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ORIGINAL RESEARCH

Metabolomics analysis and cytokine profiling in ARDS: rationale and methodology of standardized laboratory procedures for biological sample analysis

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Abstract

Background: Acute respiratory distress syndrome (ARDS) is a life-threatening condition associated with high short- and long-term morbidity and mortality. One major limitation in the management of ARDS is its biological and clinical heterogeneity, which may explain the lack of consistent benefit observed for most therapeutic interventions in unselected patient populations. Recent studies have suggested the existence of distinct ARDS subphenotypes, potentially characterized by unique inflammatory or metabolic signatures, which may respond differently to treatment. This supports the need for standardized tools to identify these subgroups and develop personalized therapeutic strategies. **Methods**: This manuscript describes a standardized protocol for metabolomic and cytokine profiling of biological samples from ARDS patients. Specifically, we outline detailed procedures for the collection and processing of serum and bronchoalveolar lavage fluid, and for the subsequent multi-omic analysis. Metabolomic profiling is performed using gas chromatography–mass spectrometry (GC–MS), following a validated sample preparation and derivatization workflow, allowing both targeted and untargeted metabolic analysis. Cytokine profiling is conducted using a Luminex® multiplex immunoassay platform, enabling the simultaneous quantification of multiple inflammatory mediators from low-volume samples. The manuscript also provides recommendations on sample quality control, data integration with clinical and imaging parameters, and multivariate statistical approaches for data interpretation. **Conclusions**: The described approach enables high-throughput, standardized, and reproducible molecular profiling of ARDS patients across different clinical studies. It is intended to support the identification of ARDS subphenotypes based on inflammatory and metabolic signatures, and to foster the integration of biological data into personalized clinical decision-making. This may serve as a methodological foundation for future prospective investigations aimed at improving outcome prediction and tailoring therapy in patients with ARDS.

Keywords

Acute respiratory distress syndrome; Cytokine profiling; Disease phenotype; Respiratory failure; Inflammation; Metabolomic; Mechanical ventilation

1. Introduction

Acute Respiratory Distress Syndrome (ARDS) is a life-threatening condition characterized by widespread inflammation in the lungs, leading to impaired gas exchange and severe respiratory failure, associated with persistently high morbidity and mortality rates (>35%). The etiology of this condition is multifactorial, with potential origins including, but not limited to, pneumonia, trauma, or aspiration [1].

Therapeutic approach to ARDS includes management of the primary cause of respiratory failure, lung-protective mechanical ventilation, prone positioning, steroids, and, for most severe cases, extracorporeal respiratory support [2–5]. However, with the exception of lung-protective mechanical ventilation, currently available therapeutic approaches did not show

convincing evidence of improvement in survival when tested in unselected populations of ARDS patients, underscoring the urgent need for novel tools to individualize therapy [6–11]. Recent studies suggested that different phenotypes of the same clinical syndrome may exist [12, 13], and may respond differently to the same therapeutic interventions [14, 15].

In light of these persistent challenges, significant attention has turned toward understanding ARDS heterogeneity through molecular analysis approaches [16, 17]. In particular, metabolomics and cytokine profiles are powerful tools for understanding the pathogenesis underlying the disease [17]. Metabolomics analyzes small molecules to uncover changes in metabolism, inflammation, and cellular stress, which are central to the pathology of ARDS [18]. The cytokines profile identifies the status and the fingerprint of local inflammation and immune response, helping to define molecular signatures of the disease [19].

Accordingly, we developed a standardized laboratory protocol to perform metabolomic analysis on bronchoalveolar lavage (BAL) and cytokine profiling on either BAL or serum in patients with ARDS. This will allow for a further understanding of the biological heterogeneity of ARDS. The analysis methodology described in our manuscript could be used in multiple research projects involving biological sampling in ARDS patients [20].

2. Methods

2.1 Study aim

This study aims to describe a methodological approach for performing metabolomic analysis of BAL and cytokine profiling in BAL and serum from patients with ARDS. By applying this approach, it will be possible to:

- Identify metabolites that correlate with disease severity or predict outcomes;
- Identify metabolic pathways that could be modulated to improve patient outcomes;
- Identify cytokines signatures that can serve as diagnostic or prognostic biomarkers;
- Discover specific cytokines that could be targeted to modulate disease progression.

The methodology described herein was developed through the integration of established protocols from prior metabolomics and cytokine profiling studies in critical care [21, 22], adapted to the ARDS population through internal validation in pilot experiments and consensus among experts (all included as Authors of this manuscript) from the participating institutions.

2.2 Sample collection and management

To obtain serum samples, whole blood can be collected in 10 mL Becton Dickinson (BD) Vacutainer Clot Activator Tube (CAT, Plus Blood Collection Tubes, Becton Dickinson, Plymouth, UK) and centrifuged at 3500 revolutions per minute (rpm) at room temperature. Thereafter, the supernatant can be stored at −80 °C until the analysis. BAL fluid can be obtained following the official recommendations [23] and adapted to the specific conditions of each patient. Bronchoalveolar fluid quality can be deemed inadequate if the sample has a purulent appearance and an increased number of erythrocytes due to an iatrogenic traumatic procedure.

