current limitations in their clinical translation, the results presented here highlight the ability of spectroscopies, particularly RS and FTIR, to generate reproducible biochemical signatures that support both diagnostic evaluation and the monitoring of therapeutic or rehabilitative responses. Indeed, these results provide a step toward overcoming existing methodological barriers and reinforce the potential of spectroscopies applied to biofluids and their EV fractions for profiling in future precision medicine applications.

Importantly, the results collectively highlight that no single biofluid or analytical technique can be considered universally optimal. Instead, the findings support a complementary and multiscale strategy, in which the choice of biofluid, analytical method, and scale of investigation must be tailored to the specific clinical question. Saliva emerged as a particularly attractive matrix for minimally invasive diagnostics and longitudinal monitoring, whereas plasma and serum provided broader systemic information, especially when combined with EV analysis.

Despite the promising results, this work also emphasizes several limitations that currently hinder the full clinical translation of spectroscopic approaches. These include the intrinsic biological variability of biofluids, the complexity of EV isolation and characterization, and the need for standardized pre-analytical and analytical workflows. Moreover, while vibrational spectroscopies provide highly reproducible molecular fingerprints, their biological interpretation often requires integration with complementary techniques and advanced data analysis strategies.

Indeed, the experimental evidence collected throughout this thesis confirms the clinical relevance of both saliva and its EV fractions. In particular, the work on salivary diagnostics demonstrated that this minimally invasive biofluid can capture biochemical markers of disease with high reproducibility, supporting its use for respiratory disorders such as COPD, asthma, and OSA. This is illustrated by the development of a patented Raman-based workflow that demonstrates the feasibility of translating spectroscopic fingerprints into diagnostic tools.

Similarly, analyses of plasma and serum, and their EV fractions, showed that vibrational spectroscopy and NMR can detect molecular and mesoscale changes associated with pathological processes. Although NMR analyses in this thesis were performed exclusively on whole plasma, ongoing experiments now extend this approach to EVs. In particular, an optimized protocol has been established for metabolomics of EVs, opening the possibility of integrating NMR at the mesoscale level in future studies.

This aspect further supports the multiscale perspective of this work, in which vibrational spectroscopies and NMR provide complementary information spanning molecular composition and mesoscale organization.

By revealing signatures associated with disease, therapeutic treatments, and rehabilitation outcomes, these studies expand the applicability of spectroscopies beyond early detection, positioning them as valuable tools for patient monitoring and stratification in complex clinical contexts.

For these reasons, a near-future perspective involves integrating artificial intelligence (AI) and machine learning (ML) into the multiscale analytical workflow, representing a step toward greater accuracy, robustness, and interpretability. Indeed, by leveraging advanced algorithms, spectral fingerprints can be directly translated into clinical outputs, enabling automated classification and supporting decision-making. Consequently, combining AI and ML with Raman and FTIR analyses could improve accessibility and reproducibility across clinical settings.

Moreover, future research could also focus on the multimodal integration of vibrational spectroscopy with other (biorthogonal) techniques such as NMR, NTA, AFM, lipidomics, and metabolomics. Combining these heterogeneous datasets using AI-driven fusion strategies would enable the reconstruction of comprehensive molecular and mesoscale signatures that can identify specific patterns related to the diseases, even in conditions lacking established biomarkers. Therefore, this multiscale approach could provide a powerful pipeline for improving diagnostic accuracy and advancing precision medicine, particularly in rare diseases or those for which no early-diagnosis biomarker currently exists.

To do this, a large-scale study across different cohorts will be necessary to confirm the diagnostic and monitoring performance of the identified spectral biomarkers. Such studies will help define clinically relevant thresholds, assess inter-individual variability, and establish the robustness of models in real-world scenarios.

Considering the evidence discussed above, the most plausible route to translating these technologies into clinical practice may involve using Raman, FTIR, and SERS platforms for

point-of-care applications. In particular, among biofluids, saliva is most suitable, as it can be collected easily, non-invasively, and at low cost. Portable devices, when combined with AI-driven analysis, could enable rapid bedside or pharmacy screening, supporting primary clinicians, and provide specialists with fast spectral reports to guide diagnosis and treatment. This evolution would allow continuous and repeated assessment of patients, facilitating early detection of disease exacerbations, evaluation of therapy adherence, and monitoring of rehabilitation outcomes.

In summary, this thesis demonstrates that biofluids and their EV fractions, when combined with vibrational spectroscopies, hold strong potential for addressing unmet clinical needs. These approaches pave the way for timelier diagnosis, improved monitoring of therapeutic response, and ultimately, more personalized patient management.

## CHAPTER 6 – APPENDIX

In addition to the main research presented in this PhD thesis, I contributed to several side projects. The following sections summarize the main scientific contributions of each work, highlighting their goals, results, and my specific role within the study.

### **Raman spectroscopy characterization of Multi-Functionalized liposomes as Drug-Delivery systems for neurological disorders**

This study focused on the biochemical characterization of functionalized liposomes designed for targeted drug delivery to the central nervous system. The study aimed to evaluate liposomal formulations engineered to cross the blood–brain barrier and release specific drugs for the treatment of neuroinflammatory conditions in Alzheimer’s disease and glioblastoma. Raman spectroscopy (RS) was performed to assess the molecular composition, stability, and synthetic reproducibility of the liposomes. Indeed, every liposome component was acquired via RS to determine how individual chemical components influenced the Raman fingerprint of each formulation. To discriminate liposomes with different functionalization, the multivariate analysis was performed, confirming their stability over time. These results demonstrated the potential of RS as a rapid, label-free quality-control tool for liposomes used in drug delivery. In this project, my contribution focused on acquiring Raman spectra and their post-processing workflow. Furthermore, I contributed to multivariate statistical analysis, helping to understand spectral variations associated with functionalization, drug loading, and batch-to-batch reproducibility. This collaboration highlighted the versatility of RS for characterizing complex nanosystems.

### **Multiplexing Biosensor for the Detection of Extracellular Vesicles as Biomarkers of Tissue Damage and Recovery after Ischemic Stroke**

This side project investigated the role of Extracellular Vesicles (EVs) as potential biomarkers for patient stratification and rehabilitation outcome prediction in subacute ischemic stroke using Surface Plasmon Resonance Imaging (SPRi). The project focused on identifying EV-associated molecules involved in post-stroke inflammatory, reparative, and neurodegenerative processes. The primary aim was to obtain information that could complement clinical scales, in particular, biomarkers able to predict recovery. Using a multiplexed SPRi biosensor, the study demonstrated that, 10 days after the ischemic event, subacute stroke patients exhibit increased expression of VEGFR2 and TSPO on EVs, with a specific contribution from microglial and endothelial EVs. These preliminary results suggested that EV profiling could support patient

characterization at the onset of rehabilitation and inform predictions of therapeutic responsiveness.

My contribution to this work was concentrated on EV isolation, their SPRi analysis, and data interpretation, ensuring the robustness and reproducibility of the analytical workflow.

### **SPRi analysis of molecular interactions of mApoE-functionalized liposomes as drug delivery systems for brain diseases**

This study focused on the development and molecular characterization of multifunctionalized liposomes designed for targeted delivery across the blood–brain barrier (BBB) in the treatment of Glioblastoma. The liposomal formulations were engineered to encapsulate a therapeutic compound and to be functionalized with two key ligands: an Apolipoprotein E-modified peptide (mApoE) to promote BBB translocation, and a metalloprotease-sensitive lipopeptide to localize drug release within the pathological microenvironment. Surface Plasmon Resonance imaging (SPRi) was optimized and applied to evaluate the binding affinity of the functionalized liposomes toward brain receptors. SPRi measurements revealed strong interactions between mApoE-LPs, while showing negligible binding to peripheral immune receptors expressed on monocytes and lymphocytes. In particular, a strong affinity for the endothelial marker VCAM-1 was observed, supporting the specificity of the functionalization. These results demonstrated the suitability of SPRi as a sensitive and multiplexed platform for the characterization of functionalized liposomes.

My expertise was relative to the EV analysis by SPRi.

### **Extracellular vesicles of different cellular origin feature distinct biomolecular corona dynamics**

As part of a side project, we investigated the formation and dynamics of the biomolecular corona (BC) on Extracellular Vesicles (EVs), a phenomenon normally observed in synthetic nanoparticles. This study focused on EVs derived from human red blood cells (REV) and amniotic membrane mesenchymal stromal cells, exploring how their surface interacts with plasma proteins under physiological conditions. The research employed fluorescence correlation spectroscopy to monitor BC formation. My contribution focused on EV separation and characterization, specifically supporting the isolation of REV and performing nanoparticle tracking analysis to assess size distribution and concentration. This work highlighted distinct BC dynamics between the two EV populations, suggesting that EV origin influences corona formation, providing insights into EV heterogeneity and informing the development of EV-based diagnostic and therapeutic applications.

## **MicroRNA-142-3p shuttling in extracellular vesicles marks regulatory T cell dysfunction in multiple sclerosis**

This study explored the role of EVs released by regulatory T cells (Treg-EVs) to maintain immune tolerance and how their dysfunction contributes to the pathogenesis of relapsing remitting multiple sclerosis (RR-MS). This study demonstrated that Treg-EVs from HC mediate immunosuppressive effects by transferring miR-142-3p to conventional CD4<sup>+</sup> T cells (Tconv). In contrast, Treg-EVs from RR-MS contained lower levels of miR-142-3p, leading to reduced suppressive activity and correlating with disease severity.

Within this project, my contribution focused on the characterization of Treg-EVs obtained from HC and RR-MS with two different isolation protocol: ultracentrifugation and Exo-spin™. Samples were analyzed through CONAN assay and AFM to assess purity and morphology. These analyses revealed that EVs isolated by ultracentrifugation exhibited a cleaner profile and lower contamination compared to those purified using the Exo-spin kit. Nonetheless, both methods did not affect the downstream functional results presented in the study. For this reason, all the analyses present in this paper were performed using the Exo-spin isolation method for its practicality and reproducibility.

## **Assessment of chronic Myeloid Leukaemia in vitro models variability: insights into extracellular vesicles**

This study aims to investigate EVs derived from Chronic Myeloid Leukaemia (CML) cell models to better understand their molecular characteristics and responses to Tyrosine Kinase Inhibitor (TKI) treatment. Indeed, the success of TKI treatment, therapeutic resistance remains an obstacle for CML. For this reason, the work compared two CML-derived cell lines, K562 and KCL22, by focusing on their EVs before and after different treatments, alongside Ki67 expression to value proliferation.

My contribution to this work focused on the EV characterization. Specifically, I performed Dot Blot analyses to confirm the presence of EV markers and sample purity, BCA assay to evaluate the protein concentration, Nanoparticle Tracking Analysis to determine EV size distribution, and Atomic Force Microscopy for morphological characterization. These analyses confirmed the presence of EV in our samples allowing us to proceed with the spectroscopic analysis.

FTIR combined with PCA analysis revealed differences between EVs derived from K562 and KCL22 cells. Under basal conditions, both cell lines released comparable amounts of EVs containing similar levels of BCR::ABL1 transcripts. However, following TKI treatment, K562

cells exhibited an increased EV release and an higher BCR::ABL1 content. In contrast, KCL22 cells showed an earlier proliferative activity.

### **Tracking Leukemic Residuals: Dissecting the Inverse Relationship Between CD26<sup>+</sup> Stem Cells and Extracellular BCR::ABL1 in CML**

This work explored the relationship between circulating CD26<sup>+</sup> leukemic stem cells (LSC) and Extracellular Vesicle–Enriched Secretome (EVES) BCR::ABL1 transcripts in CML patients under TKI therapy. CD26<sup>+</sup> LSC were quantified via flow cytometry, while EVES BCR::ABL1 levels were separated using Norgen Kit. In particular, my help was focused on EVES characterization, performing Bradford assay for total protein, Colorimetric NANoplasmonic assay for soluble protein contaminants, and Atomic Force Microscopy for morphological evaluation. Results confirmed enrichment in EVs with lipoprotein contamination, validating the EVES fractions. An inverse correlation emerged between circulating CD26<sup>+</sup> LSC counts and EVES BCR::ABL1 transcripts. Therefore, transcript release by EVs may reflect dynamic changes in LSC activity. These findings support EVES as a minimally invasive biomarker for disease monitoring and provide insight into EVs signaling in CML.

### **Red Blood Cell-derived Extracellular Vesicles as biomaterials: the opportunity of freezing-induced accelerated aging**

For this side project, we investigated how storage conditions influence the physicochemical properties of Red Blood Cell–Derived Extracellular Vesicles (RBC-EVs), which are increasingly considered as attractive building blocks for biomaterial and drug-delivery applications. Freshly isolated RBC-EV preparations showed marked heterogeneity, with two distinct nanoparticle subpopulations identifiable after density-based separation and high-resolution interferometric Nanoparticle Tracking Analysis (iNTA). The study demonstrated that prolonged storage at -80°C, as well as controlled freeze–thaw cycles, reduced this heterogeneity and drove RBC-EV toward a stable, more homogeneous population with preserved membrane integrity and cellular uptake capacity. These results support the concept of “accelerated ageing” as a practical strategy to standardize RBC-EV batches and to reproduce the biophysical features typically observed after long-term storage. In particular, for this project, my contribution involved performing the separation of RBC-EVs and standard Nanoparticle Tracking Analysis, which were essential for monitoring the evolution of RBC-EV size distribution and concentration across the different storage conditions. In summary, the work highlights how processing and storage parameters critically shape RBC-EV behaviour,

and underscores the importance of standardized workflows for their future use in bioengineering and therapeutic contexts.

### **Shared protocol to enrich and compare Extracellular Vesicles from human plasma and skeletal muscle biopsy**

In this work, we investigated extracellular vesicles (EVs) from both solid (human skeletal muscle tissue) and liquid (human plasma) matrices, with the aim of developing a standardized protocol for their separation and comparative characterization. While EV research has traditionally focused on liquid matrices due to accessibility, EVs from solid tissues (tissue-EV) provide valuable insights into the local microenvironment and tissue-specific functions. The study employed optimized separation protocols and advanced analytical techniques to assess biomolecular composition, nanomechanical properties, particle yield, size distribution, and colloidal stability, while minimizing methodological biases across sample types. My contributions included EV separation using differential centrifugation and density gradient, comprehensive EV characterization through nanoparticle tracking analysis to assess the EV concentration and size, Western Blot analysis for a biochemical characterization, COlorimetric NANoplasmonic assay in order to confirm the purity of the EV sample, and BCA assay for the total protein content. The results highlighted clear differences between muscle- and plasma-derived EVs, underscoring tissue-specific features and potential implications for EV-based diagnostics and understanding of their biological roles in different microenvironments.

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## Article

# Molecular Understanding of the Surface-Enhanced Raman Spectroscopy Salivary Fingerprint in People after Sars-COV-2 Infection and in Vaccinated Subjects

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**Abstract:** The rapid spread of SARS-COV-2 and the millions of worldwide deaths and hospitalizations have prompted an urgent need for the development of screening tests capable of rapidly and accurately detecting the virus, even in asymptomatic people. The easy collection and the biomarker content of saliva, together with the label-free and informative power of surface-enhanced Raman spectroscopy (SERS) analysis have driven the creation of point-of-care platforms capable of identifying people with COVID-19. Indeed, different salivary fingerprints were observed between uninfected and infected people. Hence, we performed a retrospective analysis of SERS spectra from salivary samples of COVID-19-infected and -vaccinated subjects to understand if viral components and/or the immune response are implicated in spectral variations. The high sensitivity of the proposed SERS-based method highlighted the persistence of molecular alterations in saliva up to one month after the first positive swab, even when the subject tested negative for the rapid antigenic test. Nevertheless, no specific spectral patterns attributable to some viral proteins and immunoglobulins involved in COVID-19 infection and its progression were found, even if differences in peak intensity, presence, and position were observed in the salivary SERS fingerprint.

**Keywords:** saliva; Raman spectroscopy; COVID-19; spike protein



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## 1. Introduction

The COVID-19 pandemic, caused by the Sars-COV-2 virus, became a worldwide health emergency that caused deaths and radical changes in our habits. Belonging to the coronavirus family, Sars-COV-2 displays an affinity towards the ACE-2 receptor, resulting in a principal involvement of the upper and lower airways. The coronas of spike proteins around the virus mediate the receptor binding through subunit 1, while subunit 2 is responsible for virus–host–cell membrane fusion [1]. Over these four years, this wild virus form has undergone spontaneous mutations, causing the appearance of five variants of concern responsible for increased transmission. It also has a variability of symptoms [2,3]. Its clinical manifestations range from fever, cough, sore throat, and muscle pain to pneumonia and death in the most severe cases. To slow down the spread of the virus and decrease

the mortality rate, anti-COVID-19 vaccines were developed. Based on mRNA technology, Comirnaty (BNT162b2) (Pfizer-BioNTech®) and Spikevax (Moderna®) vaccines were the ones mainly administered in Italy [4]. The induced immunological memory, through the production of anti-Sars-COV-2 spike protein antibodies, prevents the worsening of the disease and should reduce contagiousness [5]. Additionally, a variety of diagnostic methods for different contexts has been developed. RT-PCR molecular test and the rapid antigen immuno-based test are the two gold-standard techniques performed mainly using a nasopharyngeal swab. The presence of the virus is verified through the quantification of the genetic material and the detection of viral proteins, respectively. Although RT-PCR is a very sensitive technique, it is time-consuming and expensive, while the rapid antigen test allows fast screening at the expense of accuracy [6,7]. In this context, saliva has attracted interest as a specimen for COVID-19 diagnosis for the self because of its non-invasive and painless collection, and since it is a reservoir of a variety of molecules, diseases-related biomarkers, and viruses [8–10]. Sars-COV-2 can persist in saliva for up to about 20 days after the onset of symptoms, causing alterations in the lipid and inflammatory profile, T-cells, metabolites, and proteins [11–13]. Given the concordance of the results with the nasopharyngeal swab, saliva has been confirmed as a valid alternative and has been employed in some COVID-19 tests [14–16]. However, new diagnostic tools have been created to address some of the limitations of conventional platforms. Among them, surface-enhanced Raman spectroscopy (SERS) has emerged as a valuable analysis thanks to its high-sensitivity ability to detect the lowly concentrated molecules present in saliva. Indeed, the use of metallic substrates enhances the Raman effect, providing more accurate biomolecular information [17–21]. From 2020, about 20 papers have been published on this topic. Most studies involve the creation of SERS-based immunoassays for the detection of viruses by spike and/or nucleocapsid proteins or receptor-binding domain recognition, employing artificial or human saliva samples enriched with viral proteins or particles [22–26]. Although these methods can detect very low concentrations of viral components, they are expensive, variant-dependent (as they involve the use of antibodies), and do not fully mirror the real condition. Alternatively, the whole salivary spectrum can be considered a fingerprint of the individual's status of health, avoiding the use of reagents. The possible clinical application of a label-free SERS platform for COVID-19 diagnosis is supported by good levels of accuracy, precision, and specificity, also achieved using machine learning algorithms. Alterations of the salivary Raman spectrum mirror the modifications in saliva due to the infection. In most of the works, spectral variations are attributable to proteins, nucleic acids, and lipids. In our previous study, we were able to discriminate subjects positive for Sars-COV-2 from those with a past infection or uninfected with a diagnostic accuracy between 89% and 92% [27]. Indeed, a specific salivary Raman fingerprint was ascribable for each experimental group, with spectral variations related to O–O stretching, glucose/glycogen, C–N, and C–C protein stretching, tryptophan, phenylalanine, and secondary bands of amide III. Along the same line, Ember K. et al. found differences in the ranges of proteins, DNA, urea salt, and nitrate by comparing the spectra of the main saliva constituents [28]. Moreover, the involvement of methionine and tyrosine has been suggested due to variations in the intensity bands at 654, 720, 1320, 1443  $\text{cm}^{-1}$ , 828, and 853  $\text{cm}^{-1}$ , respectively, between positive and negative subjects [29]. However, no study has delved into the possible molecules behind these differences in the Raman spectra. Tentative assignments of the peaks were made based on the literature data and molecular alterations in the saliva after infection. Since Raman bands could derive from different molecules, the aim of this study was to better understand which molecules are involved in saliva spectra variance. Specifically, we studied the involvement of the Sars-COV-2 virus by investigating the potential implication of the spike and nucleocapsid proteins, and of the immune system. For this purpose, we performed a study on both COVID-19 subjects and healthy ones after the administration of the Comirnaty® vaccine.

## 2. Materials and Methods

### 2.1. Saliva and Serum Sample Collection

This study was approved by the Ethics Committee of IRCCS Fondazione Don Carlo Gnocchi ONLUS on 17 February 2021 and 20 May 2021. All participants gave written informed consent and filled out a brief questionnaire about smoking habits and ongoing pharmacological treatments. Eventual oral infections, recent dental surgeries, and pathologies of the respiratory system were recorded. The study involved 38 volunteers who were divided into two groups, following a different timeline in the saliva collection. The first group (Group 1), consisting of 17 people, collected saliva once a week for one month after the first positive nasopharyngeal test. The positivity of Sars-COV-2 was diagnosed by nasal rapid antigen test and hospitalisation was not needed. The second group (Group 2) consisted of 21 hospital co-workers. The saliva sampling was performed before starting the anti-COVID-19 vaccine cycle and 30 days after the second dose of the Pfizer vaccine (Comirnaty®). Stimulated saliva samples were self-collected using Salivette® (Sarstedt AG & CO, Numbrecht, Germany), processed following our previously optimised protocol, and stored at  $-20\text{ }^{\circ}\text{C}$  until use [27]. Before all the analyses, saliva aliquots were thawed and vortexed to guarantee sample homogeneity. Serum samples for immunoglobulin quantification were collected from Group 2 30 days after the second dose using collection tubes with a gel separator (BD Vacutainer® SST™ II Advance tubes, Becton-Dickinson Biosciences, San José, CA, USA). Aliquots of serum samples were stored at  $-80\text{ }^{\circ}\text{C}$  until use.

### 2.2. Preparation of Spike Protein Sample

The Sars-COV-2 spike protein (2019-nCoV Spike Protein S—Adipogen, Füllinsdorf, Switzerland) was reconstituted in sterile water, obtaining a final concentration of  $10^{-6}\text{ M}$ . Then, the stock solution was diluted in a Sars-COV-2 negative saliva sample to final concentrations of  $10^{-7}$  and  $10^{-9}\text{ M}$ , based on literature data [30].