For processing of the study samples, BAL fluids can be centrifuged at 1000 rpm at room temperature. The supernatants can be aliquoted and stored at −80 °C until use.

Timepoint for sample collections and number of collected samples will depend on protocols of individual studies using the described methodology. However, collection of the first sample within 48 h from enrolment will be strongly recommended in each study.

Standardization with protein or urea concentration will not be performed, as it has been demonstrated that both parameters increase in pro-inflammatory lung processes due to increased vascular permeability [24–26]. To overcome this limitation, and in accordance with experts' recommendations [27], we will use the smallest possible volume of saline solution to obtain BAL fluid, and will ensure precise pooling, mixing, and accurate documentation of the lavage fluid volume.

2.3 Metabolomic analysis

2.3.1 Metabolomic analysis by gas chromatography–mass spectrometry

Metabolomics research seeks to obtain a comprehensive profile of the wide range of metabolites present in biological samples. Due to the wide dynamic range of metabolite concentrations and their diverse physicochemical characteristics, careful optimization of sample preparation methods and analytical detection strategies is essential to ensure accurate and reliable measurements [28]. Bronchoalveolar lavage represents a valuable biological sample for studying and diagnosing lung conditions, including chronic obstructive pulmonary disease (COPD), ARDS, and asthma. It has been widely used in previous research to explore various disease states, including the mechanisms underlying asthma, the impact of air pollution exposure, acute nanoparticle-induced toxicity, inflammatory processes in cystic fibrosis, and ARDS pathophysiology. Compared to more systemic biofluids such as plasma, BAL offers a localized perspective, as it contains molecular constituents near the site of lung injury or stress [29]. In conditions such as ARDS, analysis of lung-associated small molecule changes in BAL can provide important insights into the molecular mechanisms that drive inflammation and tissue damage. The integration with advanced separation and detection technologies, such as GC–MS, facilitates the relatively unbiased detection of a broad spectrum of known and unknown metabolites [30]. This approach, referred to as metabolite profiling, offers valuable insights into the biological system under investigation. Considering this background, in the present methodology study, it was decided to describe a protocol for performing a GC–MS metabolomic analysis of BAL, which will be subsequently correlated with cytokine profiling performed on either BAL or serum.

Gas chromatography–mass spectrometry integrates two complementary analytical techniques: GC enables the resolution of metabolites with similar mass spectra, including structural isomers, while mass spectrometry (MS) provides detailed fragmentation patterns that help distinguish co-

eluting but chemically distinct compounds. This powerful combination allows for both qualitative and quantitative analysis and is widely applied in clinical diagnostics as well as in high-throughput profiling of complex biological matrices [31, 32]. GC–MS-based metabolite profiling typically involves a six-step workflow [30, 32]:

- Metabolite extraction: compounds are extracted from the biological matrix using methods to maximize recovery while minimizing the metabolites' degradation or chemical alteration.
- Derivatization: to make metabolites volatile and suitable for GC analysis, derivatization is performed, most commonly by trimethylsilylation, which remains the preferred method because of its efficiency and compatibility with a wide range of metabolite classes.
- Gas chromatographic separation: metabolites are separated under rigorously controlled conditions, including consistent gas flow, temperature gradients, and column specifications, to ensure reproducibility.
- Ionization: as compounds exit the GC column, they undergo ionization—typically via electron impact (EI), a robust and reproducible method that generates characteristic fragmentation patterns and molecular ions for reliable identification. Ionization represents a fundamental step in MS, as it enables the conversion of neutral molecules into charged species, which are essential for their manipulation and detection in the vacuum environment of the mass analyser. Various ionization techniques are available, including EI ionization, where molecules are exposed to a high-energy electron beam (typically 70 electronvolt (eV)), leading to efficient ion formation but often accompanied by extensive fragmentation. In contrast, soft ionization methods—such as electrospray ionization (ESI), chemical ionization (CI), and atmospheric pressure photoionization (APPI)—impart lower energy, reducing molecular fragmentation but with the trade-off that not all analytes are readily ionized. Thus, the choice of ionization technique depends on the chemical nature of the analytes and the analytical objectives. In the case of the present study, EI ionization was employed.
- Mass detection: ionized metabolites are detected using mass analysers such as quadrupole (QUAD), ion trap (TRAP), or time-of-flight (TOF) systems. In the present case, a triple quadrupole detector was used.
- Data analysis: data are processed by matching retention times and fragmentation spectra against internal or publicly available databases. The software accompanying GC–MS systems vary in capability, with advanced platforms supporting automated deconvolution, correction for co-eluting molecules, calculation of retention indices, and optimal fragment selection for quantification.