### 2.3. Surface-Enhanced Raman Spectroscopy Analysis

SERS spectra were collected using the Raman microscope Aramis (Horiba Jobin-Yvon, France) coupled with a laser source at 785 nm (150 mW power emission at source). The analysis of saliva was performed as previously described [27]. A saliva drop was dried at room temperature on a glass slide covered with commercial aluminium foil, and, for each subject, at least 15 spectra were collected in random points, given the reproducibility of the SERS spectra (Figure S1). The spectral regions of interest were 435–690 and 700–1600  $\text{cm}^{-1}$ . The silicon spectrum was measured every day for Raman shift calibration to have accurate, reliable, and reproducible measurements. The same protocol was followed for the acquisition of the 15 spectra of spike solutions and saliva in which the protein was diluted. Compared to our previous work, the contribution of the aluminium substrate was not removed to minimise the manipulation of the spectra.

### 2.4. SERS Data Processing

The raw data of the saliva samples were subjected to post-processing using Lab-Spec6 (Horiba, France). Specifically, spikes and artefact spectra were removed, and a Savitsky–Golay smoothing filter of order 3 and a fifth-degree polynomial curve were applied. After alignment to the 1252  $\text{cm}^{-1}$  band, spectra were extracted with a final resolution of 1.21  $\text{cm}^{-1}$ /step, corresponding at 939 x-axis points. The resulting spectra were normalised by dividing each curve by the dataset norm using OriginLab2021b (OriginLab, version 2021, Northampton, MA, USA). Spectra of spike proteins underwent the same processing steps. The average of each saliva sample was used as input for the principal component analysis (PCA). For the comparative study among uninfected subjects and those with current and past infection, the first 7 principal components (PCs) were extracted and used for the creation of the classification model using linear discriminant analysis (LDA). An agglomerative hierarchical analysis was carried out on the 21 average spectra

of Group 2-timepoint 1. Statistical analysis was performed using one-way ANOVA for pair-wise comparisons, and the significance is indicated in the figures as \*  $p < 0.001$ .

### 2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

Cortisol and nucleocapsid protein were quantified using a commercial ELISA salivary test (Cortisol ELISA Kit—R&D Systems, Biotechne, Minneapolis, MN, USA; SARS-COV-2 nucleocapsid protein ELISA Kit—Arigo Biolaboratories, Zhubei City, Taiwan) according to the manufacturer's protocol. A cortisol assay was performed on Group 1 saliva samples ( $n = 17$ ), whereas the nucleocapsid protein was quantified only in COVID-19-positive subjects ( $n = 35$ , as shown in Figure 1). Saliva samples were diluted 5-fold (cortisol) and 2-fold (nucleocapsid) to be within the standard curve, and the optical intensity was measured within 30 min at 450 nm with a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany).

### 2.6. Salivary and Serum IgA Quantification

The IgA antibodies specific to the recombinant receptor-binding domain (S1-RBD) of the spike protein (Wuhan-Hu-1) were measured in saliva (2-fold diluted) and serum (500-fold diluted) using a commercial ELISA kit assay (RayBio, CliniSciences, Guidonia Monticello, Rome, Italy), according to the manufacturer's instruction, as previously described [31]. IgA antibody titres were expressed as U/mL (units/mL; cut-off: 21.4 U/mL).

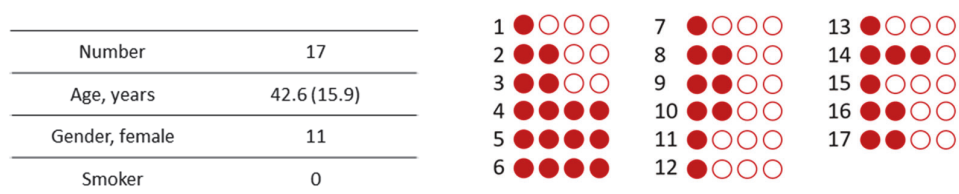
### 2.7. Serum IgG Quantification

The IgG antibodies specific to the SARS-COV-2 receptor-binding domain (RBD) of the spike protein were measured in a serum by chemiluminescence immunoassay, CLIA, (Access Immunoassay Systems; Beckman Coulter, Brea, CA, USA) as recommended by the manufacturers. IgG antibody titres were expressed as AU/mL (arbitrary units/mL; cut-off: 10 AU/mL).

## 3. Results and Discussion

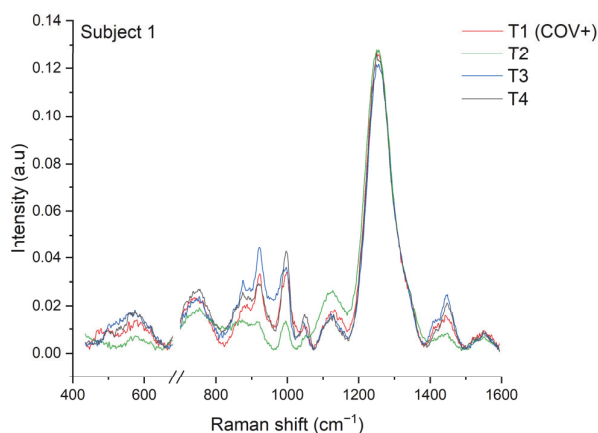
### 3.1. Longitudinal COVID-19 Fingerprint

To investigate whether the progression of the Sars-COV-2 infection resulted in changes in the SERS fingerprint of the saliva, 17 people were asked to collect saliva every week for one month after being diagnosed with COVID-19 (COVID19+; T1, T2, T3, T4). The SERS analysis was performed following our previously optimised protocol that allows us to obtain the salivary fingerprint in a fast and reproducible manner [27]. Each subject was considered separately for the spectral analysis, as each had a different timing of negativisation from COVID-19. Indeed, for some participants, the infection lasted about a week, while some subjects remained positive for the entire duration of the study. Compared to our previous work, the people included in this study were aged between 26 and 71 years and showed mild symptoms that required home-based therapy following the indications of the general medicine practitioner and were thus not hospitalised. Figure 1 summarises the demographic and clinical data of Group 1 subjects.



**Figure 1.** General information on COVID-19 subjects enrolled in this study. The standard deviation is reported in brackets. Dots indicate the saliva samples taken at time-points 1, 2, 3, 4. When the dot is filled, the subject is COVID-19 positive.

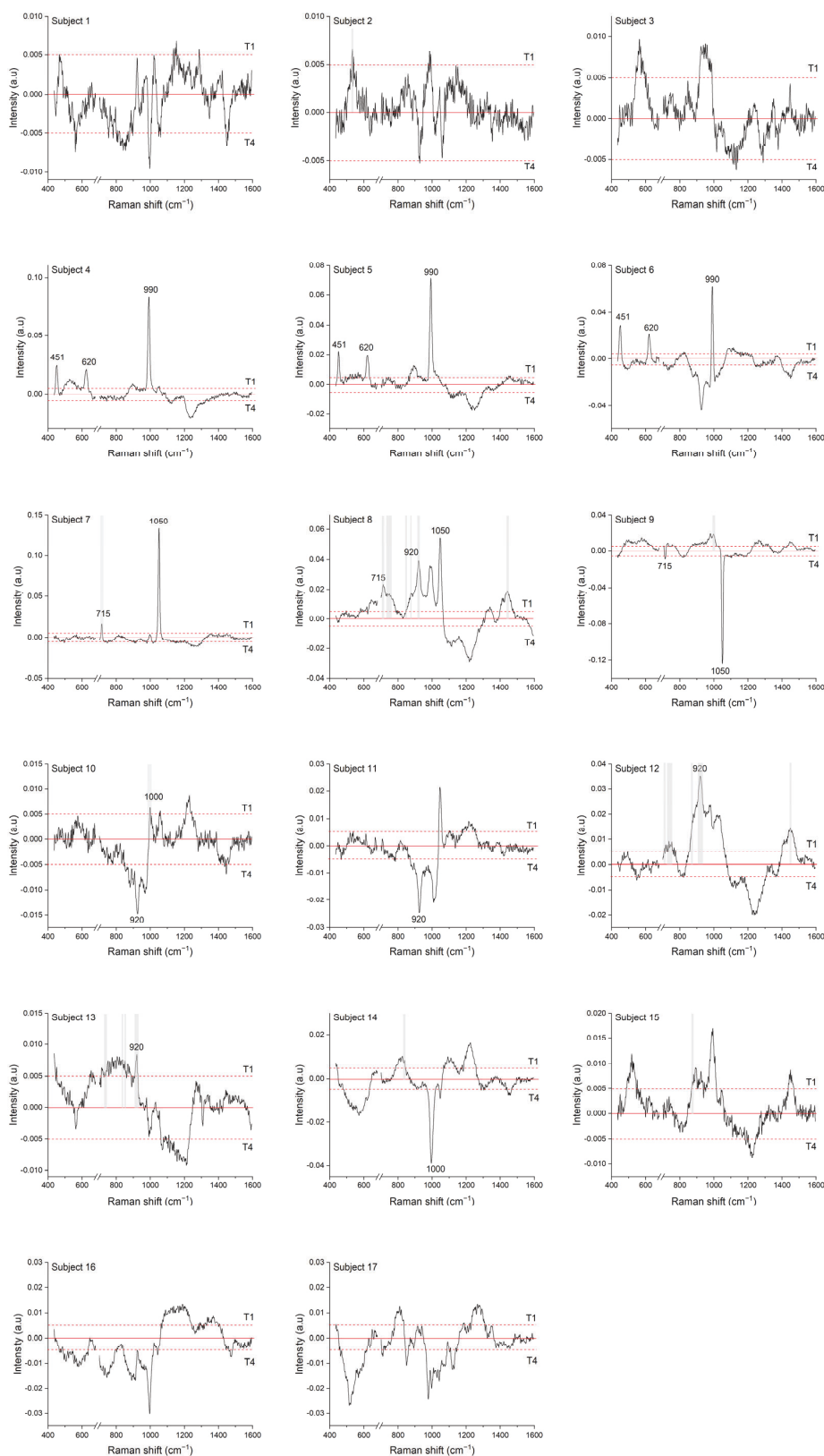
Figure 2 shows the overlaid mean spectra of the four time-points of subject 1, who tested positive for one week. The SERS spectra of samples of other subjects from Group 1 are present in the Supplemental Materials (Figure S2).



**Figure 2.** Overlapped average spectra of four time-points (T1, T2, T3, T4) of participant 1's saliva.

Comparing the four time-points, differences can be found mainly in the change in intensity and/or the presence and absence of specific peaks and bands. Some positive (#1T1, #8T1, #17T1, #12T1, #12T2) and negative (#1T3, #1T4, #8T2, #8T3, #17T2, #17T3, #1T4) saliva samples showed a band/shoulder at approximately  $1420\text{ cm}^{-1}$  that can be related to the ring breathing modes of the DNA/RNA bases [32]. The peak at  $852\text{ cm}^{-1}$ , attributable to glycogen, proline, or tyrosine, is also present indiscriminately in subjects with a current (COV+) and past (COV−) infection [33]. The same applies to the peak at  $715\text{ cm}^{-1}$  related to phospholipids, which may be derived from the virion particle [27]. To better visualise possible spectral variations characteristic of disease progression, the spectrum at T4 was subtracted from T1. We selected these two time-points because, within a month, molecular changes are expected to occur independently of whether the subject is still positive or has become negative. The resulting subtraction spectra are displayed in Figure 3. Since all the subjects were positive at T1, the spectral area above the horizontal solid line (at zero intensity) is characteristic of the initial phase of the infection when the viral load is expected to be at maximum [34,35].

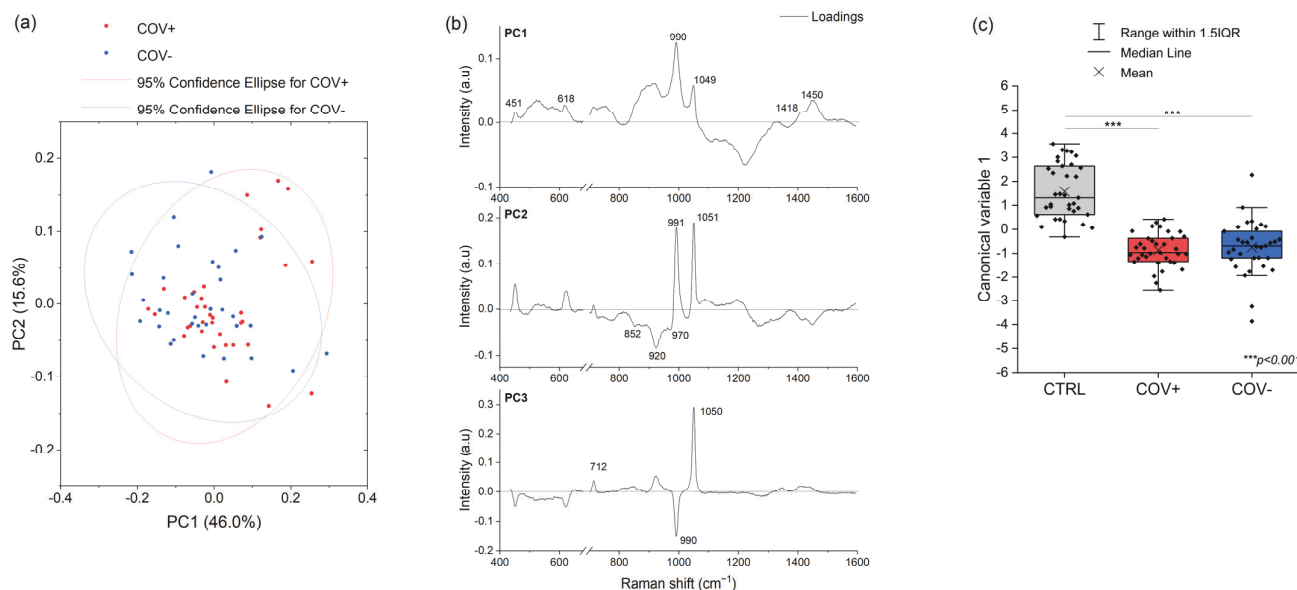
For three participants (#1/#2/#3), the subtraction spectrum is very noisy suggesting a similarity between the two time-points. Only subjects #4, #5, and #6 have three characteristic peaks at T1 ( $451$ ,  $620$ , and  $990\text{ cm}^{-1}$ ) while peaks at  $715$  and  $1050\text{ cm}^{-1}$  well differentiate COV+ from COV− saliva of subjects #7 and #8. Nevertheless, the possible relationship of these peaks to the presence of the virus in saliva is contradicted by the subtraction spectrum of subject #9, which showed the opposite pattern. Lastly, the peaks at  $920$  (carbohydrates),  $1000$  (phenylalanine), and  $1050$  (tryptophan)  $\text{cm}^{-1}$  exhibited the greatest variation between T1 and T4 [27]; however, no discernible trend is evident. Already highlighted as one of the main differentiators in our previous work [27], the peak at  $1050$  can also be attributed to nitrates so we can speculate that its fluctuation may also depend on dietary habits [36].



**Figure 3.** Subtraction spectra for each subject. The  $\pm 0.005 \Delta I$  interval was chosen as the lower limit for peak attribution.

### 3.2. Multivariate Analysis

In an effort to uncover potential differences not discernible from a simple comparison of spectra, multivariate statistical analysis was performed. After PCA, the obtained PC score distribution showed a minimal inter-group variation between the COV+ and COV− samples (Figure 4a), suggesting that the molecular alterations that occurred in saliva due to infection did not lead to major modifications in the SERS spectrum within a month. The inability to distinguish the COV+ from COV− samples, in contrast to our previous work [27], may be explained by the fact that in the present study, the COV− saliva samples were collected a short time after the negativization happened. Nevertheless, the loadings of the first three PCs, which account for about 75% of the variance, showed some variations at specific Raman shifts (Figure 4b). It has to be noted that despite the involvement of peaks at 451, 618, 712, 920, 990, 1049, 1418, and 1450  $\text{cm}^{-1}$  in the differences between COV+ and COV− spectra, the areas under the curve of these spectral regions were not significantly different between the two groups (Figure S3, Supplementary Materials). Hence, to ascertain that our SERS approach is also suitable as a diagnostic platform for non-severe COVID-19 cases, a PCA-LDA was performed including the saliva samples of 36 healthy controls (CTRLs) used in the past work [27]. The first seven PCs were extracted and used for the classification model. As expected, the CTRLs significantly differ from the other two experimental groups, while the distribution of the canonical variable 1 highlighted no differences between COV+ and COV− (Figure 4c). This result is in agreement with our previous observation that negativized patients do maintain the biomolecular composition of saliva chemical alterations related to a previous infection by Sars-COV-2, even in mild cases of COVID-19. These variations make the SERS salivary fingerprint of subjects after COVID-19 significantly different from CTRL subjects (no previous infection by Sars-COV-2 registered) but partially overlapped with COV+. Disease severity, subjects' age, and sample size are possibly responsible for the differences in the present results, compared to previous data [27].



**Figure 4.** Results of the multivariate analysis. (a): PC1 and PC2 scores distribution. Each dot represents the average spectrum of each sample; (b): the loadings of the first three PCs; (c): the statistical analysis (one-way ANOVA test) on Canonical Variable 1, derived from the LDA of the CTRL, COV+, and COV− subjects.

### 3.3. Salivary Cortisol Quantification

Salivary cortisol was quantified to exclude any potential impact in the SERS spectrum since its level follows a circadian profile and is influenced by lifestyle, stress, and pathological conditions [37,38]. The analysis was performed on all samples from Group 1 and samples at T1 from Group 2. Except for one specimen, all saliva samples had cortisol levels in the range of 0–20 ng/mL, which was in agreement with results reported in the literature [39,40]. Values are reported in Table S1 in the Supplementary Materials. Considering the Raman peaks characteristic of salivary cortisol [41], only the peak at  $1450\text{ cm}^{-1}$  is present in our spectra, and no variations in its intensity or position were found. Therefore, we can determine that differences in salivary cortisol levels did not affect the obtained spectra in the considered analysis.

### 3.4. SERS Data Interpretation

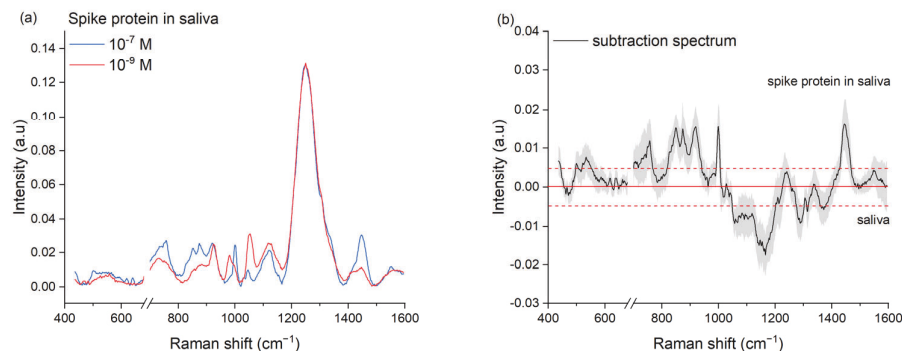
#### 3.4.1. Nucleocapsid Protein

To understand if the viral components are implicated in the discrimination of COVID-19 samples, we studied the potential contribution of the nucleocapsid protein. The COV+ saliva samples of Group 1 were tested for the presence of the Sars-COV-2 nucleocapsid protein. Unexpectedly, in most samples, the assay did not allow for nucleocapsid quantitation, as it was probably under the limit of detection of the techniques, whereas only two samples from the same subject (T1 and T2 of subject #7) contained the protein at a concentration of 15.2 and 15.6 ng/mL, respectively. Considering the Raman spectrum of the nucleocapsid protein [42], in #7T1 and #7T2, only the peak at  $1050\text{ cm}^{-1}$  could refer to its presence, even though this peak is present indifferently in the COV+ and COV− spectra, with an intensity comparable to these two samples. In all the other COV+ specimens, the Sars-COV-2 nucleocapsid protein was not detectable by ELISA, although previously reported data showed that levels of up to 100 ng/mL were detected in saliva in the first ten days of infection using a single molecule array [43].

#### 3.4.2. Spike Protein

The potential impact of the Sars-COV-2 spike protein in the SERS fingerprint was assessed through the SERS analysis of the protein itself in saliva. The averaged spectra of spike protein in saliva are presented in Figure 5a. The spectrum at  $10^{-7}\text{ M}$  is more informative in terms of peak presence and intensity. Interestingly, the shift of the peak from  $920\text{ cm}^{-1}$  to  $924\text{ cm}^{-1}$ , passing from  $10^{-7}$  to the  $10^{-9}\text{ M}$  concentration, was observed. A red shift also involves the peak at  $1046\text{ cm}^{-1}$  that moves to  $1053\text{ cm}^{-1}$  in the  $10^{-9}\text{ M}$  solution. These spectral areas were already identified as responsible for the inter-group variations. In contrast, only at the  $10^{-9}\text{ M}$  concentration is there the peak at  $980\text{ cm}^{-1}$ , which has never previously been reported for the Sars-COV-2 spike protein solution. The observed concentration-dependent spectral variations are not surprising due to the expected alteration in ion–protein and protein–protein interactions in biological samples which are rich in both salts and proteins [44,45]. Although we cannot conclude if the observed shifts are related to spike-protein aggregates or to the interaction of the spike protein with other salivary components, we decided to consider for the subsequent analyses the spectra of saliva sample added with the  $10^{-7}\text{ M}$  protein that is expected to avoid the “crowding” effect.

To study the spectral contribution of the Sars-COV-2 protein in the saliva spectrum, we subtracted the spectrum of saliva from the spectrum of the same saliva sample added to the protein ( $10^{-7}\text{ M}$ ) (Figure 5b). The resulting subtraction spectrum is expected to show which peaks and bands are related to the spike protein in saliva. Spectral differences were considered significant for  $\pm 0.005\text{ ΔI}$ . The Raman shift of the peaks related to the spike protein is listed in Table 1.



**Figure 5.** SERS analysis of spike protein added to saliva. (a) Overlapped SERS spectra of spike protein at two different concentrations; (b) subtraction spectrum of spike protein in saliva versus saliva. The grey area represents the propagation error.

**Table 1.** Peaks attributed to spike protein in saliva. Values refer to Raman shift ( $\text{cm}^{-1}$ ).

|     |     |     |     |      |
|-----|-----|-----|-----|------|
| 500 | 702 | 738 | 835 | 999  |
| 521 | 707 | 741 | 849 | 1445 |
| 533 | 712 | 749 | 874 |      |
| 538 | 728 | 758 | 884 |      |
| 542 | 733 | 828 | 919 |      |

Our data are in line with previous literature that identified 500, 542, 849, 874, 920, and 1445  $\text{cm}^{-1}$  as the featured peaks of the Sars-COV-2 spike protein [42]. Thus, to demonstrate the contribution of the spike protein in COV+ saliva samples, we compared these values with the peaks observed in the subtraction spectra and referred to the T1 time-point that was expected to have a higher viral load. The comparison demonstrated the contribution of the Sars-COV-2 spike protein to the salivary fingerprint of some COV+ saliva samples at T1 (subjects #2, #7, #8, #12, #13, #14, #15). The grey bands in Figure 3 represent these common peaks and show that the spectral region with the greatest similarity is between 700 and 920  $\text{cm}^{-1}$ . Additionally, since the 920  $\text{cm}^{-1}$  peak was identified in the subtraction spectrum as belonging to the spike protein, and its position varies according to concentration, the Raman shift of this peak was evaluated in all saliva samples. Interestingly, a red shift was observed in samples from the same subject from T1 to T4 for samples #2, #11, #12, and #13 (Table 2), suggesting a decrease in spike protein concentration over time. Even the presence and/or the intensity of the peak at 920  $\text{cm}^{-1}$  is not always visible in COV+ spectra, maybe due to the spike proteins' concentrations that are not detectable by SERS in such a complex specimen. Moreover, its correlation with Sars-COV-2 positive saliva is further strengthened by the fact that the spike protein is highly glycosylated [46]. On the other hand, albeit in a very small percentage, we observed the presence of these peaks in the T4 region of the subtraction spectrum. These observations may suggest the presence of the virus (or parts of the virus) one month after the first positive test, although, it has to be noted that a peak can result from the contribution of multiple molecules, and, thus, the spike protein, together with other molecules within saliva, might contribute to the same peak.

**Table 2.** Longitudinal variation of the Raman peak at around 920  $\text{cm}^{-1}$ . (+) indicates a COV+ sample, while (-) indicates the peak absence.

| Subject | T1      | T2      | T3      | T4      |
|---------|---------|---------|---------|---------|
| 4       | 918 (+) | 919 (+) | 919 (+) | 924 (+) |
| 5       | 918 (+) | 920 (+) | 920 (+) | 921 (+) |
| 6       | 920 (+) | 923 (+) | -       | -       |
| 9       | 920 (+) | 920 (+) | 924     | 924     |

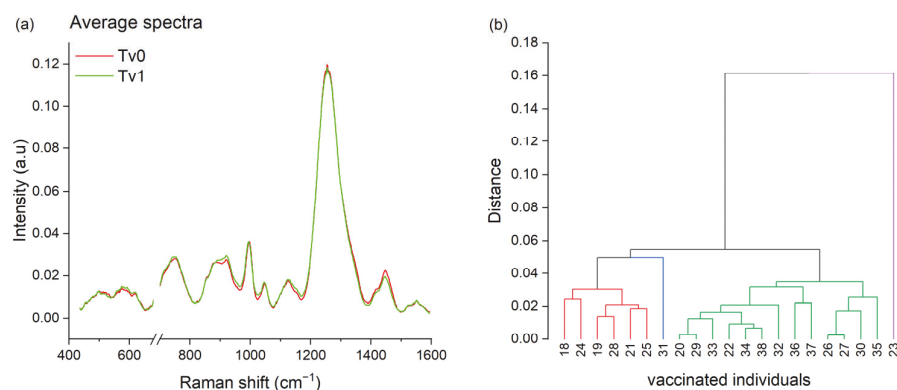
### 3.5. Vaccination Fingerprint

To investigate the reasons for the observed overlap between COV+ and COV− saliva, the possible contribution of molecules associated with the immune response to Sars-COV-2 infection was hypothesised. Since immunoglobulins G (IgG) and A (IgA) are produced in response to the virus, we evaluated their possible involvement in the SERS spectrum. For this purpose, the saliva samples of 21 healthy hospital employees were analysed before starting the anti-COVID-19 vaccine cycle and one month after the second dose of the Pfizer vaccine to understand if the immune response could be detected in the SERS fingerprint of saliva. The timeline was selected because, at the second time-point (1 month after the second vaccine administration), the concentration of circulating antibodies produced against the virus is expected to reach their maximum. All the volunteers tested negative for Sars-COV-2 at both time-points, and only three subjects reported previous COVID-19 diagnoses. Table 3 summarises the demographic and clinical parameters.

**Table 3.** Clinical and demographic characteristics of the cohort of vaccinated volunteers. The standard deviation is reported in brackets.

| Number            | 21          |
|-------------------|-------------|
| Age, years        | 42.8 (14.2) |
| Gender, female    | 13          |
| Previous COVID-19 | 3           |

The average spectra of the two time-points (Tv0 and Tv1) are presented in Figure 6a. The two spectra presented the same pattern of peaks with very slight differences in terms of intensity. Thus, to identify if there are any intra-group relationships relating to spectral regions, a hierarchical cluster analysis of the T1 dataset was conducted. The resulting dendrogram highlighted two outliers and three clusters (Figure 6b). The spectra of these sub-groups underwent PCA to extract the PCs and study which peaks/bands were implicated in data separation. To verify if the spectral variations highlighted by the PC loadings could be related to the inter-individual fluctuation of immunoglobulins, PC1 scores were tested for correlation with the measured concentration of salivary IgA and with the serum concentration of IgA and IgG, but no correlation was assessed. Table S2 in the Supplementary Materials reported the level of the immunoglobulins quantified in saliva and serum, possibly due to the overlap between the Amide III region typically associated with IgA and IgG and the aluminium substrate interference.



**Figure 6.** (a): Averaged SERS spectra of 21 saliva samples before (Tv0) and one month after the second dose of Pfizer vaccine (Tv1); (b) Hierarchical clustering dendrogram representing the unsupervised grouping of the SERS spectra at Tv1.

#### 4. Conclusions

In the present work, we tried to decrypt the biomolecules involved in the modifications of the salivary SERS fingerprint of COV+ individuals by conducting a longitudinal study and taking into account some of the molecules that are expected to be detectable in saliva during Sars-COV-2 infection and after immunisation. Although COVID-19 is no longer a current emergency, the identification of the biomolecules involved in the biochemical alterations of saliva could possibly be used for the profiling of patients diagnosed with long-COVID disorders.

Our results demonstrate that SERS can identify a salivary biochemical fingerprint of people with a mild form of COVID-19 and that saliva composition is progressively modified after COVID-19 recovery. Indeed, after negativization, i.e., when the virus is no more detectable with standard nasopharyngeal rapid testing, saliva still retains biochemical features that resemble the biochemistry of saliva during COVID-19, even mild forms of the disease. However, compared to our previous work, the lack of differentiation between COV+ and COV− may be due to the different infection severity of the people involved in the study and the timing of saliva collection. Nonetheless, the modifications observed in saliva could not be associated either to Sars-COV-2 specific components (nucleocapsid and spike protein) or to immunoglobulins that are expected to be released after the infection. Although this study was conducted on a limited cohort of subjects, because of the pandemic situation at the time of sample collection, our data support the ability of SERS applied to saliva to detect and monitor COVID-19 in a label-free and cost-effective manner. This technology holds promise as a point-of-care solution for the screening of other infectious respiratory diseases of significant global clinical importance, using a portable Raman device combined with clinician-friendly software.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemosensors12070136/s1>, Figure S1. 15 spectra of Subject 1 at timepoint 1; Figure S2. Overlapping of the average spectra for the four timepoints for each subject; Figure S3. Comparison of areas under the curve between COV+ and COV− saliva samples; Table S1: Level of salivary cortisol (ng/mL) in the enrolled groups; Table S2. IgA and IgG levels in saliva and serum samples.

**Author Contributions:** Conceptualization, F.R., A.G., S.P. and M.B.; methodology, F.R., L.F., V.M., R.M. and S.A.; validation, A.G., R.M. and M.B.; formal analysis, F.R., A.G. and P.S.; investigation, F.R., L.F. and S.A.; resources, F.R. and S.A.; data curation, R.M., P.I.B., P.P. and M.B.; writing—original draft preparation, F.R. and A.G.; writing—review and editing, S.P., L.F., V.M., R.M., S.A., P.I.B., P.P. and R.A.R.; visualisation, F.R., and S.P.; supervision, P.I.B. and M.B.; project administration, M.B.; funding acquisition, M.B. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** All data generated or analysed during this study are included in this published article.

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#### References

1. Huang, Y.; Yang, C.; Xu, X.; Xu, W.; Liu, S. Structural and Functional Properties of SARS-CoV-2 Spike Protein: Potential Antivirus Drug Development for COVID-19. *Acta Pharmacol. Sin.* **2020**, *41*, 1141–1149. [[CrossRef](#)] [[PubMed](#)]
2. Magazine, N.; Zhang, T.; Wu, Y.; McGee, M.C.; Veggiani, G.; Huang, W. Mutations and Evolution of the SARS-CoV-2 Spike Protein. *Viruses* **2022**, *14*, 640. [[CrossRef](#)] [[PubMed](#)]

3. SARS-CoV-2 Variants of Concern as of 1 March 2024. Available online: <https://www.ecdc.europa.eu/en/covid-19/variants-concern> (accessed on 12 March 2024).
4. COVID-19 Medicines | European Medicines Agency. Available online: <https://www.ema.europa.eu/en/human-regulatory-overview/public-health-threats/coronavirus-disease-covid-19/covid-19-medicines> (accessed on 12 March 2024).
5. Rahman, M.M.; Masum, M.H.U.; Wajed, S.; Talukder, A. A Comprehensive Review on COVID-19 Vaccines: Development, Effectiveness, Adverse Effects, Distribution and Challenges. *Virusdisease* **2022**, *33*, 1–22. [CrossRef] [PubMed]
6. Afzal, A. Molecular Diagnostic Technologies for COVID-19: Limitations and Challenges. *J. Adv. Res.* **2020**, *26*, 149–159. [CrossRef] [PubMed]
7. Pérez-López, B.; Mir, M. Commercialized Diagnostic Technologies to Combat SARS-CoV2: Advantages and Disadvantages. *Talanta* **2021**, *225*, 121898. [CrossRef] [PubMed]
8. Khurshid, Z.; Zafar, M.; Khan, E.; Mali, M.; Latif, M. Human Saliva Can Be a Diagnostic Tool for Zika Virus Detection. *J. Infect. Public Health* **2019**, *12*, 601–604. [CrossRef] [PubMed]
9. Zhang, C.-Z.; Cheng, X.-Q.; Li, J.-Y.; Zhang, P.; Yi, P.; Xu, X.; Zhou, X.-D. Saliva in the Diagnosis of Diseases. *Int. J. Oral Sci.* **2016**, *8*, 133–137. [CrossRef] [PubMed]
10. Chojnowska, S.; Ptaszyńska-Sarosiek, I.; Kępka, A.; Knaś, M.; Waszkiewicz, N. Salivary Biomarkers of Stress, Anxiety and Depression. *J. Clin. Med.* **2021**, *10*, 517. [CrossRef] [PubMed]
11. Saheb Sharif-Askari, N.; Soares, N.C.; Mohamed, H.A.; Saheb Sharif-Askari, F.; Alsayed, H.A.H.; Al-Hroub, H.; Salameh, L.; Osman, R.S.; Mahboub, B.; Hamid, Q.; et al. Saliva Metabolomic Profile of COVID-19 Patients Associates with Disease Severity. *Metabolomics* **2022**, *18*, 81. [CrossRef]
12. Muñoz-Prieto, A.; Rubić, I.; Gonzalez-Sanchez, J.C.; Kuleš, J.; Martínez-Subiela, S.; Cerón, J.J.; Bernal, E.; Torres-Cantero, A.; Vicente-Romero, M.R.; Mrljak, V.; et al. Saliva Changes in Composition Associated to COVID-19: A Preliminary Study. *Sci. Rep.* **2022**, *12*, 10879. [CrossRef]
13. Bernardo, R.A.; Roque, J.V.; de Oliveira Júnior, C.I.; Lima, N.M.; Machado, L.S.; Duarte, G.R.M.; Costa, N.L.; Sorgi, C.A.; Soares, F.F.L.; Vaz, B.G.; et al. Exploring Salivary Lipid Profile Changes in COVID-19 Patients: Insights from Mass Spectrometry Analysis. *Talanta* **2024**, *269*, 125522. [CrossRef] [PubMed]
14. Sapkota, D.; Søland, T.M.; Galtung, H.K.; Sand, L.P.; Giannecchini, S.; To, K.K.W.; Mendes-Correa, M.C.; Giglio, D.; Hasséus, B.; Braz-Silva, P.H. COVID-19 Salivary Signature: Diagnostic and Research Opportunities. *J. Clin. Pathol.* **2020**, *74*, 344–349. [CrossRef] [PubMed]
15. Azzi, L.; Maurino, V.; Baj, A.; Dani, M.; d’Aiuto, A.; Fasano, M.; Lualdi, M.; Sessa, F.; Alberio, T. Diagnostic Salivary Tests for SARS-CoV-2. *J. Dent. Res.* **2021**, *100*, 115–123. [CrossRef] [PubMed]
16. Tan, S.H.; Allicock, O.; Armstrong-Hough, M.; Wyllie, A.L. Saliva as a Gold-Standard Sample for SARS-CoV-2 Detection. *Lancet Respir. Med.* **2021**, *9*, 562–564. [CrossRef] [PubMed]
17. Carlomagno, C.; Gualerzi, A.; Picciolini, S.; Rodà, F.; Banfi, P.I.; Lax, A.; Bedoni, M. Characterization of the COPD Salivary Fingerprint through Surface Enhanced Raman Spectroscopy: A Pilot Study. *Diagnostics* **2021**, *11*, 508. [CrossRef] [PubMed]
18. Carlomagno, C.; Bertazioli, D.; Gualerzi, A.; Picciolini, S.; Andrico, M.; Rodà, F.; Meloni, M.; Banfi, P.I.; Verde, F.; Ticozzi, N.; et al. Identification of the Raman Salivary Fingerprint of Parkinson’s Disease through the Spectroscopic—Computational Combinatory Approach. *Front. Neurosci.* **2021**, *15*, 704963. [CrossRef] [PubMed]
19. Hardy, M.; Kelleher, L.; de Carvalho Gomes, P.; Buchan, E.; Chu, H.O.M.; Goldberg Oppenheimer, P. Methods in Raman Spectroscopy for Saliva Studies—A Review. *Appl. Spectrosc. Rev.* **2022**, *57*, 177–233. [CrossRef]
20. Chisanga, M.; Muhamadali, H.; Ellis, D.I.; Goodacre, R. Enhancing Disease Diagnosis: Biomedical Applications of Surface-Enhanced Raman Scattering. *Appl. Sci.* **2019**, *9*, 1163. [CrossRef]
21. Buchan, E.; Hardy, M.; Gomes, P.d.C.; Kelleher, L.; Chu, H.O.M.; Oppenheimer, P.G. Emerging Raman Spectroscopy and Saliva-Based Diagnostics: From Challenges to Applications. *Appl. Spectrosc. Rev.* **2022**, *59*, 277–314. [CrossRef]
22. Zhang, M.; Li, X.; Pan, J.; Zhang, Y.; Zhang, L.; Wang, C.; Yan, X.; Liu, X.; Lu, G. Ultrasensitive Detection of SARS-CoV-2 Spike Protein in Untreated Saliva Using SERS-Based Biosensor. *Biosens. Bioelectron.* **2021**, *190*, 113421. [CrossRef]
23. Tuckmantel Bido, A.; Brolo, A.G. Digital SERS Protocol Using Au Nanoparticle-Based Extrinsic Raman Labels for the Determination of SARS-CoV-2 Spike Protein in Saliva Samples. *ACS Appl. Nano Mater.* **2023**, *6*, 15426–15436. [CrossRef]
24. Mohammadi, M.; Antoine, D.; Vitt, M.; Dickie, J.M.; Sultana Jyoti, S.; Wall, J.G.; Johnson, P.A.; Wawrousek, K.E. A Fast, Ultrasensitive SERS Immunoassay to Detect SARS-CoV-2 in Saliva. *Anal. Chim. Acta* **2022**, *1229*, 340290. [CrossRef] [PubMed]
25. Lai, S.; Liu, Y.; Fang, S.; Wu, Q.; Fan, M.; Lin, D.; Lin, J.; Feng, S. Ultrasensitive Detection of SARS-CoV-2 Antigen Using Surface-Enhanced Raman Spectroscopy-Based Lateral Flow Immunosensor. *J. Biophotonics* **2023**, *16*, e202300004. [CrossRef] [PubMed]
26. Dong, Y.; Yuan, X.; Zhuang, K.; Li, Y.; Luo, X. Simultaneous and Sensitive Detection of SARS-CoV-2 Proteins Spike and Nucleocapsid Based on Long-Range SERS Biosensor. *Anal. Chim. Acta* **2024**, *1287*, 342070. [CrossRef] [PubMed]
27. Carlomagno, C.; Bertazioli, D.; Gualerzi, A.; Picciolini, S.; Banfi, P.I.; Lax, A.; Messina, E.; Navarro, J.; Bianchi, L.; Caronni, A.; et al. COVID-19 Salivary Raman Fingerprint: Innovative Approach for the Detection of Current and Past SARS-CoV-2 Infections. *Sci. Rep.* **2021**, *11*, 4943. [CrossRef] [PubMed]

28. Ember, K.; Daoust, F.; Mahfoud, M.; Dallaire, F.; Ahmad, E.Z.; Tran, T.; Plante, A.; Diop, M.-K.; Nguyen, T.; St-Georges-Robillard, A.; et al. Saliva-Based Detection of COVID-19 Infection in a Real-World Setting Using Reagent-Free Raman Spectroscopy and Machine Learning. *J. Biomed. Opt.* **2022**, *27*, 025002. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Berus, S.M.; Nowicka, A.B.; Wieruszewska, J.; Niciński, K.; Kowalska, A.A.; Szymborski, T.R.; Drózd, I.; Borowiec, M.; Waluk, J.; Kamińska, A. SERS Signature of SARS-CoV-2 in Saliva and Nasopharyngeal Swabs: Towards Perspective COVID-19 Point-of-Care Diagnostics. *Int. J. Mol. Sci.* **2023**, *24*, 9706. [\[CrossRef\]](#)
30. Yeh, Y.-J.; Le, T.-N.; Hsiao, W.W.-W.; Tung, K.-L.; Ostrikov, K.; Chiang, W.-H. Plasmonic Nanostructure-Enhanced Raman Scattering for Detection of SARS-CoV-2 Nucleocapsid Protein and Spike Protein Variants. *Anal. Chim. Acta* **2023**, *1239*, 340651. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Mancuso, R.; Agostini, S.; Citterio, L.A.; Chiarini, D.; Santangelo, M.A.; Clerici, M. Systemic and Mucosal Humoral Immune Response Induced by Three Doses of the BNT162b2 SARS-CoV-2 mRNA Vaccines. *Vaccines* **2022**, *10*, 1649. [\[CrossRef\]](#)
32. Movasaghi, Z.; Rehman, S.; Rehman, I.U. Raman Spectroscopy of Biological Tissues. *Appl. Spectrosc. Rev.* **2007**, *42*, 493–541. [\[CrossRef\]](#)
33. Szymborski, T.R.; Berus, S.M.; Nowicka, A.B.; Słowiński, G.; Kamińska, A. Machine Learning for COVID-19 Determination Using Surface-Enhanced Raman Spectroscopy. *Biomedicines* **2024**, *12*, 167. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Zhu, J.; Guo, J.; Xu, Y.; Chen, X. Viral Dynamics of SARS-CoV-2 in Saliva from Infected Patients. *J. Infect.* **2020**, *81*, e48–e50. [\[CrossRef\]](#) [\[PubMed\]](#)
35. To, K.K.-W.; Tsang, O.T.-Y.; Leung, W.-S.; Tam, A.R.; Wu, T.-C.; Lung, D.C.; Yip, C.C.-Y.; Cai, J.-P.; Chan, J.M.-C.; Chik, T.S.-H.; et al. Temporal Profiles of Viral Load in Posterior Oropharyngeal Saliva Samples and Serum Antibody Responses during Infection by SARS-CoV-2: An Observational Cohort Study. *Lancet Infect. Dis.* **2020**, *20*, 565–574. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Pannala, A.S.; Mani, A.R.; Spencer, J.P.E.; Skinner, V.; Bruckdorfer, K.R.; Moore, K.P.; Rice-Evans, C.A. The Effect of Dietary Nitrate on Salivary, Plasma, and Urinary Nitrate Metabolism in Humans. *Free Radic. Biol. Med.* **2003**, *34*, 576–584. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Hansen, A.M.; Garde, A.H.; Persson, R. Sources of Biological and Methodological Variation in Salivary Cortisol and Their Impact on Measurement among Healthy Adults: A Review. *Scand. J. Clin. Lab. Investig.* **2008**, *68*, 448–458. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Nater, U.M.; Hoppmann, C.A.; Scott, S.B. Diurnal Profiles of Salivary Cortisol and Alpha-Amylase Change across the Adult Lifespan: Evidence from Repeated Daily Life Assessments. *Psychoneuroendocrinology* **2013**, *38*, 3167–3171. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Umeda, T.; Hiramatsu, R.; Iwaoka, T.; Shimada, T.; Miura, F.; Sato, T. Use of Saliva for Monitoring Unbound Free Cortisol Levels in Serum. *Clin. Chim. Acta* **1981**, *110*, 245–253. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Heaney, J.L.J.; Phillips, A.C.; Carroll, D. Aging, Health Behaviors, and the Diurnal Rhythm and Awakening Response of Salivary Cortisol. *Exp. Aging Res.* **2012**, *38*, 295–314. [\[CrossRef\]](#)
41. Moore, T.J.; Sharma, B. Direct Surface Enhanced Raman Spectroscopic Detection of Cortisol at Physiological Concentrations. *Anal. Chem.* **2020**, *92*, 2052–2057. [\[CrossRef\]](#)
42. Sanchez, J.E.; Jaramillo, S.A.; Settles, E.; Salazar, J.J.V.; Lehr, A.; Gonzalez, J.; Aranda, C.R.; Navarro-Contreras, H.R.; Raniere, M.O.; Harvey, M.; et al. Detection of SARS-CoV-2 and Its S and N Proteins Using Surface Enhanced Raman Spectroscopy. *RSC Adv.* **2021**, *11*, 25788–25794. [\[CrossRef\]](#)
43. Shan, D.; Johnson, J.M.; Fernandes, S.C.; Suib, H.; Hwang, S.; Wuelfing, D.; Mendes, M.; Holdridge, M.; Burke, E.M.; Beauregard, K.; et al. N-Protein Presents Early in Blood, Dried Blood and Saliva during Asymptomatic and Symptomatic SARS-CoV-2 Infection. *Nat. Commun.* **2021**, *12*, 1931. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Batoulis, H.; Schmidt, T.H.; Weber, P.; Schloetel, J.-G.; Kandt, C.; Lang, T. Concentration Dependent Ion-Protein Interaction Patterns Underlying Protein Oligomerization Behaviours. *Sci. Rep.* **2016**, *6*, 24131. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Quevedo, M.; Karbstein, H.P.; Emin, M.A. Concentration-Dependent Changes in the Reaction Behavior of Whey Proteins: Diffusion-Controlled or Transition State-Controlled Reactions? *Food Hydrocoll.* **2021**, *118*, 106745. [\[CrossRef\]](#)
46. Yao, H.; Song, Y.; Chen, Y.; Wu, N.; Xu, J.; Sun, C.; Zhang, J.; Weng, T.; Zhang, Z.; Wu, Z.; et al. Molecular Architecture of the SARS-CoV-2 Virus. *Cell* **2020**, *183*, 730–738.e13. [\[CrossRef\]](#) [\[PubMed\]](#)

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## Article

# Biochemical Characterization of Human Salivary Extracellular Vesicles as a Valuable Source of Biomarkers

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**Simple Summary:** Extracellular vesicles (EVs) are an emerging source of biomarkers for a plethora of human disorders, ranging from neurodegenerative to cancer disorders. Most works focusing on biomarker discovery use blood as the EV source; however, although informative, EVs from blood are not easily separated from other particles and proteins circulating in blood vessels. Other authors have proposed the use of saliva as an EV source, while also finding limitations in the downstream analysis due to the limited amount of salivary EVs. Herein, we propose the isolation of salivary EVs and their subsequent characterization by Raman spectroscopy to obtain a comprehensive biochemical characterization that can be easily translated to diagnostics. The proposed method was successfully applied. The sensitivity of the Raman characterization of EVs was not limited by the low concentration of salivary EVs compared to serum EVs, and was able to provide a comprehensive characterization of EVs in a high throughput and repeatable manner. Consequently, our data suggest a new perspective on the use of salivary EVs when the interference of lipoproteins might hinder biomarker detection or when blood withdrawal is hampered. Raman spectroscopy can represent a turning point in the application of salivary EVs in clinics.