2.3.2 Sample preparation

Bronchoalveolar lavage samples can be prepared following the method of Yan *et al.* [33] with minor modifications. In particular, BAL samples stored at −80 °C can be thawed at ambient temperature and centrifuged at 14,000 × g for 5 minutes at 4 °C to remove residual mucus. For each sample, 100 μL of succinic acid-2,2,3,3-d_4 (30 μmol/L) can be added as an internal standard, followed by the addition of 600 μL of methanol–acetonitrile mixture (2:1, volume per volume (v/v)) containing 0.1% formic acid. The resulting mixture can be subjected to sonication for 10 minutes and subsequently incubated at −20 °C for 30 minutes. After incubation, samples can be centrifuged at 13,900 × g for 10 minutes at 4 °C to remove pellet proteins from the supernatant, thereby leaving only the metabolites of interest. 100 μL of the supernatant can be shifted in a glass vial and dried with nitrogen at room temperature; subsequently, 30 μL of methoxyamine hydrochloride dissolved in anhydrous pyridine (15 mg/mL) can be added. The resulting mixture can be vortexed for 2 minutes and incubated at 37 °C for 60 minutes. Following this, 70 μL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane can be added. The samples can be vortexed for 2 minutes and then incubated at 70 °C for 60 minutes to complete the derivatization process. Finally, the samples can be allowed to equilibrate at room temperature for 30 minutes before being subjected to GC–MS-based metabolomic analysis.

The amino acid standard mixture and alkane mixture can be prepared under the same conditions as the sample.

2.3.3 GC–MS analysis parameters

GC–MS analysis can be conducted using a Trace 1310/TSQ 9000 mass spectrometer (Thermo Scientific, Sunnyvale, CA, USA). Chromatographic separation can be achieved using a DB-5 capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA, USA). High-purity helium (≥99.999%) can be used as the carrier gas at a constant flow rate of 1 mL/min. A 1 μL aliquot of each sample can be injected in splitless mode, with a solvent delay of 3 minutes. The oven temperature program can be as follows: was set at 80 °C and held for 0.7 min, then increased to 200 °C at a rate of 8 °C/min and held for 0 min, 300 °C at a rate of 30 °C/min for 3 min.

Mass spectrometric conditions can be as follows: the injector temperature can be maintained at 270 °C, the transfer line at 250 °C, and the EI source at 270 °C. Data acquisition can be performed in Full Scan mode with a mass-to-charge (*m/z*) range of 40–600 and in selected ion monitoring (SIM) mode.

2.3.4 Metabolome analysis

Gas chromatography enables the resolution of complex derivatized compound mixtures into individual components sequentially introduced into the mass spectrometer. Once inside the MS system, each compound undergoes ionization and fragmentation, producing a distinct set of ion fragments with specific mass-to-charge ratios. These fragment patterns (mass-spectral tags) act as unique molecular signatures, allowing for the identification and characterization of individual metabolites. Each mass-spectral tag possesses distinct characteristics that support the unambiguous identification of the corresponding parent metabolite when compared to a pure reference standard [34]. These identifying features include: (1) the chromatographic retention behavior, typically expressed as a retention index (RI), and (2) a unique profile of fragment ions, each defined by its specific mass-to-charge ratio (*m/z*). For quantitative analysis, it is essential to select fragment ions that are specific to the target mass-spectral tag. Only

unique fragments—those not shared with co-eluting mass-spectral tags or compounds with similar retention indices—should be used to ensure selective and accurate quantification. The use of non-specific fragments may compromise data reliability due to interference from structurally related or simultaneously eluting metabolites [30].

Data acquisition for BAL metabolomic analysis can be performed in SIM mode for targeted analysis and SCAN (Full Scan analysis) mode for untargeted analysis. Selected ion monitoring is a data acquisition mode in which the mass spectrometer focuses on detecting specific ions of interest rather than scanning the entire mass range. This approach offers greater sensitivity for targeted analytes and is particularly useful for quantitative analysis of trace components. Selected ion monitoring is often preferred for identifying and quantifying known compounds because it allows more efficient use of instrument time and improves the signal-to-noise ratio. On the other hand, SCAN involves the mass spectrometer monitoring a wide range of mass-to-charge ratios (*m/z*) over the time course of the GC separation. This mode is used for qualitative profiling and untargeted screening, identifying unknown compounds, and confirming results from other GC detectors. It scans across a broad *m/z* range, typically from 50–500 or even 600 *m/z*, capturing the full spectrum of ions produced by the sample.

2.3.5 Targeted screening

In target screening by GC–MS, known standards are used to identify and quantify specific compounds within a sample by comparing the retention times and mass spectra of the standards with those obtained from the sample. This approach is highly accurate for quantifying target compounds and provides a valuable method for confirming their identity. By focusing on known ions, targeted analysis can minimize interference from other compounds that may co-elute in the GC; this allows researchers to precisely identify and quantify specific compounds in complex mixtures, making it a powerful tool for various analytical applications. The amino acid standard mixture can be used to perform target analysis. Table 1 lists the amino acids analyzed in standard mixtures, along with their corresponding quantifier and confirmation ions. The identity of each amino acid is confirmed by comparing the amino acid MS spectra with the corresponding present in the National Institute of Standards and Technology 02 (NIST02) library (Institute of Standards and Technology, Gaithersburg, USA) (Fig. 1).