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**Abstract:** Extracellular vesicles (EVs) are natural nanoparticles secreted under physiological and pathological conditions. Thanks to their diagnostic potential, EVs are increasingly being studied as biomarkers of a variety of diseases, including neurological disorders. To date, most studies on EV biomarkers use blood as the source, despite different disadvantages that may cause an impure isolation of the EVs. In the present article, we propose the use of saliva as a valuable source of EVs that could be studied as biomarkers in an easily accessible biofluid. Using a comparable protocol for the isolation of EVs from both liquid biopsies, salivary EVs showed greater purity in terms of co-isolates (evaluated by nanoparticle tracking analysis and Conan test). In addition, Raman spectroscopy was used for the identification of the overall biochemical composition of EVs coming from the two different biofluids. Even considering the limited amount of EVs that can be isolated from saliva, the use of Raman spectroscopy was not hampered, and it was able to provide a comprehensive characterization of EVs in a high throughput and repeatable manner. Raman spectroscopy can thus represent a turning point in the application of salivary EVs in clinics, taking advantage of the simple method of collection of the liquid biopsy and of the quick, sensitive and label-free biophotonics-based approach.

**Keywords:** saliva; Raman spectroscopy; biomarkers; extracellular vesicles; diagnostics

## 1. Introduction

Extracellular vesicles (EVs) are known to be natural nanoparticles released by all cells in the body under physiological and pathological conditions. EVs perform their biological

functions by delivering proteins, metabolites and nucleic acids to the recipient cells, thereby ensuring cell communication. It has been shown that EVs carry and express the protein markers typical of the cell of origin, both inside the vesicles and on their membrane surface. Indeed, the main characteristic of EVs is to reflect the condition and content of the parental cell [1]. As a result, EVs have been increasingly studied over the years as biomarkers of a variety of diseases, including neurological disorders [2].

To date, most studies on EVs use blood as the liquid biopsy and EV source. Disadvantages related to the use of serum and plasma to isolate EVs are the presence of a high protein concentration (for example, albumin) and of lipoproteins that share dimensions and density with EVs, thus representing co-isolates in most isolation procedures [3]. Moreover, there are a plethora of pre-analytical variables that can affect the quality of the blood preparation, hamper the reproducibility of EV isolation and represent an obstacle in the validation of blood EVs as biomarkers [4]. As an example, it is not yet clear whether a fasting situation is necessary for the isolation of EVs; the plasma concentration of EVs has been measured using Nanoparticle Tracking Analysis (NTA) before and after eating a meal, and it showed that concentration did not vary significantly after eating a high-fat meal [5]. However, the plasma concentration of EVs is strongly correlated with the concentration of very low density lipoproteins (VLDL) in plasma and triglyceride in serum after meals, suggesting that the number of particles measured through NTA may be affected by the presence of these macromolecules. Furthermore, attention should also be paid to the type of test tube used. Indeed, samples obtained using tubes with Ethylenediaminetetraacetic Acid (EDTA) as blood anticoagulant showed the highest plasma concentrations of EVs, supporting the idea that EDTA promotes the formation of micro-vesicles due to platelet activation [5].

A biological fluid that could help in overcoming some of the limits of the use of blood as the EV source is saliva. Saliva is an optimal biological fluid for diagnostics as it contains a mixture of systemic molecules able to give information on the physiological and pathological state of a subject [6–9]. Furthermore, it presents the advantage of being a non-invasive and low-cost sampling method. Indeed, salivary samples are more easily collected compared to blood, as no specialized professionals are required [10], even though the composition of saliva can change according to the circadian cycle [11]. Due to its remarkable diagnostic potential, human saliva has been widely explored for the identification of oral [12,13] as well as systemic diseases [6–9,14,15]. Among its components, it has been reported that human saliva harbors EVs, namely salivary extracellular vesicles (SEVs) [16,17] that have a higher degree of purity compared to EVs from serum or plasma [18]. Due to the low amount of co-isolated proteins and the absence of lipoproteins, SEVs have been proposed as potential biomarkers of diseases ranging from cancer [19,20] to traumatic brain injury (TBI) [21]. Moreover, knowing that salivary glands release  $\alpha$ -synuclein in saliva [22], SEVs have been investigated in people with Parkinson's disease (PD) [23], suggesting the possibility of using SEVs as potential diagnostic biomarkers in multiple diseases, including neurodegenerative diseases, where early diagnosis is urgently needed. Nonetheless, the main limitation for the use of SEVs seems to be the paucity of EVs in saliva and, consequently, the limit of detection for the downstream method.

For these reasons, in this study, we propose the use of Raman spectroscopy (RS), a biophotonics-based method, for the characterization of the EV salivary content and the comparison of the physico-chemical features of EVs isolated from both saliva and serum from the same subjects. RS allows the evaluation of the overall biochemical composition from a tiny volume of sample without labelling, providing a unique spectral fingerprint of each sample with peaks corresponding to specific molecular bond vibrations. This technique has already been applied for the analysis of blood, saliva or EVs isolated from biofluids such as plasma or serum, or from specific cells, with diagnostic purposes, demonstrating the ability of RS to identify a biochemical profile of the sample, showing simultaneously its content in proteins, lipids and nucleic acids [24–27].

In this preliminary study, we describe a straightforward procedure for the collection of salivary samples, the isolation of SEVs and the subsequent analysis by RS [6–9]. The

concomitant analysis of SEVs and serum-derived EVs from the same subjects demonstrates differences that might relate to the cell source of the EV samples, to the protein corona and/or to co-isolated macromolecules. To the best of our knowledge, this is the first time that the biochemical characterization of EVs from saliva has been performed, and that RS analysis of EVs from serum and saliva were performed in parallel for the same subject, suggesting a new perspective for the use of SEVs when the interference of lipoproteins might hinder biomarker detection or when blood withdrawal is hampered.

## 2. Materials and Methods

### 2.1. Subject Recruitment and Sample Collection

In this study, five volunteers with good health status, and with no previous diagnosis of cardiovascular diseases, neurological or severe systemic disorders, were enrolled (average age 33.4 y/o; all subjects were female and non-smokers). According to the Helsinki Declaration, ethical approval was obtained from the Institution Ethical Committee and all recruited subjects signed informed consent forms before the collection of serum and saliva samples. Serum was obtained by the centrifugation of blood at 2500 g for 10 min at room temperature, and then stored at  $-80^{\circ}\text{C}$  before analysis. For the collection of saliva, Salivette<sup>®</sup> swabs were purchased from Sarstedt (Sarstedt AG & CO, Numbrecht, Germany). Each Salivette<sup>®</sup> contains a cotton swab that can filter saliva and retain any food residues. The saliva collection procedure was performed following the manufacturer's instructions. Briefly, the swab was placed in the mouth and chewed for one minute, at least two hours after the last meal and teeth brushing. Then, the swab was centrifuged at  $1000\times g$  for 5 min in order to recover the biofluid. Saliva samples were stored at  $-20^{\circ}\text{C}$  before analysis. This method was previously reported for the collection of saliva for Raman analysis, demonstrating no interference in the subsequent spectroscopic evaluation [6–9].

### 2.2. Isolation of Extracellular Vesicles

EVs were isolated from the serum and saliva of the enrolled subjects by Size Exclusion Chromatography (SEC) (qEV/70 nm series; Izon Science, Christchurch, New Zealand). Prior to loading onto the SEC column, serum and saliva samples were thawed and centrifuged at  $10,000\times g$  for 10 min. Saliva samples were also concentrated using Amicon 3K filters (Amicon Ultra, Sigma-Aldrich, St. Louis, MO, USA). A total of 500  $\mu\text{L}$  of each serum or saliva sample was loaded in the SEC column and fractions (500  $\mu\text{L}$  each) from 4 to 16 were collected.

### 2.3. EV Size Distribution

SEC fractions from 7 to 11 obtained from both serum and saliva were pooled together and characterized by NTA (NanoSight NS300; Malvern, Panalytical LTD, Malvern, UK) to evaluate the size distribution and concentrations of EVs. For the analysis, all samples were diluted in fresh filtered phosphate buffered saline (PBS) (saliva 1:10–serum 1:100) and then injected in the sample chamber through a syringe pump that provides a continuous flow of new particles into the sample chamber. Recordings of the movements of particles were collected for 60 s, five times for each sample. In parallel, the total protein content of the EV-containing fractions (7–11) was measured by BCA assay (Pierce BCA assay kit, Scientific, Waltham, MA, USA) after EV lysis in 1X RIPA buffer.

### 2.4. EVs Purity Assessment

In order to determine the purity of both serum EVs and SEVs, the Colorimetric NANoplasmonic (CONAN) Method was performed [28,29]. To start the CONAN assay, spherical gold nanoparticles (GNPs) were prepared using the classic Turkevich's citrate reduction method [30]. Briefly, 20 mL of tetrachloroauric acid solution ( $\text{HAuCl}_4$ ) 1 mM was prepared and boiled under stirring. Then, 2 mL of 1% sodium citrate was added and left to stir for 15 min. Thereafter, the solution was placed in an ice and  $\text{H}_2\text{O}$  bath. Finally, the diameter and concentration values of GNPs were calculated from the UV spectrum values

by referring to the tables in the literature [31]. GNPs with a concentration of 6nM were used for the CONAN assay, mixing EV samples with GNPs in a ratio of 1:1. The aggregation index (AI) % for each sample was calculated in order to assess the purity of the EVs. GNPs in H<sub>2</sub>O and GNPs in PBS were used as controls. For EV samples, the preparation can be considered as having an acceptable degree of purity when the AI is not greater than 50% [28].

### 2.5. Raman Analysis

Before the Raman analysis, the EV-containing fractions (7–11) obtained by SEC were ultra centrifuged at  $100,000 \times g$  for 70 min (L7-65; Rotor SW60; Beckman Coulter, Brea, CA, USA). A drop of freshly isolated and concentrated EVs (4  $\mu$ L of serum EVs and 20–30  $\mu$ L of SEVs) was deposited on a CaF<sub>2</sub> disk and dried at room temperature. The Raman analysis was performed using a Raman microscope Aramis (Horiba Jobin-Yvon, Palaiseau, France), equipped with a 532 nm laser source, and following a previously described method for the bulk characterization of EVs [32–34]. First, the instrument was calibrated using the silicon reference band at  $520.7 \text{ cm}^{-1}$ . For all the analysis, a  $50\times$  objective (Olympus, Tokyo, Japan) was used, with acquisition time of 30 s for two accumulations, diffraction grating at 1800 grooves/mm, 400  $\mu$ m entrance slit and hole at 600  $\mu$ m. Spectra were acquired using LabSpec6 software (Horiba Scientific) in the spectral ranges  $500\text{--}1800 \text{ cm}^{-1}$  and  $2700\text{--}3200 \text{ cm}^{-1}$  with a spectral resolution of  $0.8 \text{ cm}^{-1}/\text{step}$  for the first range and  $0.6 \text{ cm}^{-1}/\text{step}$  for the second range. For all samples, about 20 spectra were acquired for each drop, both at the edges and in the center of the drop area. Afterwards, all the acquired spectra were fit with a polynomial baseline, resized on the reference band at  $1554 \text{ cm}^{-1}$  for the first range and  $2938 \text{ cm}^{-1}$  for the second range. Normalization was performed through unit vector normalization in order to compensate for autofluorescence and background interference. Finally, adapting the Mihály's protocol for IR spectroscopy [35], Raman spectra were used to measure the spectroscopic ratios of nucleic acids–proteins (AC/P) and proteins–lipids (P/L) for both serum EVs and SEVs, as previously described [33].

### 2.6. Statistical Analysis

Raman spectra were analyzed by descriptive and multivariate statistical analysis using OriginPro2022 (OriginLab, Northampton, MA, USA) as previously described [32,33]. Average Raman spectra were obtained for serum-derived EVs and SEVs. The Mann–Whitney non-parametric test was used to compare data obtained from EV characterization (i.e., NTA analysis). The index was identified as statistically relevant for  $p$ -values  $< 0.05$ .

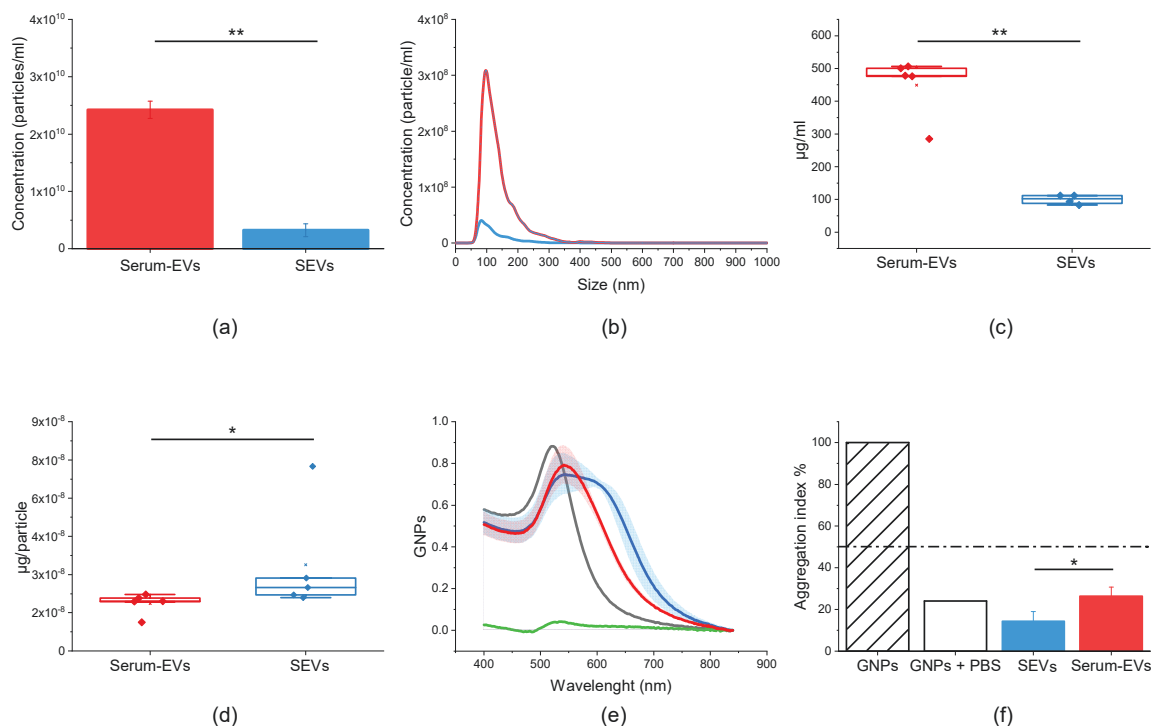
## 3. Results

### 3.1. EV Physico-Chemical Characterization

The EVs were isolated from all considered serum and saliva samples by SEC. The NTA analysis was performed on the pooled SEC fractions (from 7 to 11) containing EVs to evaluate their dimensions and concentrations. As expected, serum EVs (mean concentration:  $2.4 \times 10^{10}$  particles/mL) proved to be more concentrated than SEVs (mean concentration:  $3.2 \times 10^9$  particles/mL) ( $p = 0.012$ , Mann–Whitney test; Figure 1a). On the other hand, serum EVs and SEVs showed comparable size distribution (Figure 1b) and approximately the same mean sizes at 136 nm and 135 nm, respectively. Considering the total amount of proteins measured by BCA, EV samples isolated from serum contained a higher concentration of proteins compared to SEVs (Figure 1c) ( $p = 0.019$ , Mann–Whitney test), while the amount of proteins per particle was increased in SEVs compared to serum EVs, with a statistically significant difference ( $p = 0.036$ , Mann–Whitney test) (Figure 1d).

To investigate the source of the increased protein concentration per particle, the CONAN assay [28] was performed to evaluate the degree of purity of serum EVs and SEVs and to verify whether the protein corona could account for the increased protein concentration. As shown in Figure 1e, GNPs in contact with SEVs and serum EVs changed their localized surface plasmon resonance (LSPR) peak indicating aggregation. The CONAN results

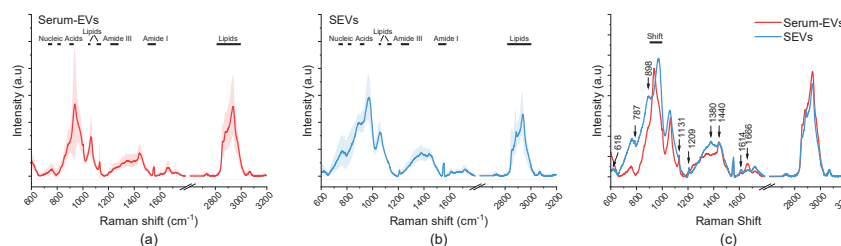
showed a mean AI equal to 14.33% for the SEV preparation, while serum EVs presented an AI equal to 26.4%, indicating a higher purity for the SEV preparation compared to the serum-derived EVs (Figure 1f).



**Figure 1.** Physico-chemical characterization of extracellular vesicles isolated from serum (serum EVs, red) and saliva (SEVs, blue). (a,b) Data obtained by NTA analysis allow the comparison of particle concentration (a) and size distribution (b) of vesicles obtained from serum and saliva. (c) Box plot showing the values of protein concentration obtained by colorimetric microBCA protein assay. (d) Purity of EV preparation expressed as the ratio of  $\mu\text{g}$  of protein per particle, calculated by colorimetric microBCA protein assay and NTA, respectively. (e) UV spectra of aqueous dispersion of GNPs 6nM (black), GNPs dispersed in 10 mM PBS (green), GNPs with SEVs (blue; mean and standard deviation) and GNPs with serum EVs (red; mean and standard deviation). (f) Aggregation index (AI%) for each sample obtained by the nanoplasmonic assay. \* indicates  $p < 0.05$ , \*\*  $p < 0.01$  after Mann–Whitney test.

### 3.2. Raman Analysis

The Raman analysis was performed starting from previously optimized parameters for EV characterization [32–34]. The biochemical fingerprint of EVs was obtained investigating two spectral ranges:  $500\text{--}1800\text{ cm}^{-1}$  and  $2700\text{--}3200\text{ cm}^{-1}$ . For the EVs isolated from serum ( $n = 5$ ) and saliva ( $n = 5$ ) samples, the average spectra ( $\pm$ standard deviation) were calculated demonstrating (i) a good signal-to-noise ratio in both samples and (ii) the applicability of the proposed spectroscopic procedure for saliva-derived EVs (Figure 2a,b). The fingerprint of EVs from both biofluids showed peaks in the Amide I and Amide III regions as well as bands in the spectral ranges accounting for lipid components and nucleic acids, as expected from previous studies [32,36,37]. For an easier visualization of the major spectral differences, the average spectra of serum-derived EVs and SEVs are overlapped in Figure 2c. As shown, the differences between the experimental groups are more prominent in the first spectral range ( $500\text{--}1800\text{ cm}^{-1}$ ). Indeed, some peaks overlap and others are shifted, in particular the average spectrum of SEVs presents a shifted peak at  $971\text{ cm}^{-1}$ , relating to the C–C stretch of carbohydrates.



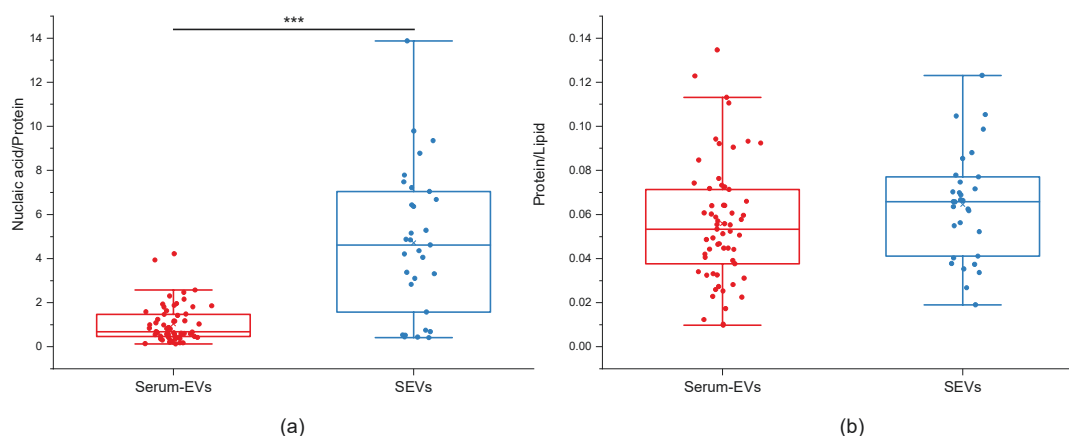
**Figure 2.** Raman fingerprint of serum- and saliva-derived EVs obtained using 532 nm laser line. (a) Average Raman spectrum of EVs isolated from serum samples of human healthy volunteers. (b) Average Raman spectrum of SEVs isolated from saliva samples of human healthy volunteers. In (a) and (b) shadow represent the standard deviation. (c) Overlapped spectra obtained from EVs isolated from serum (red) and saliva (blue) from the same subjects. Black lines on top of the graphs indicate some of the main macromolecular assignments.

Besides the presence of differences in the relative intensities of some peaks, serum EVs and SEVs presented characteristic peaks in their average spectra, as indicated by arrows in Figure 2c. As for SEVs, in the nucleic acids region, peaks at 618 cm<sup>-1</sup> and 787 cm<sup>-1</sup> are present that are absent in the mean spectrum of serum EVs, as well as peaks related to carbohydrates (898 cm<sup>-1</sup>) and proteins (1209 cm<sup>-1</sup>). Furthermore, the peak at 1380 cm<sup>-1</sup> relating to C-H bending is well evident in the SEV spectrum, but not in the one for serum EVs. As regards the spectral range attributable to Amide I, in the SEV spectrum there is a peak at 1616 cm<sup>-1</sup>, while a peak at 1666 cm<sup>-1</sup> is present for serum EVs. In the second range (2700–3200 cm<sup>-1</sup>), the main peaks related to lipids were observed both in the serum and saliva EV spectra, with a higher intensity in the spectrum of the serum EVs. The peak assignments reported in Table 1 were based on previous literature [32,38,39].

**Table 1.** Raman peak assignments. The table reports the assignments of the main peaks that can be identified in the overlapped spectra of serum-derived EVs and SEVs.

| Serum            |                                     | Saliva           |                                     |
|------------------|-------------------------------------|------------------|-------------------------------------|
| cm <sup>-1</sup> | Assignment                          | cm <sup>-1</sup> | Assignment                          |
| 760              | Tryptophan                          | 618              | C-C twisting (protein)              |
| 937              | C-C stretch                         | 760              | Tryptophan                          |
| 1002             | Phenylalanine                       | 787              | Phosphatidylserine                  |
| 1064             | Lipids                              | 898              | Monosaccharides                     |
| 1131             | Fatty acid                          | 971              | C-C stretch                         |
| 1207             | Hydroxyproline, Tyrosine            | 1060             | Lipids                              |
| 1440             | CH <sub>2</sub> lipids              | 1209             | Tryptophan and phenylalanine mode   |
| 1600–1800        | Amide I                             | 1290–1400        | CH-bending                          |
| 2700–3500        | Stretching vibrations of CH, NH, OH | 2700–3500        | Stretching vibrations of CH, NH, OH |
| 2800–3050        | Contributions from acyl chains      | 2800–3050        | Contributions from acyl chains      |
| 2929–2940        | CH <sub>2</sub> asymmetric stretch  | 2929–2940        | CH <sub>2</sub> asymmetric stretch  |

To better elucidate the differences between the serum EVs and SEVs, the spectroscopic ratios NA/P and P/L were calculated. The NA/P and P/L were obtained by dividing the relative intensities of the nucleic acids band (720–800 cm<sup>-1</sup>) by the Amide I band (1600–1690 cm<sup>-1</sup>), and the Amide I band (1600–1690 cm<sup>-1</sup>) by lipids band (2750–3040 cm<sup>-1</sup>), respectively. As shown in Figure 3a, the spectroscopic NA/P ratio obtained from SEV was statistically higher than the ratio obtained from serum EVs ( $p = 1.7 \times 10^{-7}$ —Mann–Whitney test) suggesting a difference in the nucleic acids load between the two experimental groups of samples. On the other hand, the spectroscopic L/P ratio showed no significant differences (Figure 3b,  $p > 0.05$ —Mann–Whitney test).



**Figure 3.** Spectroscopic nucleic acid-to-protein and protein-to-lipid ratios. Box plots showing the spectroscopic nucleic acid-to-protein ratio (nucleic acid/protein) in (a) and protein-to-lipid ratio (protein/lipid) in (b). Differences in the nucleic acid/protein and protein/lipid values obtained for serum-derived EVs and salivary EVs (SEVs) were compared. \*\*\* indicates  $p < 0.001$  after Mann–Whitney test.

#### 4. Discussion

In recent years, EVs have become an important object of study as potential disease biomarkers. However, one of the main difficulties is related to their small size, which makes it difficult to efficiently separate and prepare EVs, especially from complex liquid biopsies such as blood [4,5,19]. To date, there are still few studies that have used saliva as the biofluid for the isolation of EVs. It was previously reported that SEVs can carry cancer-cell-specific proteins and enter saliva from the blood in patients with lung cancer [19], and brain-derived EVs (L1CAM+) have also been found in the saliva of people with Parkinson's disease [23], as were amyloid-beta and p-tau markers of Alzheimer's disease [40]. However, one of the major limits for the use of saliva as a source of EVs for diagnostic purposes is the common knowledge that saliva EV concentration is significantly lower than in blood [41], although it contains most of the major soluble constituents of blood.

In the present preliminary study, we proposed the comparison of EVs isolated from serum and from saliva, in order to deepen the understanding of their biochemical features and reconsider the possibility of using SEVs as potential biomarkers in diagnostics. First of all, the collection of the two biological fluids took place, respectively, through blood sampling and through the mastication of salivary swabs (Salivette®). The use of a cotton swab for saliva collection allows the removal of any food residues and the filtering of any viscous salivary component in the saliva, which would be obstacles for the isolation of EVs [19]. We herein report that serum EVs are more concentrated than SEVs, confirming the data in the literature [41], although the concentration of serum EVs observed by NTA could be overestimated due to the co-isolation of lipoproteins, especially very low density lipoproteins (VLDL) that are smaller in size than EVs but have the same density. On the other hand, saliva does not contain lipoproteins, thus resulting in a lower concentration of isolated particles in saliva. In addition, we registered a higher concentration of total protein content in serum EVs compared to SEVs, allowing us to hypothesize that either serum EVs are loaded with a consistently higher amount of proteins, or that protein co-isolation occurs, or both. Indeed, it is well known that EVs that circulate in blood have the so-called protein corona, which consists of proteins that can be found anchored to the vesicles and cannot be separated from the outer membrane of EVs during isolation, some of them being part of the protein cargo adsorbed to EVs, but also partly recruited in body fluids after vesicle shedding [42,43]. The hypothesis of a more consistent protein corona on serum EVs compared to SEVs was supported by the CONAN assay results that showed a higher level

of purity for SEVs than for serum EVs, i.e., a reduced amount of co-isolated proteins. The principle of the CONAN assay is based on the high surface energy of the GNPs. Indeed, when the GNPs reacted with SEVs, the color of the solution immediately changed from red to blue, indicating an aggregation with the membrane proteins of the EVs that would not happen if other contaminants/co-isolates were present in the sample. Considering a 100% AI for a solution of not aggregated GNPs (GNPs in water), a lower AI% referred to a sample with few contaminants and we found that the serum EV samples were richer in co-isolates than the SEV samples. Finally, it is worth noting that the calculated ratio  $\mu\text{g}$  of protein per particle was greater for SEVs than for serum EVs, suggesting that SEVs could have a limited protein corona but a major protein load in their core.

To evaluate the biochemical features of EVs from blood and saliva, we took advantage of RS, a vibrational spectroscopy method that, in recent years, has been proven to ensure high chemical specificity and a fast analytical procedure also in the EV field. Specifically, RS allowed us to rapidly and accurately characterize EVs, providing detailed information on the average composition of external and inner molecules, demonstrating its remarkable potential for both basic research and translational EV analyses. In the present study, we applied a previously optimized acquisition protocol that was already proven to be able to comprehensively evaluate the biochemistry of a sample starting from tiny volumes of EV suspension [32–34]. The RS analysis revealed biochemical differences between the serum EVs and SEVs, both in terms of variations in the intensity and in the presence of some characteristic peaks. Indeed, the differences in the biochemical composition found in the Raman spectra of SEV and serum EVs could explain differences in the cellular origin of EVs circulating in the two biofluids and also differences in the molecules loaded and/or co-isolated with EVs. Major differences were found in the Amide regions, thus accounting for differences both in the internal cargo and in the protein corona, and in the carbohydrate components, that could be explained by differences in the glycosylation of proteins and lipids present within vesicles. Concerning the latter observation, it has to be noted that most EVs in saliva are expected to be originated from the respiratory tract, and thus the observed differences in the carbohydrates could account for the presence of mucins within or adsorbed to SEVs. Mucin is a highly glycosylated protein that is produced by the cells of ciliated epithelium of upper airways and it is known that vesicles originated from these cells express different forms of mucin on their surface (i.e., MUC1, MUC4 and MUC16) that protect against pathogens and maintain the structure of vesicles [44]. For this reason, we can speculate that mucin might explain some of the major differences in the Raman spectra of SEVs and serum EVs, although further studies are needed to verify its presence and the cellular origin of SEVs. Furthermore, it was observed that the spectroscopic ratio NA/P was statistically different between the mean spectra of SEV and serum EVs, while the P/L ratio was comparable in the two sample preparations. In this regard, we may speculate that SEVs may have a higher cargo of nucleic acids than serum EVs, being potentially valuable cargo also for RNA biomarkers. This is in line with previous literature demonstrating that SEVs have been found to carry tRNA-derived small RNAs (tsRNAs) which represent important biomarkers for the diagnosis and prognosis of esophageal squamous cell carcinoma [20], as well as carriers of genetic biomarkers for traumatic brain injury (TBI) [21].

## 5. Conclusions

In conclusion, we propose the use of RS for the characterization of salivary EVs, even with diagnostic purposes. Using a comparable protocol for both liquid biopsies, SEVs showed greater purity in terms of co-isolates, including both lipoproteins and protein corona. Moreover, the sensitivity of the Raman bulk characterization of EVs is not limited by the low concentration of SEVs compared to serum EVs, and it is able to provide a comprehensive characterization of EVs in a high throughput and repeatable manner. Consequently, our preliminary data suggest a new perspective on the use of SEVs when the interference of lipoproteins might hinder biomarker detection or when blood withdrawal is hampered. Raman spectroscopy can represent a turning point in the application of salivary

EVs in clinics, taking advantage of the simple method of collection of the liquid biopsy and of this quick, sensitive and label-free biophotonics-based approach.

**Author Contributions:** Conceptualization, A.G. and S.P.; formal analysis, V.M., A.G. and S.P.; investigation, V.M., F.R., A.D.P. and L.F.; writing—original draft preparation, V.M. and A.G.; writing—review and editing, S.P., F.R., A.D.P., L.F., R.A.R. and M.B.; supervision and funding acquisition, M.B. All authors have read and agreed to the published version of the manuscript.

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## References

- Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Théry, C. Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-to-Cell Communication. *Nat. Cell Biol.* **2019**, *21*, 9–17. [\[CrossRef\]](#) [\[PubMed\]](#)
- Hill, A.F. Extracellular Vesicles and Neurodegenerative Diseases. *J. Neurosci.* **2019**, *39*, 9269–9273. [\[CrossRef\]](#) [\[PubMed\]](#)
- Martins, T.S.; Vaz, M.; Henriques, A.G. A Review on Comparative Studies Addressing Exosome Isolation Methods from Body Fluids. *Anal. Bioanal. Chem.* **2022**, 1–25. [\[CrossRef\]](#) [\[PubMed\]](#)
- Clayton, A.; Boilard, E.; Buzas, E.I.; Cheng, L.; Falcón-Perez, J.M.; Gardiner, C.; Gustafson, D.; Gualerzi, A.; Hendrix, A.; Hoffman, A.; et al. Considerations towards a Roadmap for Collection, Handling and Storage of Blood Extracellular Vesicles. *J. Extracell. Vesicles* **2019**, *8*, 1647027. [\[CrossRef\]](#)
- Jamaly, S.; Ramberg, C.; Olsen, R.; Latysheva, N.; Webster, P.; Sovershaev, T.; Brækkan, S.K.; Hansen, J.-B. Impact of Preanalytical Conditions on Plasma Concentration and Size Distribution of Extracellular Vesicles Using Nanoparticle Tracking Analysis. *Sci. Rep.* **2018**, *8*, 17216. [\[CrossRef\]](#)
- Carlomagno, C.; Banfi, P.I.; Gualerzi, A.; Picciolini, S.; Volpato, E.; Meloni, M.; Lax, A.; Colombo, E.; Ticozzi, N.; Verde, F.; et al. Human Salivary Raman Fingerprint as Biomarker for the Diagnosis of Amyotrophic Lateral Sclerosis. *Sci. Rep.* **2020**, *10*, 1–13. [\[CrossRef\]](#)
- Carlomagno, C.; Bertazioli, D.; Gualerzi, A.; Picciolini, S.; Banfi, P.I.; Lax, A.; Messina, E.; Navarro, J.; Bianchi, L.; Caronni, A.; et al. COVID-19 Salivary Raman Fingerprint: Innovative Approach for the Detection of Current and Past SARS-CoV-2 Infections. *Sci. Rep.* **2021**, *11*, 4943. [\[CrossRef\]](#)
- Carlomagno, C.; Gualerzi, A.; Picciolini, S.; Rodà, F.; Banfi, P.I.; Lax, A.; Bedoni, M. Characterization of the COPD Salivary Fingerprint through Surface Enhanced Raman Spectroscopy: A Pilot Study. *Diagnostics* **2021**, *11*, 508. [\[CrossRef\]](#)
- Carlomagno, C.; Bertazioli, D.; Gualerzi, A.; Picciolini, S.; Andrico, M.; Rodà, F.; Meloni, M.; Banfi, P.I.; Verde, F.; Ticozzi, N.; et al. Identification of the Raman Salivary Fingerprint of Parkinson's Disease Through the Spectroscopic—Computational Combinatory Approach. *Front. Neurosci.* **2021**, *15*, 704963. [\[CrossRef\]](#)
- Kaczor-Urbanowicz, K.E.; Carreras-Presas, C.M.; Aro, K.; Tu, M.; Garcia-Godoy, F.; Wong, D.T.W. Saliva Diagnostics—Current Views and Directions. *Exp. Biol. Med.* **2017**, *242*, 459–472. [\[CrossRef\]](#)
- Dawes, C. Circadian Rhythms in Human Salivary Flow Rate and Composition. *J. Physiol.* **1972**, *220*, 529–545. [\[CrossRef\]](#)
- Hu, S.; Arellano, M.; Boontheung, P.; Wang, J.; Zhou, H.; Jiang, J.; Elashoff, D.; Wei, R.; Loo, J.A.; Wong, D.T. Salivary Proteomics for Oral Cancer Biomarker Discovery. *Clin. Cancer Res.* **2008**, *14*, 6246–6252. [\[CrossRef\]](#)
- Hu, S.; Wang, J.; Meijer, J.; Jeong, S.; Xie, Y.; Yu, T.; Zhou, H.; Henry, S.; Vissink, A.; Pijpe, J.; et al. Salivary Proteomic and Genomic Biomarkers for Primary Sjögren's Syndrome. *Arthritis Rheum* **2007**, *56*, 3588–3600. [\[CrossRef\]](#)
- Zhang, L.; Xiao, H.; Karlan, S.; Zhou, H.; Gross, J.; Elashoff, D.; Akin, D.; Yan, X.; Chia, D.; Karlan, B.; et al. Discovery and Preclinical Validation of Salivary Transcriptomic and Proteomic Biomarkers for the Non-Invasive Detection of Breast Cancer. *PLoS ONE* **2010**, *5*, e15573. [\[CrossRef\]](#)
- Xiao, H.; Zhang, Y.; Kim, Y.; Kim, S.; Kim, J.J.; Kim, K.M.; Yoshizawa, J.; Fan, L.-Y.; Cao, C.-X.; Wong, D.T.W. Differential Proteomic Analysis of Human Saliva Using Tandem Mass Tags Quantification for Gastric Cancer Detection. *Sci. Rep.* **2016**, *6*, 22165. [\[CrossRef\]](#)
- Winck, F.V.; Prado Ribeiro, A.C.; Ramos Domingues, R.; Ling, L.Y.; Riaño-Pachón, D.M.; Rivera, C.; Brandão, T.B.; Gouvea, A.F.; Santos-Silva, A.R.; Coletta, R.D.; et al. Insights into Immune Responses in Oral Cancer through Proteomic Analysis of Saliva and Salivary Extracellular Vesicles. *Sci. Rep.* **2015**, *5*, 16305. [\[CrossRef\]](#)
- Xiao, H.; Wong, D.T.W. Proteomic Analysis of Microvesicles in Human Saliva by Gel Electrophoresis with Liquid Chromatography-Mass Spectrometry. *Anal. Chim. Acta* **2012**, *723*, 61–67. [\[CrossRef\]](#)

18. Deutsch, O.; Fleissig, Y.; Zaks, B.; Krief, G.; Aframian, D.J.; Palmon, A. An Approach to Remove Alpha Amylase for Proteomic Analysis of Low Abundance Biomarkers in Human Saliva. *Electrophoresis* **2008**, *29*, 4150–4157. [\[CrossRef\]](#)
19. Sun, Y.; Xia, Z.; Shang, Z.; Sun, K.; Niu, X.; Qian, L.; Fan, L.-Y.; Cao, C.-X.; Xiao, H. Facile Preparation of Salivary Extracellular Vesicles for Cancer Proteomics. *Sci. Rep.* **2016**, *6*, 24669. [\[CrossRef\]](#)
20. Li, K.; Lin, Y.; Luo, Y.; Xiong, X.; Wang, L.; Durante, K.; Li, J.; Zhou, F.; Guo, Y.; Chen, S.; et al. A Signature of Saliva-Derived Exosomal Small RNAs as Predicting Biomarker for Esophageal Carcinoma: A Multicenter Prospective Study. *Mol. Cancer* **2022**, *21*, 21. [\[CrossRef\]](#)
21. Cheng, Y.; Pereira, M.; Raukar, N.; Reagan, J.L.; Queseneberry, M.; Goldberg, L.; Borgovan, T.; LaFrance, W.C.; Dooner, M.; Deregis, M.; et al. Potential Biomarkers to Detect Traumatic Brain Injury by the Profiling of Salivary Extracellular Vesicles. *J. Cell. Physiol.* **2019**, *234*, 14377–14388. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Devic, I.; Hwang, H.; Edgar, J.S.; Izutsu, K.; Presland, R.; Pan, C.; Goodlett, D.R.; Wang, Y.; Armaly, J.; Tumas, V.; et al. Salivary  $\alpha$ -Synuclein and DJ-1: Potential Biomarkers for Parkinson's Disease. *Brain* **2011**, *134*, e178. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Rani, K.; Mukherjee, R.; Singh, E.; Kumar, S.; Sharma, V.; Vishwakarma, P.; Bharti, P.S.; Nikolajeff, F.; Dinda, A.K.; Goyal, V.; et al. Neuronal Exosomes in Saliva of Parkinson's Disease Patients: A Pilot Study. *Park. Relat. Disord.* **2019**, *67*, 21–23. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Rodà, F.; Bedoni, M. Biophotonics for Diagnostic Detection of Extracellular Vesicles. *Adv. Drug Deliv. Rev.* **2021**, *174*, 229–249. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Carlomagno, C.; Cabinio, M.; Picciolini, S.; Gualerzi, A.; Baglio, F.; Bedoni, M. SERS-based Biosensor for Alzheimer Disease Evaluation through the Fast Analysis of Human Serum. *J. Biophotonics*. **2020**. [\[CrossRef\]](#)
26. Krafft, C.; Popp, J. The Many Facets of Raman Spectroscopy for Biomedical Analysis. *Anal. Bioanal. Chem.* **2015**, *407*, 699–717. [\[CrossRef\]](#)
27. Hardy, M.; Kelleher, L.; Gomes, P.d.C.; Buchan, E.; Chu, H.O.M.; Oppenheimer, P.G. Methods in Raman Spectroscopy for Saliva Studies—A Review. *Appl. Spectrosc. Rev.* **2022**, *57*, 177–233. [\[CrossRef\]](#)
28. Zendrini, A.; Paolini, L.; Busatto, S.; Radeghieri, A.; Romano, M.; Wauben, M.H.M.; van Herwijnen, M.J.C.; Nejsun, P.; Borup, A.; Ridolfi, A.; et al. Augmented Colorimetric Nanoplasmonic (CONAN) Method for Grading Purity and Determine Concentration of EV Microliter Volume Solutions. *Front. Bioeng. Biotechnol.* **2020**, *7*, 452. [\[CrossRef\]](#)
29. Maiolo, D.; Paolini, L.; Di Noto, G.; Zendrini, A.; Berti, D.; Bergese, P.; Ricotta, D. Colorimetric Nanoplasmonic Assay To Determine Purity and Titrate Extracellular Vesicles. *Anal. Chem.* **2015**, *87*, 4168–4176. [\[CrossRef\]](#)
30. Turkevich, J.; Stevenson, P.C.; Hillier, J. A Study of the Nucleation and Growth Processes in the Synthesis of Colloidal Gold. *Discuss. Faraday Soc.* **1951**, *11*, 55. [\[CrossRef\]](#)
31. Haiss, W.; Thanh, N.T.K.; Aveyard, J.; Fernig, D.G. Determination of Size and Concentration of Gold Nanoparticles from UV–Vis Spectra. *Anal. Chem.* **2007**, *79*, 4215–4221. [\[CrossRef\]](#)
32. Gualerzi, A.; Niada, S.; Giannasi, C.; Picciolini, S.; Morasso, C.; Vanna, R.; Rossella, V.; Masserini, M.; Bedoni, M.; Ciceri, F.; et al. Raman Spectroscopy Uncovers Biochemical Tissue-Related Features of Extracellular Vesicles from Mesenchymal Stromal Cells. *Sci. Rep.* **2017**, *7*, 1–11. [\[CrossRef\]](#)
33. Gualerzi, A.; Kooijmans, S.A.A.; Niada, S.; Picciolini, S.; Brini, A.T.; Camussi, G.; Bedoni, M. Raman Spectroscopy as a Quick Tool to Assess Purity of Extracellular Vesicle Preparations and Predict Their Functionality. *J. Extracell. Vesicles* **2019**, *8*, 1568780. [\[CrossRef\]](#)
34. Gualerzi, A.; Picciolini, S.; Carlomagno, C.; Terenzi, F.; Ramat, S.; Sorbi, S.; Bedoni, M. Raman Profiling of Circulating Extracellular Vesicles for the Stratification of Parkinson's Patients. *Nanomed. Nanotechnol. Biol. Med.* **2019**, *22*, 102097. [\[CrossRef\]](#)
35. Mihály, J.; Deák, R.; Szigyártó, I.C.; Bóta, A.; Beke-Somfai, T.; Varga, Z. Characterization of Extracellular Vesicles by IR Spectroscopy: Fast and Simple Classification Based on Amide and C[Sbnd]H Stretching Vibrations. *Biochim. Et Biophys. Acta Biomembr.* **2017**, *1859*, 459–466. [\[CrossRef\]](#)
36. Tatischeff, I.; Larquet, E.; Falcón-Pérez, J.M.; Turpin, P.-Y.; Kruglik, S.G. Fast Characterisation of Cell-Derived Extracellular Vesicles by Nanoparticles Tracking Analysis, Cryo-Electron Microscopy, and Raman Tweezers Microspectroscopy. *J. Extracell. Vesicles* **2012**, *1*, 19179. [\[CrossRef\]](#)
37. Krafft, C.; Wilhelm, K.; Eremin, A.; Nestel, S.; von Bubnoff, N.; Schultze-Seemann, W.; Popp, J.; Nazarenko, I. A Specific Spectral Signature of Serum and Plasma-Derived Extracellular Vesicles for Cancer Screening. *Nanomed. Nanotechnol. Biol. Med.* **2017**, *13*, 835–841. [\[CrossRef\]](#)
38. Movasaghi, Z.; Rehman, S.; Rehman, I.U. Raman Spectroscopy of Biological Tissues. *Appl. Spectrosc. Rev.* **2007**, *42*, 493–541. [\[CrossRef\]](#)
39. Ricciardi, A.; Piuri, G.; Porta, M.D.; Mazzucchelli, S.; Bonizzi, A.; Truffi, M.; Sevieri, M.; Allevi, R.; Corsi, F.; Cazzola, R.; et al. Raman Spectroscopy Characterization of the Major Classes of Plasma Lipoproteins. *Vib. Spectrosc.* **2020**, *109*, 103073. [\[CrossRef\]](#)
40. Rastogi, S.; Rani, K.; Kumar, S. Progression of Cognitive Impairment to Alzheimer's Disease: Through the Lens of Salivary Extracellular Vesicles. *J. Exp. Neurosci.* **2021**, *16*, 263310552110586. [\[CrossRef\]](#)
41. Yuana, Y.; Böing, A.N.; Grootemaat, A.E.; van der Pol, E.; Hau, C.M.; Cizmar, P.; Buhr, E.; Sturk, A.; Nieuwland, R. Handling and Storage of Human Body Fluids for Analysis of Extracellular Vesicles. *J. Extracell. Vesicles* **2015**, *4*, 29260. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Radeghieri, A.; Alacqua, S.; Zendrini, A.; Previcini, V.; Todaro, F.; Martini, G.; Ricotta, D.; Bergese, P. Active Antithrombin Glycoforms Are Selectively Physiosorbed on Plasma Extracellular Vesicles. *J. Extracell. Biol.* **2022**, *1*, e57. [\[CrossRef\]](#)