2.3.6 Untargeted screening

Untargeted screening of BAL samples can be performed based on alkane RIs since it allows compounds in complex samples to be identified by comparing their retention time with that of known alkanes. This approach, often referred to as "non-target analysis", is critical for identifying unknown metabolites within a matrix. While mass spectral matching is a powerful and essential tool for metabolite identification, it is not sufficient on its own to achieve unambiguous identification, particularly in complex biological matrices. A key limitation arises from the presence of numerous structural isomers that produce highly similar or identical mass spectra. To address this challenge, RI values—typically calibrated using a homologous series of n-alkanes—are recommended as complementary data, enhancing the specificity and confidence of compound

TABLE 1. Amino acid standard mixture used for target analysis performed in selected ion monitoring mode.

RT[1]	Amino acid	MW[2] (g/mol)	Derivatized Amino acid	Derivatized MW (g/mol)	Quantifier ion (*m/z*)	Confirmation ion (*m/z*)
8.95	Alanine	89.09	Alanine, 2TMS derivative	233.45	116	147
9.20	Glycine	75.07	Glycine, 2TMS derivative	219.43	102	174
10.90	Valine	117.15	Valine, 2TMS derivative	261.51	144	218
11.85	Leucine	131.17	Leucine, 2TMS derivative	275.53	158	147
12.24	Isoleucine	131.17	Isoleucine, 2TMS derivative	275.53	158	218
12.36	L-Proline	115.13	L-Proline, 2TMS derivative	259.49	142	216
13.34	Serine	105.09	Serine, 3TMS derivative	321.63	204	218
13.81	L-Threonine	119.12	L-Threonine, 3TMS derivative	335.66	218	217
15.90	L-Methionine	149.21	L-Methionine, 2TMS derivative	293.58	176	128
15.92	Aspartic Acid	133.10	Aspartic Acid, 3TMS derivative	349.64	218	232
16.39	L-Cysteine	240.30	L-Cysteine, 3TMS derivative	337.70	220	218
17.07	Glutamic acid	147.13	Glutamic acid, 3TMS derivative	363.67	246	128
17.24	Phenylalanine	165.19	Phenylalanine, 2TMS derivative	309.55	218	192
17.89	L-Lysine	146.19	L-Lysine, 3TMS derivative	362.73	84	156
18.75	Arginine	174.20	Arginine, 3TMS derivative	390.23	157	256
19.38	Histidine	155.16	Histidine, TMS derivative	241.36	154	254
19.45	Tyrosine	181.19	Tyrosine, 3TMS derivative	397.70	354	218

[1]*RT: retention time;* [2]*MW: molecular weight; TMS: trimethylsilyl.*

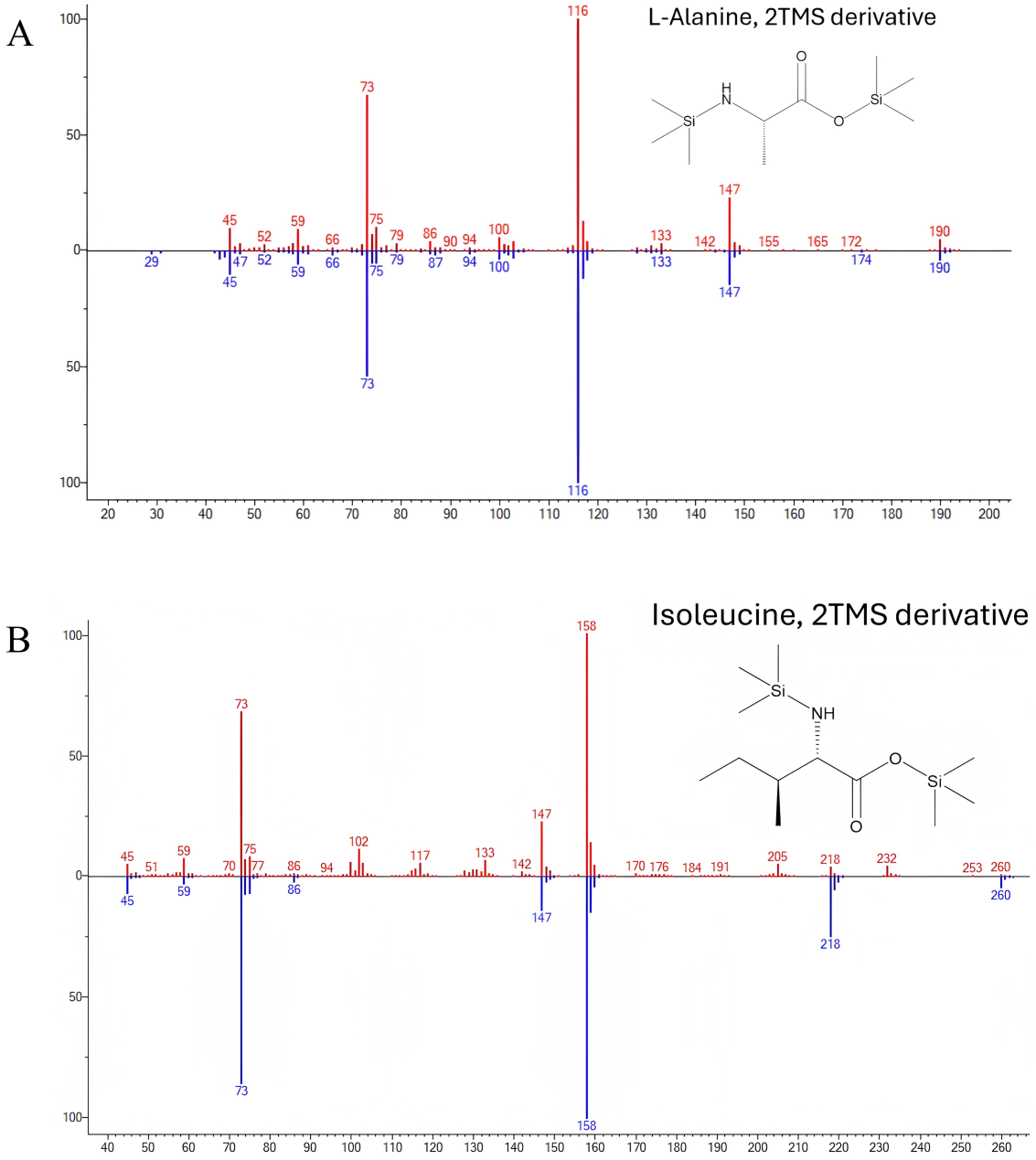

FIGURE 1. Example of comparison between amino acid mass spectrometry (MS) spectra (in red) with the corresponding present in the National Institute of Standards and Technology MS Search 2.4 library (in blue). (A) Fragmentation pattern of L-Alanine, 2TMS derivative. (B) Fragmentation pattern of Isoleucine, 2TMS derivative. TMS: trimethylsilyl.

identification [35]. In particular, for calculating the RI, the Kovats RI is used:

$$I_x = 100n + 100[log(t_x) - log(t_n)]/[log(t_{n+1}) - log(t_n)]$$

where t_n and t_{n+1} are retention times of the reference n-alkane hydrocarbons eluting immediately before and after chemical compound "X"; t_x is the retention time of compound "X". Therefore, The Kovats RI of an unknown compound is determined by interpolation between the RI of the n-alkanes that elute immediately before and after it in the chromatogram. Once the RI for an unknown molecule is calculated based on the mass spectrum, several possible molecules are identified in the National Institute of Standards and Technology (NIST) database, and then the RI for each is compared with that calculated for the unknown molecule. The NIST database is, indeed, the most extensive and widely utilized repository of reference RI values, containing information on over 100,000 compounds. Updated every three years, it serves as a critical resource for metabolite identification. Furthermore, this database underlies machine learning models designed to predict RI values for compounds lacking experimental RI data, thus expanding its utility in analytical workflows where reference standards are unavailable [36]. Generally, a difference of ±30 units of Kovats RI between the calculated value for an unknown compound and the value in the NIST database is considered a good starting point for potential identification. Table 2 reported examples of metabolites identified using RI.

TABLE 2. Example of metabolites identified using Kovats retention Index.

RT[1]	Name	RI[2] Calculated	RI NIST non-polar
8.25	Lactic Acid, 2TMS derivative	1067	1067
9.35	2-Hydroxybutyric acid, 2TMS	1133	1131
9.59	Oxalic acid, 2TMS derivative	1145	1139
9.89	3-Hydroxybutyric acid, 2TMS derivative	1164	1163
10.02	2-Hydroxy-3-methylbutyric acid, 2TMS derivative	1171	1171
10.14	2-Aminobutanoic acid, 2TMS derivative	1178	1177
10.36	Urea, 3TMS derivative	1189	1182
11.22	Urea, 2TMS derivative	1241	1237
14.79	Decanoic acid, TMS derivative	1457	1454
16.49	Creatinine enol N1, N3, O-tris(trimethylsilyl)	1577	1594
19.89	Palmitic Acid, TMS derivative	2047	2050
20.71	Oleic Acid, (Z)-, TMS derivative	2219	2208
20.81	Stearic acid, TMS derivative	2241	2239

[1]*RT: retention time;* [2]*RI: retention index; TMS: trimethylsilyl; NIST: National Institute of Standards and Technology.*

2.4 Cytokines profiling

The following 15-inflammation related biomarkers can be measured in either BAL fluid and serum of patients on the base of what reported in bibliography [21]: Interleukin-1 receptor antagonist (IL-1Ra), Interleukin-1β (IL-1β), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Interleukin-12 (IL-12p70), Interleukin-17A (IL-17-A), interferon g (IFN g), Tumor Necrosis Factor-alpha (TNF-α), Monocyte Chemoattractant Protein-1/Chemokin (C-C motif) ligand 2 (CCL2) type a (MCP-1a), Granulocyte colony-stimulating factor (G-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Macrophage Inflammatory Protein beta (MIP-1β), Intercellular Adhesion Molecule 1 (ICAM-1).