43. Rai, A.; Fang, H.; Claridge, B.; Simpson, R.J.; Greening, D.W. Proteomic Dissection of Large Extracellular Vesicle Surfaceome Unravels Interactive Surface Platform. *J. Extracell. Vesicles* **2021**, *10*, e12164. [[CrossRef](#)] [[PubMed](#)]
44. Kesimer, M.; Scull, M.; Brighton, B.; DeMaria, G.; Burns, K.; O'Neal, W.; Pickles, R.J.; Sheehan, J.K. Characterization of Exosome-like Vesicles Released from Human Tracheobronchial Ciliated Epithelium: A Possible Role in Innate Defense. *FASEB J.* **2009**, *23*, 1858–1868. [[CrossRef](#)]

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# Circulating particles carotenoids are associated with rehabilitation recovery in Parkinson's disease

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## ABSTRACT

Circulating anoparticles include Extracellular vesicles (EVs) and other circulating EV-like particles that are known mediators of the trafficking of pathologic proteins associated with Parkinson's disease (PD) progression as well as of products of the oxidative damage that characterizes the disease. In the framework of the VIRTREAD-PD trial protocol, circulating particles were isolated from the serum of 30 subjects with PD before and after an intensive rehabilitation program using a treadmill. Raman spectroscopy was used to verify modifications in the biochemistry of circulating particles and proved that an intensive motor rehabilitation program could affect the molecular composition of EV-like particles in the blood of people with PD (pwPD). A analysis performed before and after 8 weeks of intensive rehabilitation demonstrate significant changes in the biomolecules associated with EV-like particles and in carotenoid content. The presence of antioxidants linked to circulating particles was proved to be informative for the profiling of pwPD at admission and prognostic of the rehabilitation recovery.

In conclusion, our data support the hypothesis that circulating particles have a dual role in PD, both in the neurodegenerative processes and in the response to rehabilitation. Besides, the Raman fingerprint and the spectral signature of carotenoids can represent measurable biomarkers of rehabilitation.

## 1. Introduction

Parkinson's disease (PD) is a complex neurodegenerative disorder characterized by bradykinesia, tremor, rigidity, and postural instability [1]. It is the second most common neurodegenerative disorder and it is estimated to affect about 10 million people worldwide with a prevalence of about 2 % in the over-80 population.

From a biological point of view, the mechanisms underlying PD progression can be summarized into two main events: i) accumulation and aggregation of misfolded protein strains of  $\alpha$ -synuclein ( $\alpha$ syn) within the brain tissue with neurotoxic effect; ii) disruption of the

physiologic redox potential and oxidative damage to key cellular component leading to cell death [2]. These two mechanisms are closely interconnected, with the deposition of pathological protein aggregates of  $\alpha$ syn strictly related to brain oxidative damage and increased apoptotic events, that ultimately contribute to tissue degeneration and disease progression.

Clinically, treatments include dopamine-based pharmacologic therapies that can ameliorate patients' motor symptoms but cannot stop either neurodegeneration or disease progression that may lead to serious complications. In selected advanced cases, Deep Brain Stimulation represents a surgical treatment option for delivering an adjustable signal inside the dysfunctional nigrostriatal circuitry that characterizes the

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**Abbreviations**

|           |                                               |
|-----------|-----------------------------------------------|
| AUC       | Area Under the Curve                          |
| AVR       | Augmented Virtual Reality                     |
| BDI       | Beck's Depression Inventory                   |
| CV        | Canonical variable                            |
| EVs       | Extracellular Vesicles                        |
| HY scale  | Hoehn and Yahr scale                          |
| LDA       | Linear Discriminant Analysis                  |
| LOOCV     | Leave-One-Out Cross-Validation                |
| mBI       | Modified Barthel Index                        |
| MDS-UPDRS | Movement Disorder Society-Unified Parkinson's |

|        |                                                    |
|--------|----------------------------------------------------|
|        | Disease Rating Scale                               |
| MoCA   | Montreal Cognitive Assessment                      |
| NTA    | Nanoparticle Tracking Analysis                     |
| PCA    | Principal Component Analysis                       |
| PD     | Parkinson's Disease                                |
| POMA   | Performance Oriented Mobility Assessment           |
| POMA-B | Performance Oriented Mobility Assessment – Balance |
| POMA-G | Performance Oriented Mobility Assessment – Gait    |
| wpd    | People with Parkinson's Disease                    |
| ROS    | Reactive Oxygen Species                            |
| RS     | Raman Spectroscopy                                 |
| TT     | Treadmill Training                                 |

disease [3].

Rehabilitation has long been considered an adjuvant to pharmacological treatments with the aim of maximizing functional abilities, improving quality of life, and minimizing secondary complications. More recently, rehabilitation has been recognized as a pivotal intervention for people with PD (pwPD), with intensive exercise improving walking speed and balance [4]. However, PD is characterized by a relatively large phenotypic heterogeneity, and it is unclear whether specific clinical subtypes may adapt differently to the rehabilitative intervention. Clinical evidence on rehabilitation efficacy has long relied on poor quality studies, largely heterogeneous, based on empirical experience. A pressing need in rehabilitation medicine is the identification of specific biomarkers (“rehabilomics”), i.e., measurable indicators, that can resume individuals' biological characteristics to tailor a personalized approach in rehabilitation and/or verify the therapeutic effect of a particular intervention or clinical decision [5].

In this regard, Extracellular vesicles (EVs) are membrane-bound nanoparticles released within the bloodstream by all body tissues, known to be characterized by typical protein markers (i.e. tetraspanins) that can help recapitulate their biogenetic pathway, and by common physicochemical properties [6]. Nonetheless, EV is a general term that includes nanoparticles with molecular and structural heterogeneity that make them difficult to be separated by particles (EV-like particles) with similar size and density in the bloodstream, including lipoproteins [6,7]. The role of EVs and EV-like particles in PD pathogenesis has been extensively proposed [8], envisioning circulating nanoparticles as vehicles and key players in the transfer of  $\alpha$ syn throughout the body, in the spreading of misfolded  $\alpha$ syn, tau, and other factors and in the disease propagation [9–11]. However, EV-like particles do not carry just pathological proteins, but also bioactive mediators of brain remodelling, and were suggested as valuable sources of biomolecular information about muscle-brain crosstalk in rehabilitation [12,13]. Specifically, in wPD undergoing rehabilitation, circulating nanoparticles can have a dual role being involved in both the neurodegenerative processes and in the response to rehabilitation therapy. For example, physical exercise can dramatically change the concentration and biochemistry of blood-derived EVs [14,15], reduce chronic oxidative stress (increased mitochondria biogenesis and autophagy up-regulation), and stimulate the synthesis of neurotransmitters and trophic factors [16]. The release of multiple factors, like protein, microRNA, and DNA, can also be shuttled as cargo of circulating nanoparticles [17]. Interestingly, changes in EV cargo were specifically attributed to oxidative stress as reactive oxygen species (ROS) reduction, and detoxification can be mediated by EV-like particles, but ROS can play a role also in the reduction of EVs themselves [18].

In the effort to understand the complex interplay among biomarkers, rehabilitation, and neurodegeneration, the primary aim of this study was to identify markers of neuroplasticity and brain remodelling potentially associated with the beneficial effects of an intensive motor rehabilitation program, by detecting changes in EVs and EV-like

particles isolated from the bloodstream of pwPD. The present work was conducted within the wider framework of the VIRTREAD-PD protocol (Clinicaltrials ID: NCT05902065), a randomized controlled single-blind trial that compares intensive treadmill training (TT) rehabilitative program with and without augmented virtual reality (AVR) in wPD characterized by gait disturbances [19]. Taking into account previous considerations, one of the main questions was to assess whether and how the biochemical content of EV-like particles may change according to the rehabilitation program and its outcome, exploring also the identification of potential prognostic markers.

## Materials and methods

### 2.1. Patient recruitment and sample processing

In the framework of the VIRTREAD-PD trial protocol (Clinicaltrials ID: NCT05902065), a cohort of 42 pwPD was recruited at IRCCS Don Gnocchi of Fondazione Don Gnocchi (Florence, Italy), after approval by the local Institutional Review Board (Comitato Etico Regione Toscana – Area Aosta Centro) on the July 5, 2022 (Approval Number: 20915 spe). Adult patients were enrolled after signing written informed consent, according to previously published inclusion/exclusion criteria [19]. Recruited subjects were randomized into two groups to take part in different types of rehabilitative intervention: the conventional TT (control group) and the TT integrated with AVR (the TT + AVR arm; experimental group). Blood was collected at recruitment (T0) and post-rehabilitation (T1, after 8 weeks of treatment) for all subjects. Serum was separated by centrifugation for 10 min at 2500×g at room temperature, then aliquoted and stored at –80 °C until use. EV-like particles were isolated from 30 pwPD with a combinatorial rocedure based on size exclusion chromatography and ultracentrifugation (see [Supplementary Material](#)). The isolation could not be performed on all recruited subjects due to limited volumes of serum collected.

The characterization of EV-like particles was performed by Nanoparticle Tracking Analysis (NTA) to evaluate their size distribution and their concentration. Mean concentration and mean size were calculated based on NTA results for all samples at both time points, to determine any statistical difference between T0 and T1 samples, without clustering data according to the rehabilitation protocol. EV-associated molecules were detected by dot blot to verify the enrichment of blood-derived EVs in the nanoparticle suspension (Detailed method description in [Supplementary Material](#)).

### 2.2. Clinical and instrumental assessment

For a comprehensive overview of the clinical and instrumental assessments, along with their respective administration timelines, please refer to the study protocol published by Lombardi et al. [19]. The objective of this investigation was to explore the correlation between biological, clinical, and functional outcomes. To address this objective,

data from the assessments conducted at T0 and T1 using the following scales and tests was analysed.

1. The Performance Oriented Mobility Assessment (POMA) [20] in both its gait (POMA-G) and balance (POMA-B) subscales.
2. The MDS-UPDRS part III [21,22].
3. The modified Barthel Index (mBI) [23].
4. The Montreal Cognitive Assessment (MOCA) [24].
5. The Beck's Depression Inventory (BDI) [25].

Furthermore, the study utilised the OptoGait system (Optogait, Microgate S.r.l., Bolzano, Italy) in conjunction with two pairs of photocells (WITTY, Microgate Srl; Bolzano, Italy; 0.001-s accuracy) to assess the overground gait parameters. Participants were instructed to walk at both a self-selected speed and a high speed, defined as the maximal speed that can be safely maintained over a limited distance. All gait parameters were measured using the central 4 m corridor, excluding the acceleration and deceleration periods registered at the commencement and cessation of walking phases, respectively. This methodological approach has been employed to ensure the measurement of steady-state walking data. In the present study, the following gait parameters were considered: gait speed at self-selected walking speed (Gait Speed Self) and at maximal speed (Gait Speed Max), step and stride lengths at self-selected walking speed (Step Length Self and Gait Cycle Self) and at maximal speed (Step Length Max and Gait Cycle Max), and cadence, defined as the number of steps per minute at self-selected (Cadence Self) and maximal (Cadence Max) speeds.

### 2.3. Raman analysis

Samples were prepared for Raman spectroscopy (RS) analysis by depositing 5 µl of freshly isolated EV-like particles onto a calcium fluoride slide and left at room temperature until completely dried. Drops were then analysed using a Raman spectroscope (LabRAM Aramis, Horiba Jobin Yvon S.A.S, Lille, France) equipped with a 532 nm laser source, daily calibrated with a silicon reference substrate. Particle characterization was performed following a previously optimized protocol [26]. Briefly, acquisition parameters were as follows: 30 s acquisition time, 1800 grooves/mm diffraction grating, 400 µm entrance slit and 600 µm hole, in the spectral ranges 600–1800 cm<sup>-1</sup> and 2600–3200 cm<sup>-1</sup>. An average of 10 spectra were acquired for each sample at the border of the dried drop, in random spots, taking advantage of the “coffee ring” effect, allowing each spectrum to describe the biochemical features of small particle clusters and possible co-isolated factors. In parallel, Raman spectra of the pure molecules of β-carotene and lutein were obtained using the same acquisition parameters here described (Supplementary Material and Supplementary Fig. 4).

### 2.4. Data analysis

Data were analysed using LabSpec6 (Horiba, France) and Origin-Lab23b software (Origin Lab, Northampton). Raman data were processed to remove the contribution of fluorescence, baseline corrected, and normalized to allow comparison between subjects.

The area under the curve (AUC) of the most relevant Raman peaks was also calculated. The angular coefficients of the line joining the corresponding AUC values at T0 and T1 (AUC slope) for each sample were also calculated. Multivariate statistical analysis was performed to reduce data dimensions and evaluate the ability of the Raman fingerprint to discriminate between the considered samples. Briefly, Principal Component Analysis (PCA) was used to reduce data dimensions. To evaluate the diversity between pre- and post-rehabilitation samples, the first 15 principal components (PCs) were extracted and used to obtain a classification model by linear discriminant analysis (LDA), which provided the Canonical Variate (CV) scores. A leave-one-out cross-validation (LOOCV) analysis was performed on the resulting CV. To further

evaluate statistical differences between T0 and T1 mean spectra, a non-parametric Wilcoxon paired signed rank test was applied to the CV scores.

Then, keeping age and disease duration as co-variables, partial correlation analysis was performed between Raman data and the main clinical scales used to profile patients before (to assess their status) and after (to monitor their status and evaluate the prognostic significance) rehabilitation. Detailed description of clinical assessments and statistical methods is reported in [Supplementary Material](#).

ROC curve analysis was used to evaluate the accuracy of our model to distinguish between responders and non-responders to rehabilitation by observing the AUC of carotenoid peaks in relation to functional improvement (MBI).

## 3. Result

### 3.1. Baseline characteristics of subjects

A cohort of 30 pwPD was successfully recruited and considered for the identification of the biochemical profile of circulating particles. For all subjects, demographic and clinical information were obtained as summarized in [Table 1](#).

### 3.2. Clinical results

[Table 2](#) presents the median values (with minimum, maximum, and interquartile range) for clinical, cognitive, and gait-related outcomes at two time points (T0 and T1) in the considered cohort of participants, along with their associated p-values. None of the variables was normally distributed thus non-parametric statistics Wilcoxon signed-rank test for paired samples was employed. The median (IQR) MDS-UPDRS part III score was found to be 33 (14/50; 15) at T0 and 35 (9/57; 13.5) at T1 ( $p = 0.23$ ), while the median BDI score remained unchanged at 11 (T0: 3/28; 8; T1: 2/38; 10,  $p = 0.44$ ). The median MBI score remained at 100, with minor alterations in range ( $p = 0.31$ ), and the median MoCA score shifts from 23 (19.1/29.2; 2.56) to 23.3 (14.8/30.2; 5.46) ( $p = 0.47$ ). Minor alterations were evident in the POMA-B and POMA-G scores, with POMA-B at 13 (5/16) at both time points ( $p = 0.566$ ) and POMA-G showing a slight increase from 9 (5/11) to 10 (5/12) ( $p = 0.211$ ). The total median POMA score shifts from 22 (13/26; 4) to 22.5 (11/27; 4.17) ( $p = 0.18$ ). Several gait parameters showed significant improvement. Specifically, the median self-selected cadence escalates from 108 (89.4/128; 16.5) steps per minute at T0 to 112 (82.7/134; 8.96) at T1 ( $p = 0.007$ ), while median maximum cadence raised from 125 (102/164; 16.9) to 128 (99.6/178; 14.1) ( $p = 0.029$ ). Median self-selected gait speed increased from 0.99 m/s (0.53/1.42; 0.44) to 1.15 m/s (0.450/

**Table 1**

**Subject recruitment.** The main demographic parameters of recruited subjects are reported. Information about their clinical history is provided. MDS-UPDRS III: Movement Disorder Society- Unified Parkinson's Disease Rating Scale third part; HY: Hoehn and Yahr scale; TT: treadmill training; AVR: augmented virtual reality.

| Subject recruitment          |                                                                                |
|------------------------------|--------------------------------------------------------------------------------|
| Mean age ± 1SD               | Years: 73.2 ± 8.9                                                              |
| Sex                          | Males: 10<br>Females: 20                                                       |
| Disease form at onset        |                                                                                |
| Bradykinesia                 | 7                                                                              |
| Resting tremor               | 15                                                                             |
| Mixed form                   | 8                                                                              |
| MDS - UPDRS III              | 14–57                                                                          |
| HY                           | HY2: 18 subjects;<br>HY3: 12 subjects;                                         |
| Mean Disease duration ± 1SD  | Years: 8.55 ± 5.34                                                             |
| Rehabilitation treatment arm | TT (control group): 15 subjects;<br>TT + AVR (experimental group): 15 subjects |

**Table**

**Clinical and instrumental assessments before and after the treatment.** Data from 30 participants are reported. Median values with minimum, maximum, and interquartile range for clinical, cognitive, and gait-related outcomes at two time points (T0 and T1) are reported. \* Significance is set for  $p < 0.05$ . MDS-UPDRS = Movement Disorder Organization - Unified Parkinson's Disease Rating Scale; BDI = Beck's Depression Inventory; MBI = Modified Barthel Index; POMA-B = Performance Oriented Mobility Assessment Balance; POMA-G = Performance Oriented Mobility Assessment Gait.

| Outcomes                 | T0                      | T1                     | p      |
|--------------------------|-------------------------|------------------------|--------|
| MDS-UPDRS III            | 33 (14/50; 15)          | 35 (9/57; 13.5)        | 0.23   |
| BDI                      | 11 (3/28; 8)            | 11 (2/38; 10)          | 0.44   |
| MBI                      | 100 (38/100; 2.75)      | 100 (88/100; 2)        | 0.31   |
| MoCA                     | 23 (19.1/29.2; 2.56)    | 23.3 (14.8/30.2; 5.46) | 0.47   |
| POMA-B                   | 13 (5/16; 3)            | 13 (5/16; 2)           | 0.57   |
| POMA-G                   | 9 (5/11; 2)             | 10 (5/12; 3)           | 0.21   |
| POMA tot                 | 22 (13/26; 4)           | 22.5 (11/27; 4.17)     | 0.18   |
| Cadence Self (steps/min) | 108 (89.4/128; 16.5)    | 112 (82.7/134; 8.96)   | *0.007 |
| Cadence Max (steps/min)  | 125 (102/164; 16.9)     | 128 (99.6/178; 14.1)   | *0.029 |
| Gait Speed Self (m/s)    | 0.995 (0.53/1.42; 0.44) | 1.15 (0.45/1.49; 0.42) | *0.03  |
| Gait Speed Max (m/s)     | 1.34 (0.61/2.70; 0.54)  | 1.50 (0.55/2.22; 0.58) | *0.04  |
| Step Length Self (cm)    | 56.2 (34.6/75.3; 18.7)  | 62.4 (31.9/76; 19.5)   | 0.06   |
| Step Length Max (cm)     | 66 (33.6/101; 23)       | 70.6 (33.4/97.3; 25.3) | 0.13   |
| Gait Cycle Self (% s)    | 1.11 (0.93/1.35; 0.18)  | 1.07 (0.9/1.55; 0.08)  | *0.03  |
| Gait Cycle Max (% s)     | 0.970 (0.73/1.19; 0.14) | 0.93 (0.66/1.25; 0.11) | *0.05  |

1.49; 0.42) ( $p = 0.03$ ). Median maximum gait speed exhibited a marked improvement, rising from 1.34 m/s (0.61/2.7; 0.54) to 1.50 m/s (0.55/2.22; 0.58) ( $p = 0.04$ ). According to Baudendistel et al. [27], the gain obtained in gait speed reached the minimally clinically important difference. While other gait parameters demonstrated trends toward enhancement, these did not attain statistical significance. Median step length (self-selected) increased from 56.2 cm (34.6/75.3; 18.7) to 62.4 cm (31.9/76; 19.5) ( $p = 0.06$ ), and median maximum step length increased from 66 cm (33.6/101; 23) to 70.6 cm (33.4/97.3; 25.3) ( $p = 0.129$ ). Furthermore, the median gait cycle self-selected time decreased from 1.11 (0.93/1.35; 0.183) to 1.07 (0.9/1.55; 0.082) ( $p = 0.03$ ). Conversely, the median maximum time of the gait cycle decreased from 0.970 (0.73/1.19; 0.135) to 0.93 (0.66/1.25; 0.108) ( $p = 0.05$ ).