The Luminex® Multiplex Immunoassay is an advanced and high-efficiency approach for the simultaneous detection and quantification of multiple analytes, such as proteins, cytokines antibodies, and growth factors, within a single biological sample. Luminex® multiplex immunoassays are magnetic microparticle-based immunoassays that operate on the same sandwich immunoassay principles as conventional enzyme-linked immunosorbent assays (ELISAs). This technology enables the simultaneous quantification of up to 100 distinct analytes from a single sample, requiring significantly less sample volume compared to traditional plate-based methods. Other advantages include significant time and reagent savings compared to traditional monoplex methods, as well as good sensitivity and specificity, making them suitable for both research and diagnostic applications. Each microsphere, or bead, is internally dyed with a defined ratio of red and infrared fluorophores, producing a unique spectral signature that allows for the identification of specific bead regions and, thereby, of the corresponding analytes. For each analyte, a specific capture antibody is covalently coupled to a designated bead region. Upon incubation with the biological sample, target analytes bind to their respective beads. Following a wash step to remove unbound components, a mixture of biotinylated detection antibodies and streptavidin-phycoerythrin (PE) is added. The PE serves as a fluorescent reporter. The addition of a streptavidin-phycoerythrin conjugate, which binds to the biotinylated antibodies, generates a quantifiable signal proportional to the analyte concentration in the sample. The bead mixture is then analyzed on a Luminex instrument, which uses one laser (or light emitting diodes (LED), depending on the model) to excite the internal dyes and identify the bead region (*i.e.*, the analyte), while a second laser/LED excites PE to quantify the bound analyte based on fluorescence intensity. Multiple readings per bead region ensure high sensitivity and robustness of detection. This multiplexing capability provides comprehensive and efficient profiling of cytokines and other biomarkers, delivering valuable insights into biological mechanisms and disease-related processes. In our studies, the assays used for analytes quantification were designed and purchased from Bio-Techne S.r.l. On the day of the experiment, BAL fluid and serum samples were centrifuged at 16,000 rpm for 10 minutes at +4 °C immediately prior to use. Samples require a minimum 2-fold dilution using the assay-specific diluent provided with the assay. Dilution is also important to limit interference due to factors in complex matrices. However, high-abundance biomarkers may require additional dilution for samples to fall within the dynamic range of the assay. Typically, 40 samples can be assayed in duplicate. This will depend on the number of points being evaluated for the standard curve and the inclusion of any controls. The R&D Systems® Luminex® Assay is typically run with a six-point standard curve. The values of the unknown samples are assigned in relation to the standard curve. Samples were run at least in duplicate. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard (blank) median fluorescence intensity. The coefficient of variation (CV) of duplicates should be $\leq$20%. Standard curves for each analyte were generated using a five-parameter logistic (5-PL) curve fit. The instrument used for signal acquisition in our experiments was the Luminex® Bio-Plex 200 system.

Raw data were exported and analyzed using the dedicated xPONENT software (Version 4, Luminex Corporation, Austin, TX, USA). Final analyte concentrations were normalized to the volume of fluid used for bronchoalveolar lavage recovery.

2.5 Data analysis methods

2.5.1 Integration of clinical, radiological and laboratory data

Clinical, radiological and laboratory data will undergo quality control, normalization, and reduction using unsupervised clustering techniques (*e.g.*, hierarchical clustering, principal component analysis (PCA)) to identify biologically distinct patient subgroups. These molecular clusters will then be compared with clinical and imaging features to evaluate associations with specific ARDS phenotypes and outcomes (*e.g.*, mortality, ventilator-free days, need for extracorporeal membrane oxygenation (ECMO)).

Supervised machine learning models (*e.g.*, logistic regression, random forests) will be applied to test the predictive value of omics-derived features on predefined endpoints. All analyses will prioritize clinical interpretability and reproducibility, using validated frameworks in R and Python. The integration of metabolomic, clinical, and radiological data is expected to enable a more precise, phenotype-driven approach to ARDS management.

2.5.2 Multivariate analysis and metabolic pathway enrichment analysis

Multivariate analysis is a fundamental technique in metabolomics, as it enables the simultaneous examination of multiple variables to identify patterns and correlations in biological data. Specifically, it is used to distinguish sample groups, identify biomarkers, and understand metabolic changes in response to physiological or pathological conditions. One of the most common approaches is PCA, which reduces the dimensionality of the data and facilitates the interpretation of metabolic variations. Additionally, methods such as partial least squares discriminant analysis (PLS-DA) are used to classify samples and identify the most influential variables [37]. Multivariate analysis is particularly useful when working with complex data from techniques such as GC coupled with a mass spectrometer and cytokine profiling, which generate large amounts of information. With these tools, it is possible to gain a more detailed view of biochemical processes and their interconnections. In particular, PCA helps to reduce the dimensionality of the data by identifying the most significant directions of variation. This facilitates visualization and interpretation, enabling the identification of significant patterns in biological data. On the other hand, PLS-DA is used to build predictive models and classify groups of samples, a useful approach for detecting signs of disease [37]. In the present study, patients' medical records will be supplemented with metabolomics results to perform multivariate analyses and identify potential markers of ARDS.

Although multivariate analysis is effective in identifying patterns and biomarkers, it does not provide detailed information about the biological processes involved by itself. Therefore, it is complemented by metabolic pathway enrichment analysis, a technique that determines whether groups of metabolites are over-represented in specific metabolic pathways. The enrichment analysis of metabolic pathways can be conducted using the web-based tools available on MetaboAnalyst (https://www.metaboanalyst.ca). Hence, the altered metabolites will be used as input, referencing the Kyoto Encyclopedia of Genes and Genomes (KEGG) human metabolic pathways database [38]. This approach will allow to:

- Make a clearer biological interpretation: moving from a list of identified metabolites to a functional analysis of the metabolic pathways involved.
- Reduce data noise and complexity: by focusing on relevant biological pathways, less significant metabolites are eliminated.
- Improve the ability to identify meaningful biomarkers: through biological contextualization, the likelihood of identifying clinically relevant markers is increased.
- Validate obtained results: consistency between multivariate patterns and known biological processes increases the robustness of conclusions.

3. Discussion

3.1 Key findings

In this methodological manuscript, we described a standardized process and techniques for cytokine expression profiling in serum and BAL samples, as well as metabolomic analysis of BAL. These methods can be used in clinical studies of patients with ARDS.

3.2 Relationship to previous studies

Cytokine profiling and metabolomic analysis are becoming increasingly popular for defining specific disease phenotypes and better understanding molecular mechanisms of disease, also in critical care medicine [16, 17, 19, 39–44].

It is well recognized that ARDS is marked by complex biological mechanisms and significant variability in individual patient responses. This variability is influenced by factors such as the primary cause of ARDS, whether it is due to sepsis, pneumonia, trauma, or other insults, as well as individual differences in genetics and immune system function, all of which shape how the disease presents and progresses. Moreover, ARDS varies within the same individual over time, exhibiting dynamic progression and, thus, a changing response to treatment.

Indeed, the cytokines and metabolomic profiling in ARDS patients would be necessary for stratifying patients in ARDS subphenotypes, staging the disease in each patient (early phase of the inflammatory process or transition to fibrotic lung remodeling in resolving ARDS), and correlating cytokines profiles in the BAL fluid with diagnostic parameters for supporting current diagnostic and prognostic approaches in the field [7].

Metabolism is increasingly recognized as a valuable target for immunomodulatory therapies aimed at amplifying or dampening immune responses. Given the intricate interplay between metabolic pathways and inflammation, the concept of

the "inflammation-immunity-metabolism axis" has emerged as a promising framework for exploring new therapeutic strategies and insights into ARDS. Metabolomics, a rapidly evolving branch of systems biology, enables the comprehensive profiling of low-molecular-weight metabolites within biological samples. This approach is particularly well-suited for studying the pathophysiological complexity of acute lung diseases, such as pneumonia and ARDS, and has shown strong potential for biomarker discovery through either non-targeted or targeted metabolomic analyses [45]. In 2014, Evans and colleagues conducted a metabolomic investigation of BAL fluid from patients with ARDS (n = 18) and healthy individuals (n = 8) using liquid chromatography-mass spectrometry (LC-MS). Their analysis revealed elevated concentrations of lactic acid and metabolites associated with purine degradation—specifically guanosine, xanthine, and hypoxanthine—alongside reduced levels of phosphatidylcholines in ARDS patients [46]. This distinct metabolic profile is indicative of cellular injury linked to inflammatory processes and oxidative stress [47]. Another clinical study on preterm infants with respiratory distress syndrome detected throughout GC–MS analysis the presence of 25 metabolites in BAL fluid, 10 of which were structurally characterized. These included undecane, decanoic acid, dodecanoic acid, hexadecanoic acid, octadecanoic acid, hexadecanoic acid methyl ester, 9-octadecenoic acid, tetracosanoic acid, myristic acid, and phosphate. Notably, these metabolites exhibited elevated expression levels in BAL fluid samples obtained during mechanical ventilation following surfactant administration [48]. Hence, starting from previous investigations on metabolomic analysis in ARDS [22, 49–55], the present clinical trial aims to find new markers indicative of ARDS, making a metabolomic analysis through a target and untargeted GC–MS screening. Furthermore, compared to previous studies, this will be the first to include metabolomic analysis on BAL samples and cytokine profiling on both serum and BAL samples.

Notably, BAL fluid had higher sensitivity for biomarker detection compared with matched serum in terms of pulmonary diseases due to its proximity to the lung tissue, thus emphasizing the biological value of the cytokine profiles in BAL fluid. Few studies have been performed to measure cytokines in the BAL fluid of patients, and even fewer have utilized the Luminex technology proposed in our study [56–59]. The Luminex assay we describe in this manuscript is based on magnetic microparticle-based immunoassay, which utilizes the same sandwich principles as traditional ELISA tests. The essay offers the same benefits of the ELISA, however enabling higher throughput, reducing sample volume, and lowering costs for measurements, since up to 48 cytokines can be measured simultaneously in each well of the assay plate. The throughput of this analysis could indeed be easily translated into current clinical practice.

3.3 Implications of study findings

Metabolomics involves comprehensive analysis of metabolites, small molecules involved in metabolism, in biological samples. In the context of ARDS, metabolomics can reveal alterations in metabolic pathways that reflect disease severity, organ dysfunction, and treatment responses [17].