### 3.3. Circulating particles characterization

Blood samples were collected before and after the treadmill-based rehabilitation protocol from 30 wPD and circulating particles were successfully isolated from all samples. Although the proposed protocol based on commercial size exclusion chromatography isolation columns is often reported as valuable method for EV isolation, the presence of co-isolates is well known and particles of similar size and density can be isolated in combination or associated to EVs [7]. For this reason, the presence of small EVs and lipoproteins was verified using specific markers, as well as broader characterization techniques like NTA analysis. The isolation was achieved with high reproducibility, as attested by the characterization results reported herein.

NTA analysis was used to verify the size distribution and the concentration of EV-like particles in people recruited before and after rehabilitation (Supplementary Fig. 2). Comparing T0 and T1 samples, a significant variation was observed in the mean concentration of EV-like particles (T0:  $9.6 \times 10^9 \pm 1.1 \times 10^{10}$  particles/ml; T1:  $1.3 \times 10^{10} \pm 1.4 \times 10^{10}$  particles/ml;  $p < 0.05$  after Wilcoxon Signed Pair Rank non-parametric test). No difference in the mean size of circulating particles

was observed (T0:  $145 \pm 32$  nm; T1:  $153 \pm 23$  nm) for the considered patients (Supplementary Fig. 2). The presence of small EVs (such as exosomes) in both T0 and T1 samples was also confirmed by the positivity of the dot blot for FLOT-1, TSG101, Alix and CD9, well-known EV markers (Supplementary Fig. 3). In parallel, the valuation of ApoA1 was also performed, confirming the presence of lipoproteins in the considered nanoparticle suspension (Supplementary Fig. 3).

### 3.4. Changes in the Raman fingerprint of particles after rehabilitation

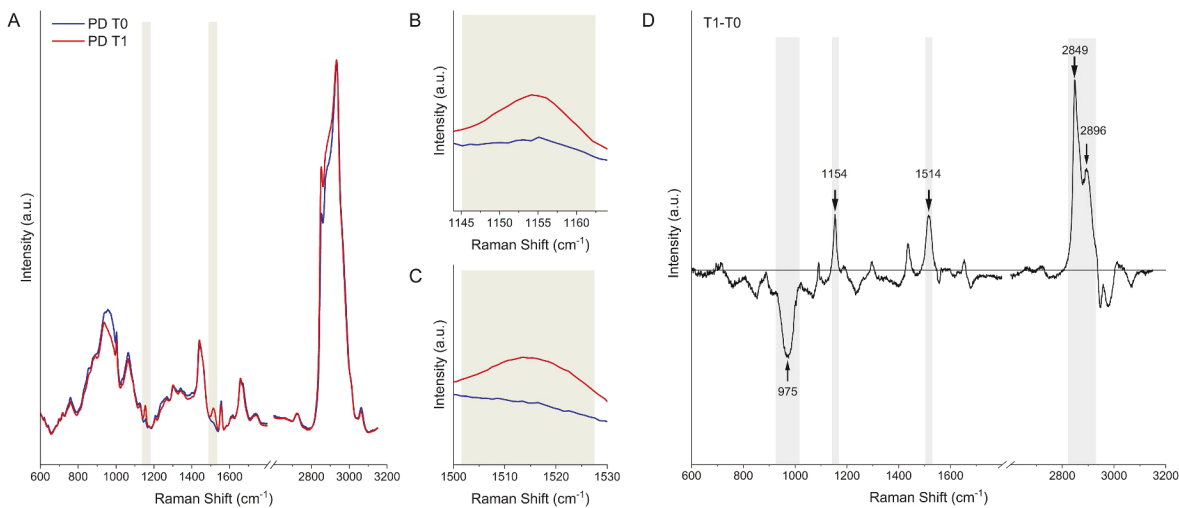
RS analysis was successfully performed on 30 T0-T1 pairs of samples from wPD undergoing intensive rehabilitation.

The average Raman spectrum for each of the 30 samples from wPD before and after intensive rehabilitation was obtained in the spectral range 600–1800  $\text{cm}^{-1}$  and 2600–3200  $\text{cm}^{-1}$ . All samples showed a good signal-to-noise ratio and the expected peaks and bands related to proteins (Amide I 1600–1690  $\text{cm}^{-1}$ ; Amide III 1200–1300  $\text{cm}^{-1}$ ) and nucleic acids (720–820  $\text{cm}^{-1}$ ), with the prominent spectral contribution of the lipidic component of EV-like particles in the spectral range 2600–3200  $\text{cm}^{-1}$  (Fig. 1A). Data confirm the repeatability of the analysis compared to the data reported by our group in 2019 [26].

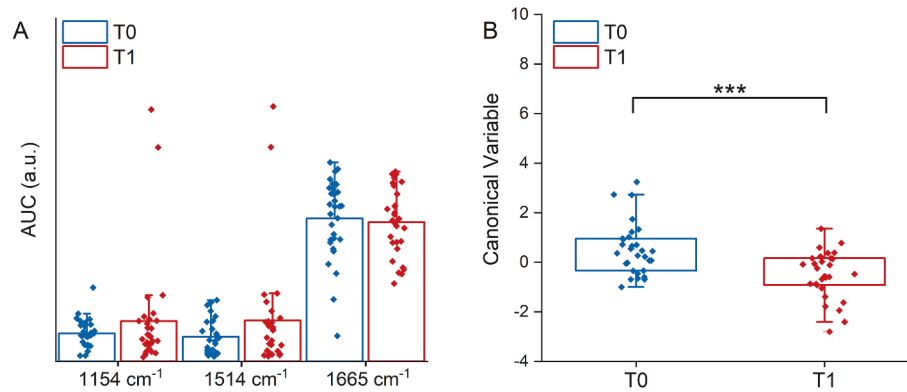
To verify changes in the biomolecular cargo of particles in pwPD before and after rehabilitation, differences in the molecular fingerprints were extracted by subtracting the T0 average spectrum from the T1 average spectrum (Fig. 1D). Functional chemical groups were assigned based on previously consolidated findings [28]. The data showed differences in the presence and intensity of peaks related to nucleic acids (975  $\text{cm}^{-1}$ ) which were found to be more represented in T0 average spectrum. Peaks related to lipids (2849  $\text{cm}^{-1}$  and 2896  $\text{cm}^{-1}$ ) and carotenoids (1154  $\text{cm}^{-1}$  and 1514  $\text{cm}^{-1}$ ; Fig. 1B and C) seemed to be predominant in the spectrum of T1 samples (Fig. 1D). Besides, the comparison of the present data to those published in 2019 [26], confirmed that the contribution of carotenoids was not detectable nor in pwPD before the rehabilitation intervention, nor in the control subjects of comparable age in the circulating EV-like particles. On the contrary, peaks become clearly visible after the completion of the intensive rehabilitation program (Fig. 1B and C).

Based on these observations, on the remarkable variations in the carotenoid peaks (Fig. 1B and C), and on the theoretical role of oxidative stress in PD pathogenesis, to further investigate the contribution of carotenoids to the particle spectra, the area under curve (AUC) of the peaks related to carotenoids ( $1154 \pm 10$   $\text{cm}^{-1}$  and  $1514 \pm 10$   $\text{cm}^{-1}$ ) were calculated for each subject (Supplementary Fig. 1). In parallel, the AUC of the peak of Amide I ( $1665 \pm 20$   $\text{cm}^{-1}$ ) was also calculated and considered as a reference band as it accounts for the secondary structure of proteins (Fig. 2A). The AUC of both carotenoid peaks showed a higher mean value at T1 when comparing T0 and T1 data, but no significant differences were observed between the calculated areas. The AUC of the Amide I peak resulted to be unchanged in all subjects, as expected (Fig. 2A). The angular coefficient of the line joining the corresponding AUC values at T0 and T1 for each sample was also calculated, showing considerable variability in the changes related to the carotenoid content (Supplementary Fig. 4).

Finally, to verify the possibility of discriminating EV-like particles isolated from wPD before and after rehabilitation by their Raman spectrum, multivariate statistical analysis was performed. The calculated PCs describe the differences between the considered Raman spectra and represent the percentage of data variance (PC1 = 76.5 %; PC2 = 9.6 %). The comparison by Wilcoxon Paired Signed Rank Test of the CV scores resulting from the LDA classification model demonstrated significant differences when comparing T0 and T1 time-points ( $p < 0.001$ ; Fig. 2B), thus demonstrating a significant biochemical change in the EV-like particles composition after rehabilitation in the considered cohort of wPD.



**Fig. 1. Raman fingerprint of EV-like particles before and after rehabilitation.** (A) Average Raman spectra of circulating EV-like particles obtained from serum of 30 pwPD before (T0; blue line) and after (T1; red line) rehabilitation. (B) Magnification of peak at 1154 cm<sup>-1</sup>. (C) Magnification of peak at 1514 cm<sup>-1</sup>. (D) Subtraction spectrum (T1-T0) of mean Raman spectra. Arrows indicate the most relevant peaks in the subtraction spectrum.



**Fig. 2. Area under curve and multivariate analysis.** (A) Comparison of the average area under curve (AUC) of peaks related to carotenoids (1154 cm<sup>-1</sup> and 1514 cm<sup>-1</sup>) and Amide I (1665 cm<sup>-1</sup>) before (T0) and after (T1) the rehabilitative treatment. (B) Box plot reporting the score of the canonical variable obtained after the multivariate statistical analysis comparing Raman spectra of EV-like particles before (T0) and after (T1) rehabilitation. \*\*\*p < 0.001 after Wilcoxon Paired Signed Rank Test. Each dot represents one subject. T0: blue bars and symbols; T1: red bars and symbols.

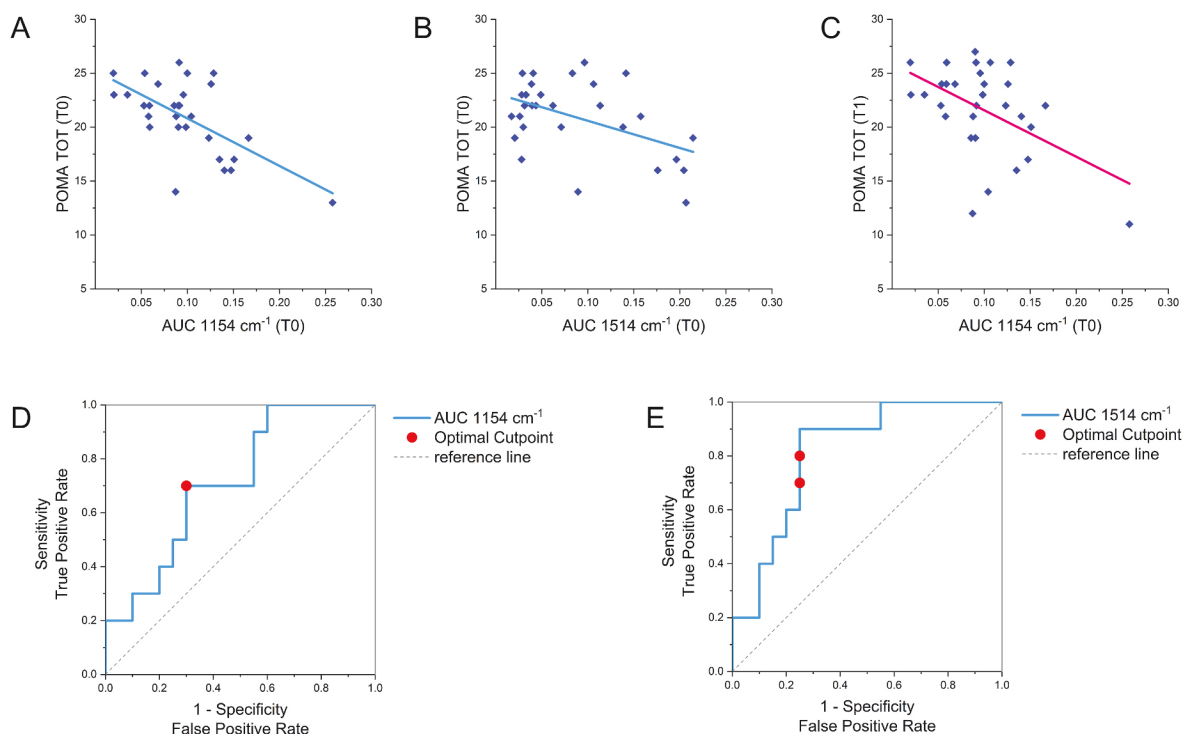
### 3.5. Correlation between Raman data and clinical assessment

The correlation analysis was performed to better interpret the Raman results in the framework of the clinical assessment of the participants. First, the contribution of carotenoids in the spectrum of EV-like particles in the considered cohort at T0 was evaluated by correlating it with age, sex and disease stage for all subjects, showing a direct correlation between the intensity of the peaks at 1154 cm<sup>-1</sup> and the individual age (Pearson correlation coefficient: 0.38;  $p = 0.03$ ). No correlation was found with sex, subtype of PD at onset, and staging of the disease measured by HY scale.

Then, keeping age and disease duration as co-variables, partial correlation analysis was performed between the AUC of carotenoid and Amide I peaks and the main clinical scales used to assess pwPD before (profiling the patients' status) and after (monitoring of patients' status and prognostic significance) rehabilitation. As shown in [Supplementary Table 1](#), the AUC of the carotenoid peaks (1154 cm<sup>-1</sup> and 1514 cm<sup>-1</sup>) both in T0 and T1 samples was significantly correlated with some of the major scales used to profile pwPD, with significant results in the correlation with the Performance-Oriented Mobility Assessment (POMA scale, measure of patient's gait/POMA-G and balance/POMA-B abilities) as well as to the modified Barthel Index (MBI scale, measure of patient's disability). Specifically, the AUC of carotenoids at T0 was found to be

negatively correlated to the POMA-B scores at T0 (Partial Corr. Coeff.  $-0.59$ ,  $p < 0.001$  for AUC 1154 cm<sup>-1</sup> and  $-0.48$ ,  $p = 0.01$  for AUC 1514 cm<sup>-1</sup>). Both carotenoid peak intensities correlated with the total POMA score at T0 (Partial Corr. Coeff.  $-0.55$ ,  $p < 0.001$  for AUC 1154 cm<sup>-1</sup> and  $-0.46$ ,  $p = 0.02$  for AUC 1514 cm<sup>-1</sup>) ([Fig. 3A](#) and [B](#)), but not with the POMA-G score at T0. Considering the clinical relevance of the POMA scores, it is worth noting that the carotenoid cargo of EV-like particles measured at T0 (AUC 1154 cm<sup>-1</sup> T0) correlates with the total POMA score at T1 (Partial Corr. Coeff.  $-0.43$ ,  $p = 0.02$ ), and with the POMA-G at T1 (Partial Corr. Coeff.  $-0.43$ ,  $p = 0.03$ ), suggesting a potential prognostic value of the antioxidants for pwPD ([Fig. 3C](#)).

Moreover, the antioxidant content of EV-like particles (AUC 1154 cm<sup>-1</sup> and AUC 1514 cm<sup>-1</sup>) at T0 correlated negatively with the MBI at T0 (Partial Corr. Coeff.  $-0.61$ ,  $p < 0.001$  for AUC 1154 cm<sup>-1</sup> and  $-0.43$ ,  $p = 0.02$  for AUC 1514 cm<sup>-1</sup>), suggesting that low levels of carotenoids in the bloodstream are indicative of a less severe disability of the patient, before starting the rehabilitation treatment. Of note, the amount of carotenoids measured in pwPD at T1 still negatively correlates with the disability status of the patient (Partial Corr. Coeff.  $-0.45$ ,  $p = 0.02$  for both AUC 1154 cm<sup>-1</sup> and AUC 1514 cm<sup>-1</sup>), confirming the possibility to monitor the disability profile of the patient by the antioxidant cargo of EV-like particles. Even more relevant from a clinical point of view, the measure of the spectral contribution of carotenoids associated with



**Fig. 3. Correlation analysis between Spectroscopic Raman data and clinical assessment.** A–B: Scatter plots representing the negative correlation between the total POMA scale scores at T0 and the intensity of the 1154 cm<sup>-1</sup> (A) and 1514 cm<sup>-1</sup> (B) peaks of carotenoids related to EV-like particles. C: Scatter plots representing the positive correlation between the total POMA scale scores at T1 (after rehabilitation) and the intensity of the 1154 cm<sup>-1</sup> peak of carotenoids related to EV-like particles. D–E: ROC curves calculated for the AUC of carotenoids to discriminate between responders and non-responders based on the variation in the disability scale MBI.

circulating articles at T0 actively correlates with the MBI at discharge (Partial Corr. Coeff.  $-0.46$ ,  $p = 0.01$  for AUC 1154 cm<sup>-1</sup> and  $-0.42$ ,  $p = 0.03$  for AUC 1514 cm<sup>-1</sup>) meaning that the low levels of carotenoids at admission could be predictive of the less severe disability of the patient both at admission and at discharge. Moreover, the antioxidant content of EV-like particles measured with AUC 1154 cm<sup>-1</sup> at T0 correlates positively (Partial Corr. Coeff.  $0.58$ ,  $p < 0.0001$  for AUC 1154 cm<sup>-1</sup>) with the variation in the MBI score between admission and discharge, i.e. a measure of the patient recovery after rehabilitation, but the correlation is not significant for AUC 1514 cm<sup>-1</sup>. This last observation is in agreement with the correlation found between the spectral variation in the carotenoid content (angular coefficient of the line joining the integrated values – SLOPE 1154 and SLOPE 1514) and the clinically measured change in disability profile (variation in the MBI scores -  $\Delta$ MBI): Partial Corr. Coeff. of  $0.71$  ( $p < 0.0001$ ) and of  $0.67$  ( $p < 0.0001$ ) for the angular coefficient of the AUC 1154 cm<sup>-1</sup> and AUC 1514 cm<sup>-1</sup>, respectively. These data make us hypothesize that an increment in the antioxidant content can be related to increased disability of pwPD.

Thus, we divided pwPD in responders and non-responders to rehabilitation treatment according to the variation in the MBI scores (responders:  $\Delta$ MBI  $> 0$ , 10 subjects, median 0.021; non-responders:  $\Delta$ MBI  $\leq 0$ , 20 subjects, median 0) and we used the spectral measure of carotenoids obtained at T0 (AUC), to calculate ROC curves and test the ability of carotenoids in discriminating between responders and non-responders, as defined. The use of carotenoid AUC values at T0 is essential to establish their predictive role. In parallel, the use of MBI scores for the ROC curve analyses allowed us to distinguish between similar but clinically different functional levels, observing variations in the global assessment that follows rehabilitation, not restricted only to the motor or functional aspect.

As shown in Fig. 3D and E, the AUC of 1154 cm<sup>-1</sup> and 1514 cm<sup>-1</sup> can be considered indicative of the response or non-response to rehabilitation with an accuracy of 72 % (95 % Confidence interval: 51 %–92 %)

and 82 % (95 % Confidence interval: 63 %–100 %), respectively. These measures allowed us also to identify the cut-off values for AUC 1154 cm<sup>-1</sup> and AUC 1514 cm<sup>-1</sup> that correspond to 0.1 and 0.09, respectively. Based on the present data, these measurements indicate that pwPD who start the rehabilitation program with a Raman fingerprint of EV-like particles with calculated AUC of carotenoids lower than the identified cut-off values are expected to be better responders to the rehabilitation intervention than people with higher carotenoid content.

Finally, the AUC of both carotenoid peaks in T0 samples actively correlates with the instrumental assessments of the performance of patients described by Cadence Max, Gait Speed Max, and Step Length Max measured at T0 and are prognostic of the bad performance for the same parameters evaluated at the end of the rehabilitation program (Supplementary Table 1).

Considering the AUC related to Amide I, that describes the secondary structure of proteins associated with the EV-like particles, it resulted to be actively correlated to MDS-UPDRS III at T0 (Partial Corr. Coeff  $-0.67$ ,  $p < 0.001$ ) and to the BDI score at T0 (Partial Corr. Coeff  $-0.5$ ,  $p = 0.01$ ), that measure motor symptoms and mood of the patient, respectively. Although these data require further investigation to clarify the molecules involved in the reported observation, they are not surprising if we take into consideration that all proteins, including pathological and misfolded proteins (i.e.  $\alpha$ syn), can contribute to the spectral signal of Amide I. On the contrary, it was unexpected that the AUC 1665 cm<sup>-1</sup> (Amide I) at T0 negatively correlates with the POMA-G value at T0 (Partial Corr. Coeff  $-0.49$ ,  $p = 0.01$ ), suggesting that the spectral signature of circulating articles comprises the information regarding proteins that might be determinants of the gait imbalance of pwPD. The patient's profiling based on the Amide I region seems also to be prognostic of a bad performance of the subject after the rehabilitation training evaluated by the total POMA score at T1 (Partial Corr. Coeff  $-0.42$ ,  $p = 0.03$ ), and balance worsening (Partial Corr. Coeff  $-0.39$ ,  $p = 0.04$ ), but these data need further investigation.