The energy-metabolism disruption during ARDS often leads to mitochondrial dysfunction and impaired energy production, as evidenced by altered levels of adenosine triphosphate, lactate, and other metabolites.

Alterations in inflammatory pathways often lead to changes in metabolites, such as acylcarnitines and amino acids, suggesting a shift in inflammatory and immune responses.

At the same time as organ-specific signatures, metabolomic profiles can differ between lung, liver, and kidney tissues in ARDS, highlighting organ-specific metabolic disturbances.

It has been demonstrated that inflammatory mediators with elevated levels of cytokines and chemokines in the lungs and bloodstream correlate with disease severity.

We strongly believe that the combination of metabolomics and cytokine analysis offers a more comprehensive understanding of ARDS severity and phenotype identification. Integrated analyses can reveal how changes at the metabolite and protein levels interact to drive disease processes. This holistic view can enhance biomarker discovery and development of therapeutic interventions [54].

Several studies already suggested that different phenotypes of ARDS may exhibit different responses to therapeutic interventions, such as fluid restriction or statin administration [14, 15]. Some recent studies have raised the hypothesis that also radiologic features may be used to individualize therapy in ARDS [7]. The combination of metabolomic cytokines and imaging analysis can further increase the possibility of identifying a specific phenotype and, thus, better individualized therapy.

For instance, we hypothesize that patients exhibiting a hyperinflammatory cytokine profile in bronchoalveolar lavage fluid—characterized by elevated IL-6, IL-8, and TNF-α—and radiological evidence of air-leak syndromes (*e.g.*, Macklin effect or pneumomediastinum) may represent a distinct, high-risk ARDS phenotype prone to barotrauma [7]. Identifying such profiles could support early selection for ultra-protective ventilation or extracorporeal support strategies [7, 60]. Indeed, ARDS phenotyping has been recognized as a key research topic by recent reviews and expert consensus [9, 10]. Our methodology can be used in prospective studies enrolling ARDS patients [20] and may provide key insights into this view and increase the possibility of providing individualized treatment for ARDS patients, as suggested and recommended by experts [6, 10, 16, 17].

3.4 Limitations of current methods

Cytokine profiling in serum has been widely used to understand immune dysregulation in several infectious and chronic diseases. However, several limitations must be acknowledged:

- Lack of site-specific information: serum cytokine levels do not distinguish the source or site of inflammation, which may limit their diagnostic utility;
- Biological variability: inter-individual variability, circadian rhythms, and pre-analytical handling can introduce significant noise, potentially masking meaningful trends in serum cytokine profiles.

While the analysis of cytokines in BAL fluid provides valu-

able insights into local pulmonary immune responses, several limitations must also be considered when interpreting the results:

- Dilution effect: the lavage procedure involves instillation and retrieval of saline, which dilutes the native alveolar secretions. This dilution can obscure true cytokine concentrations, particularly for low-abundance analytes, and complicate comparisons across samples or individuals;
- Procedure-induced artefacts: bronchoscopy and lavage can induce transient mechanical stress or minor inflammation, potentially influencing cytokine levels independently of the underlying disease process.

The analysis of both serum and BAL fluid cytokines has common pitfalls:

- The time points of the measurements: cytokine levels can rise and fall rapidly over hours; thus different time-point cytokine measurements in the patients may miss peak cytokine responses in some patients, increasing the complexity of the data interpretation;
- Cytokine stability: many cytokines are unstable and prone to degradation if samples are not promptly processed and stored under appropriate conditions. Variability in sample handling can lead to inconsistent results.

These limitations can be mitigated through standardized protocols across study centers, ensuring the following setup:

- A uniform timeline for BAL fluid and serum collection among enrolled patients;
- Use of the smallest possible volume of saline solution to obtain BAL fluid;
- Precise pooling, mixing, and accurate documentation of the lavage fluid volume;
- Established protocols for processing and storing serum and BAL fluid to minimize cytokine degradation;
- Integration of data from both serum and BAL fluid to identify the most ARDS subphenotype-specific cytokine profiles.

4. Conclusions

Metabolomic and cytokine analysis of serum and BAL samples from ARDS patients enrolled in prospective randomized studies can be used and combined with clinical and radiological data. Data obtained through multi-omic analysis can help define different subphenotypes of ARDS.

AVAILABILITY OF DATA AND MATERIALS

No data was used for the research described in the article.

AUTHOR CONTRIBUTIONS

GP, AB, GM, MC, LM, NDT—Designed the research study. SM, FMO, GG, MCI, VC, NDT, MP, MSS—Performed the research. MCI, VC, NDT, MP, MSS, BC—Formal Analysis. GP, MC, LM, AB, EM, MSS, ER, DP—Investigation. ER, MC, BF, BC, NDP, GG—Data Curation. GP, AB, EM, MCI, DP—Wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. Alessandro Belletti and Giacomo Monti are serving as the Editorial Board members of this journal. We declare that Alessandro Belletti and Giacomo Monti had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to ZQZ.

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