Finally, CV values at T0 showed a relationship with the max cadence at T0 and gait cycle max at T0, suggesting again a direct connection between the cargo of EV-like particles in blood and the clinical status of wPD.

#### 4. Discussion

In PD, oxidative stress is one of the leading mechanisms associated with neurodegeneration, even though the complex mechanisms that contribute to the loss of dopaminergic neurons remain to be fully elucidated [2].

In the present work, data demonstrate that rehabilitation can modify the oxidative state of the organism, and changes in the oxidative cargo of circulating particles are related to the individual response to intensive rehabilitation programs. Specifically, the present work highlights that intensive rehabilitation impacts the biochemical composition of circulating particles in wPD, demonstrating their role in the complex systemic response to rehabilitation. In this regard, our data suggests that rehabilitation impacts the oxidative cargo of blood-derived particles that can be related to the individual response to an intensive rehabilitation program. Summarizing our main findings and focusing on the POMA-G scale (primary clinical outcome of the present study), the AUC 1665  $\text{cm}^{-1}$  at T0 negatively correlates with POMA-G at T0 whereas the AUC 1154  $\text{cm}^{-1}$  at T0 negatively correlates with POMA-G at T1 (but not at T0), suggesting that the peak of Amide I (AUC 1665) might be an indicator of the disease pathology, whereas the peak of carotenoids might represent a prognostic factor for gait improvement after rehabilitation. Moreover, both carotenoid peaks (1154  $\text{cm}^{-1}$  and 1514  $\text{cm}^{-1}$ ) at T0 actively correlate with instrumental gait parameters (in gait speed and functional ability at T0 and T1), supporting the hypothesis that the carotenoid concentration can reflect the disease severity. Despite the limited cohort considered, it is worth noting that we were able to identify a cut-off value for the spectral signal of carotenoids that can discriminate wPD responders and non-responders to the rehabilitation treatment, with moderately good accuracy (>70 %).

In PD, EVs and EV-like particles are known to be carriers of molecules associated with neurodegeneration. The biochemical profile of circulating nanoparticles was already proven to be related to the disease stage with the Raman fingerprint of blood-derived EVs representing a biomarker for the profiling of pwPD. Previously, we demonstrated that the Raman fingerprint of circulating nanoparticles in the serum of wPD is significantly different from that of age- and sex-matched healthy controls. In that work, the presence of carotenoids associated to EV-like particles isolated using the same protocol proposed in the present study was not observed [26]. The present data confirm our previous observations and shed light on the ability of EV-like particles to mediate the response to rehabilitation by mobilizing molecules involved in oxidative homeostasis.

The cargo of EVs or EV-like particles released in oxidative conditions was proved to include both oxidized molecules and antioxidant molecules, representing a potential source of oxidative stress biomarkers [29]. Our results suggest that the antioxidant reservoir of individual subjects and their association with circulating nanoparticles can be related to their clinical status and to their response to an intensive rehabilitation treatment. Besides, a change in the antioxidant cargo of EV-like particles can be induced by intensive rehabilitation. The most interesting result is related to the role of carotenoids, known scavengers of ROS, that can circulate associated with EV-like particles and whose concentration was proved to be related to the individual responsiveness to the proposed rehabilitation program for wPD.

Carotenoids are a huge class of molecules that can be subdivided into different groups based on their chemical structure. Many of them are included in the human diet and directly linked to fruit and vegetable consumption. Carotenoids exert fundamental antioxidant and anti-apoptotic effects in neurodegenerative disorders, resulting to be the

main lipophilic antioxidants counteracting lipid-oxidating molecules in brain tissue. Carotenoids are known to be lipophilic molecules, typically circulating in blood together with lipoproteins. Recent literature has also demonstrated that carotenoids can be stabilizing agents for aggregated proteins like amyloids [30]. It was reported that the carotenoid precursor, vitamin A, has depolymerizing effects on  $\alpha$ -syn aggregates *in vitro* [31], whereas retinoic acid (RA; i.e. vitamin A metabolite) seems to be directly involved in dopaminergic neurotransmission control and in regulation of the nigrostriatal pathway [31]. A study by Zhou and colleagues, demonstrated that RA administration attenuates both degeneration and loss of dopaminergic neurons in substantia nigra, alleviating motor dysfunctions in mice models of PD [31–33]. Also, the concentration of serum carotenoids was proved to decrease with PD progression [34], although a detailed nutritional assessment is often lacking in these studies.

It has to be noted that we cannot conclude whether carotenoids are transferred in the bloodstream by small EVs or by lipoproteins of comparable size and density, or even by lipoproteins associated with EVs (the so-called biomolecular corona). Association of carotenoids to EVs or lipoproteins should be further investigated and could shed light on the mechanisms underlying our observations. Nonetheless, from a clinical point of view, antioxidants associated with circulating particles were shown to be involved in the patients' response to the rehabilitation treatment and their abundance in the bloodstream seems to be related to a more severe disease status and a better prognosis for the rehabilitation recovery. Although these observations need further validation in wider independent cohort, our data are in line with previous literature about the oxidative stress related to PD progression making us hypothesize that the mobilization of the carotenoids is indicative of a more oxidative status of the patient [35]. Further experimental studies are needed to verify this hypothesis and clarify the mechanisms underlying the present observations and the molecular basis of the reported correlations between redox status and rehabilitation outcomes. Indeed, the role of carotenoids in the considered cohort can have multiple explanations, including the role of carotenoids as biomarkers of oxidative load or their protective role in the disease progression and rehabilitation response. On the contrary, considering the connection between oxidative stress and rehabilitation recovery, a work by Cocco and colleagues recently proved changes in oxidative stress parameters between baseline and at the end of the rehabilitation program in people with a subacute stroke [36]. However, to the best of our knowledge, measurable changes in antioxidant cargo of EV-like particles were not associated with the patient status and to the rehabilitation outcome in wPD. Further studies should be designed to better characterize carotenoids in the blood of wPD, taking into account multiple quantification methods, such as mass spectrometry and chromatographic analysis, and a wider cohort of subjects, in order to validate the current results in a independent population.

Despite the limited cohort of considered subjects, our data suggest a correlation between the biomolecular profile of EV-like particles, their antioxidant content and the individual response to rehabilitation. We can speculate that the biomolecular profile of circulating particles can be predictive of the effectiveness of the rehabilitation treatment in terms of changes in the disability profile (variation in the MBI scores), and in an improvement in the gait pattern. Although the mechanisms underlying the present observations are difficult to be interpreted, we can speculate that circulating carotenoids are involved in the response of wPD to intensive rehabilitation and that the amount of mobilized antioxidant reservoir can impact a patient's recovery, being a potential tool for monitoring individual clinical status and rehabilitation's effectiveness. However, future investigation about the role of carotenoids associated to circulating particles, as well as a in-depth characterization of their mechanism of action in motor and cognitive rehabilitation is needed.

Future multicentered clinical trials on the impact of multiple rehabilitation interventions on wider cohorts of wPD are necessary to further validate our observations and verify the proposed cut-off values

for carotenoids. Besides, it is worth noting that no changes in the nutrition habits were reported in the present cohort, and pharmacological treatment was stable according to the study protocol. Future studies should consider nutrition data as part of the patients' clinical profiling at enrolment, and follow up.

The present study supports the role of circulating particles in the neurodegenerative processes of PD, but also in the individual response to motor rehabilitation. Moreover, Raman spectroscopy was effective in the identification of specific molecular changes related to the rehabilitation response and allowed us to identify carotenoids as crucial mediators of the treatment response. Our data provide evidence about the possibility to use the Raman fingerprint of EV-like particles and the spectral signature of carotenoids as a measurable biomarker of rehabilitation efficacy, even though future studies should consider also disease progression as a variable by including longer follow up periods. Considering that previous studies proved variations in carotenoid concentration in serum during PD progression [34], the variability in the Raman data could be due to PD pathogenesis and further studies should be dedicated to the monitoring of neurodegeneration during disease progression.

In conclusion, the present study leads to two major conclusions: i) the effectiveness of the rehabilitation treatment is linked to the redox status of the patient and ii) changes in the redox status may be related to changes in the rehabilitation outcomes. It is also possible that modifying the cargo of circulating particles may impact the redox and clinical status of the patients, opening the way towards pharmacological treatments, but this observation goes beyond the scope of our study and should be addressed by dedicated trials.

#### CRediT authorship contribution statement

**Alice Gualerzi:** Writing – original draft, Visualization, Supervision, Project administration, Formal analysis, Data curation, Conceptualization. **Martina Gerli:** Writing – original draft, Visualization, Investigation, Formal analysis. **Aurora Angelini:** Writing – review & editing, Investigation. **Silvia Piccolini:** Writing – review & editing, Visualization, Supervision, Formal analysis. **Francesca Rodà:** Writing – review & editing, Visualization, Supervision. **Valentina Angelini:** Writing – review & editing, Investigation. **Stefano Doronzio:** Writing – original draft, Investigation, Data curation. **Diego Longo:** Writing – original draft, Investigation, Data curation. **Giulio Cherubini:** Writing – review & editing, Investigation. **Cristina Polito:** Writing – review & editing. **Chiara De Santis:** Writing – review & editing. **Silvia Ramat:** Writing – review & editing. **Francesca Cecchi:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Gemma Lombardi:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Marzia Bedoni:** Writing – review & editing, Funding acquisition, Supervision, Resources.

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#### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesca Cecchi reports financial support was provided by Florence Savings Bank Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The Graphical Abstract was created with [BioRender.com](https://BioRender.com) with permission.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2025.103841>.

#### Data availability

Data will be made available on request.

#### References

- [1] E.M. Rocha, B.D. Miranda, L.H. Sanders, Alpha-synuclein: pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease, *Neurobiol. Dis.* 109 (2018) 249–257, <https://doi.org/10.1016/j.nbd.2017.04.004>.
- [2] J. Dias, E. Junn, M.M. Mouradian, The role of oxidative stress in Parkinson's disease, *J. Parkinsons Dis.* 3 (2013) 461–491, <https://doi.org/10.3233/JPD-130230>.
- [3] A.M. Lozano, N. Lipsman, H. Bergman, P. Brown, S. Chabardes, J.W. Chang, K. Matthews, C.C. McIntyre, T.E. Schlaepfer, M. Schulder, Y. T. Mel, J. Volkmann, J.K. Krauss, D. Brain stimulation: current challenges and future directions, *Nat. Rev. Neurol.* 15 (2019) 148–160, <https://doi.org/10.1038/s41582-018-0128-2>.
- [4] G. Abbruzzese, R. Marchese, L. Avanzino, E. Pelosin, Rehabilitation for Parkinson's disease: current outlook and future challenges, *Park. Relat. Disord.* 22 (2016) S60–S64, <https://doi.org/10.1016/j.parkreldis.2015.09.005>.
- [5] W. Cao, X. Zhang, H. Qiu, Rehabiliomics: a state-of-the-art review of framework, application, and future considerations, *Front. Neurol.* 14 (2023) 1103349, <https://doi.org/10.3389/fneur.2023.1103349>.
- [6] J.A. Welsh, D.C.I. Goberdhan, L. O'Driscoll, E.I. Buzas, C. Blenkiron, B. Bussolati, Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches, *J. Extracell. Vesicles* (2023).
- [7] S. Busatto, A. Zandrini, A. Radeghieri, L. Paolini, M. Romano, M. Presta, P. Brgese, The nanostructured secretome, *Biomater. Sci.* 8 (2020) 39–63, <https://doi.org/10.1039/C9BM01007F>.
- [8] A. Gualerzi, S. Piccolini, M. Bedoni, F.R. Guerini, M. Clerici, C. Agliardi, Extracellular vesicles as biomarkers for Parkinson's disease: how far from clinical translation? *IJMS* 25 (2024) 1136, <https://doi.org/10.3390/ijms25021136>.
- [9] B.M. Coleman, A.F. Hill, Extracellular vesicles – their role in the packaging and spread of misfolded proteins associated with neurodegenerative diseases, *Semin. Cell Dev. Biol.* 40 (2015) 89–96, <https://doi.org/10.1016/j.semcdb.2015.02.007>.
- [10] G.K. Tofaris, A critical assessment of exosomes in the pathogenesis and stratification of Parkinson's disease, *JPD* 7 (2017) 569–576, <https://doi.org/10.3233/JPD-171176>.
- [11] P.R. Tomlinson, Y. Zheng, R. Fischer, R. Heidasch, C. Gardiner, S. Evetts, M. Hu, R. Wade-Martins, M.R. Turner, J. Morris, K. Talbot, B.M. Kessler, G.K. Tofaris, Identification of distinct circulating exosomes in Parkinson's disease, *Ann. Clin. Transl. Neurol.* 2 (2015) 353–361, <https://doi.org/10.1002/acn3.175>.
- [12] A. Sahu, Z.J. Clemens, S.N. Shinde, S. Sivakumar, A. Pius, A. Bhatia, S. Piccolini, C. Carlomagno, A. Gualerzi, M. Bedoni, B. Van Houten, M. Lovalekar, N.F. Fitz, I. Lefterov, A. Barchowsky, R. Koldamova, F. Ambrosio, Regulation of aged skeletal muscle retraction by circulating extracellular vesicles, *Nat. Aging* 1 (2021) 1148–1161, <https://doi.org/10.1038/s43587-021-00143-2>.
- [13] S. Piccolini, V. Mangolini, F. Rodà, A. Montesano, F. Arnaboldi, P. Luzzi, A. Mannini, M. Bedoni, A. Gualerzi, Multiplexing biosensor for the detection of extracellular vesicles as biomarkers of tissue damage and recovery after ischemic stroke, *IJMS* 24 (2023) 7937, <https://doi.org/10.3390/ijms24097937>.
- [14] V. Lisi, G. Senesi, N. Bertola, M. Pecoraro, S. Bolis, A. Gualerzi, S. Piccolini, A. Raimondi, C. Fantini, E. Moretti, A. Parisi, P. S. R. Di Luigi, R. Giger, S. Ravera, G. Vassalli, D. Caporossi, C. Balbi, Plasma-derived extracellular vesicles released after endurance exercise exert cardioprotective activity through the activation of a antioxidant pathways, *Redox Biol.* 63 (2023) 102737, <https://doi.org/10.1016/j.redox.2023.102737>.
- [15] I.J. Echetti, T. Valentini, C.B. Mobley, J.J. McCarthy, The role of extracellular vesicles in skeletal muscle and systematic adaptation to exercise, *J. Physiol.* 599 (2021) 845–861, <https://doi.org/10.1113/JP278929>.
- [16] R.S. Monteiro-Junior, T. Cevada, B.R.R. Oliveira, E. Lattari, E.M.M. Portugal, A. Carvalho, A.C. Deslandes, We need to move more: neurobiological hypotheses of physical exercise as a treatment for Parkinson's disease, *Med. Hypotheses* 85 (2015) 537–541, <https://doi.org/10.1016/j.mehy.2015.07.011>.
- [17] V. Lisi, G. Senesi, C. Balbi, Converging protective pathways: exploring the linkage between physical exercise, extracellular vesicles and oxidative stress, *Free Radic. Biol. Med.* 208 (2023) 718–727, <https://doi.org/10.1016/j.freeradbiomed.2023.09.021>.
- [18] G. Bodega, M. Alique, L. Puebla, J. Carracedo, R.M. Ramírez, Microvesicles: ROS scavengers and ROS producers, *J. Extracell. Vesicles* 8 (2019) 1626654, <https://doi.org/10.1080/20013078.2019.1626654>.

- [19] G. Lombardi, M. Baccini, A. Gualerzi, S. Pancani, S. Campagnini, S. Doronzio, D. Longo, A. Maselli, G. Cherubini, M. Piazzini, T. Ciapetti, C. Polito, S. Pinna, C. D. Santis, M. Bedoni, C. Macchi, S. Ramat, F. Cecchi, Comparing the effects of augmented virtual reality treadmill training versus conventional treadmill training in patients with stage II-III Parkinson's disease: the VIRTREAD-PD randomized controlled trial protocol, *Front. Neurol.* 15 (2024) 1338609, <https://doi.org/10.3389/fneur.2024.1338609>.
- [20] M.E. Tinetti, Performance-oriented assessment of mobility problems in elderly patients, *J. Am. Geriatr. Soc.* 34 (1986) 119–126, <https://doi.org/10.1111/j.1532-5415.1986.tb05480.x>.
- [21] C.G. Goetz, B.C. Tilley, S.R. Shaftman, G.T. Stebbins, S. Fahn, P. Martinez-Martin, W. Poewe, C. Sampaio, M.B. Stern, R. Dodel, B. Dubois, R. Holloway, J. Jankovic, J. Kulisevsky, A.E. Lang, A. Lees, S. Leurgans, P.A. LeWitt, D. Nyenhuis, C. W. Olanow, O. Rascol, A. Schrag, J.A. Teresi, J.J. van Hilten, N. LaPelle, Movement disorder Society-sponsored revision of the unified Parkinson's disease rating scale (MDS-UPDRS): Scale presentation and clinimetric testing results, *Mov. Disord.* 23 (2008) 2129–2170, <https://doi.org/10.1002/mds.22340>.
- [22] A. Antonini, G. Abbruzzese, L. Ferini-Strambi, B. Tilley, J. Huang, G.T. Stebbins, C. G. Goetz, P. Barone, M.B. di Poggio, G. Fabbri, F.D. Stasio, M. Tinazzi, T. Bovi, S. Ramat, S. Meoni, G. Pezzoli, M. Canesi, P. Martinelli, C.L.M. Scaglione, A. Rossi, N. Tambasco, G. Santangelo, M. Picillo, L. Morgante, F. Morgante, R. Quatrate, M. Sensi, M. Pilleri, R. Biundo, G. Nordera, A. Caria, C. Pacchetti, R. Zangaglia, L. Lopiano, M. Zibetti, M. Zappia, A. Nicoletti, A. Quattrone, M. Salzone, G. Cossu, D. Murgia, A. Albanese, F.D. Sorbo, Validation of the Italian version of the movement disorder society—unified Parkinson's disease rating scale, *Neurol. Sci.* 34 (2013) 683–687, <https://doi.org/10.1007/s10072-012-1112-z>.
- [23] C. Simon, H. Everitt, F. van Dorp, K. Schroeder, *Oxford Handbook of Geriatric Practice*, third ed., Oxford University Press, Oxford ; New York, 2010.
- [24] Z.S. Nasreddine, N.A. Phillips, V. Bédirian, S. Charbonneau, V. Whitehead, I. Collin, J.L. Cummings, H. Chertkow, The Montreal cognitive assessment, MoCA: a brief screening tool for mild cognitive impairment, *J. Am. Geriatr. Soc.* 53 (2005) 695–699, <https://doi.org/10.1111/j.1532-5415.2005.53221.x>.
- [25] A.T. Beck, R.A. Steer, G. Brown, Beck depression Inventory–II, <https://doi.org/10.1037/t00742-000>, 2011.
- [26] A. Gualerzi, S. Picciolini, C. Carlomagno, F. Terenzi, S. Ramat, S. Sorbi, M. Bedoni, Raman profiling of circulating extracellular vesicles for the stratification of Parkinson's patients, *Nanomed. Nanotechnol. Biol. Med.* 22 (2019) 102097, <https://doi.org/10.1016/j.nano.2019.102097>.
- [27] S.T. Baudendistel, A.M. Haussler, K.S. Rawson, G.M. Earhart, Minimal clinically important differences of spatiotemporal gait variables in Parkinson disease, *Gait Posture* 108 (2024) 257–263, <https://doi.org/10.1016/j.gaitpost.2023.11.016>.
- [28] Z. Movasaghi, S. Rehman, I.U. Rehman, Raman spectroscopy of biological tissues, *Appl. Spectrosc. Rev.* 42 (2007) 493–541, <https://doi.org/10.1080/05704920701551530>.
- [29] E. Chiaradia, B. Tancini, C. Emiliani, F. Delo, R.M. Pellegrino, A. Tognoloni, L. Urbanelli, S. Buratta, Extracellular vesicles under oxidative stress conditions: biological properties and physiological roles, *Cells* 10 (2021) 1763, <https://doi.org/10.3390/cells10071763>.
- [30] A.P. De Oliveira, W. Chase, M.P. Confer, S. Walker, D. Baghel, A. Ghosh, Colocalization of  $\beta$ -Sheets and carotenoids in A $\beta$  plaques revealed with multimodal spatially resolved vibrational spectroscopy, *J. Phys. Chem. B* 128 (2024) 33–44, <https://doi.org/10.1021/acs.jpcc.3c04782>.
- [31] L. Zhou, Z. Ma, X. Gao, Retinoic acid prevents  $\alpha$ -Synuclein preformed fibril-induced toxicity via inhibiting STAT1-PARP1 signaling, *Mol. Neurobiol.* 60 (2023) 4828–4841, <https://doi.org/10.1007/s12035-023-03376-x>.
- [32] H.-A. Park, M.M. Hayden, S. Bannerman, J. Jansen, K.M. Crowe-White, A ti-apoptotic effects of carotenoids in neurodegeneration, *Molecules* 25 (2020) 3453, <https://doi.org/10.3390/molecules25153453>.
- [33] P. Si, C. Zhu, Biological and neurological activities of astaxanthin, *Mol. Med. Rep.* 26 (2022) 300, <https://doi.org/10.3892/mmr.2022.12816> (Review).
- [34] J.H. Kim, J. Hwang, E. Shim, E.-J. Chung, S.H. Jang, S.-B. Koh, Association of serum carotenoid, retinol, and tocopherol concentrations with the progression of Parkinson's disease, *Nutr. Res. Pract.* 11 (2017) 114, <https://doi.org/10.4162/rp.2017.11.2.114>.
- [35] N.M. Elsayed, Antioxidant mobilization in response to oxidative stress: a dynamic environmental–nutritional interaction, *Nutrition* 17 (2001) 828–834, [https://doi.org/10.1016/S0899-9007\(01\)00646-3](https://doi.org/10.1016/S0899-9007(01)00646-3).
- [36] C. Cocco, M. Siotto, A. Guerrini, M. Germanotta, C. Galluccio, V. Cipollini, L. Cortellini, A. Pavan, S. Lattanzi, S. Insalaco, E. Ruco, R. Mosca, B. Campana, I. Aprile, Systemic oxidative stress in subacute stroke patients undergoing rehabilitation treatment, *Antioxidants* 13 (2024) 354, <https://doi.org/10.3390/antiox13030354>.