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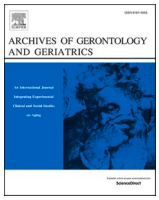

Mitochondrial dysfunction as a biomarker of frailty: The FRAMITO study protocol

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HIGHLIGHTS

- Mitochondrial dysfunction may support frailty development in older age.
- Frailty and multimorbidity share similar pathophysiological mechanisms.
- The FRAMITO study assesses mitochondrial dysfunction in adults living with frailty.
- Mitochondrial biomarkers will be tested in frailty with and without multimorbidity.

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ABSTRACT

Frailty syndrome often coexists with multimorbidity, sharing several risk factors and outcomes. Therefore, considering multimorbidity when exploring frailty biomarkers may deepen our understanding of these conditions' pathophysiology. In this regard, most studies focused on inflammation, but markers of mitochondrial dysfunction, such as mitochondrial DNA damage, cell respiratory impairment, and oxidative stress, are less explored. The FRAMITO project aims to evaluate mitochondrial dysfunction in frailty, with and without multimorbidity. This cross-sectional study will enroll 75 individuals aged ≥65 years from inpatient and outpatient clinics at the Geriatrics Units of the University Hospital of Ferrara (Ferrara, Italy) and Fondazione IRCCS San Gerardo dei Tintori (Monza, Italy). Participants will be categorized into three groups: 25 without frailty and multimorbidity, 25 with frailty but not multimorbidity, and 25 with frailty and multimorbidity. Blood samples will be collected to isolate Peripheral Blood Mononuclear Cells. Frailty biomarkers will be identified using untargeted metabolomics and functional studies on mitochondrial dysfunctions in PBMCs and their subpopulations, evaluating mitochondrial DNA damage, mitochondrial and glycolytic cellular bioenergetics, and

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intracellular reactive oxygen species. This project will advance our understanding of mitochondrial dysfunctions in frailty, particularly when combined with multimorbidity, revealing potential synergistic effects.
Clinicaltrial.gov registration number: NCT06433427.

1. Introduction

Frailty is a geriatric syndrome characterized by progressive combination of homeostasis disturbances in multiple systems leading to a reduction in physiological reserves and increased vulnerability to stressors. This makes individuals more susceptible to adverse events such as falls, acute illness, hospitalizations, and mortality (Dent et al., 2019, Lang, Michel, and Zekry, 2009). Estimates suggest that one in ten older community dwellers is living with frailty and this prevalence rises to over 50 % in long-term care facilities (Kojima, 2015, Collard, Boter, Schoevers, and Oude Voshaar, 2012). The natural course of frailty typically involves a gradual progression from robustness to frailty, ultimately leading to the development of disability and adverse health-related outcomes (Lang, Michel, and Zekry, 2009). Therefore, improving our understanding of pathophysiological pathways that contribute to the development of frailty, which may be targets of preventive actions, is crucial. In recent years, several biomarkers of frailty have been proposed; however, their specificity and sensitivity remain poor (Kane and Sinclair, 2019, Zampino et al., 2022). For instance, mild chronic inflammation associated with ageing, named *inflammaging*, is a promising frailty biomarker, and robust evidence has confirmed the presence of elevated inflammatory cytokines in older adults living with frailty (Franceschi et al., 2000).

Several studies found that the innate immune response plays a crucial role in frailty, with alterations leading to compromised function across multiple systems (Bleve et al., 2022). In analyzing inflammatory mediators in plasma, a distinctive cytokine profile was observed in frail compared to non-frail and young individuals. In particular, seven cytokines (Interleukin (IL-) -6, IL-8, IL-1α, IL-15, Cluster of differentiation (CD) 40, tumor necrosis factor (TNF-) -β, and C-X3-C Motif Chemokine Ligand 1 (CX3CL1)) exhibited significantly higher levels among people living with frailty, and five cytokines (TNF-β, IL1β, IL-2, IL-15, and IL-17) were exclusively detected in this specific population (Soysal et al., 2016, Zhang, Zeng, He, and Huang, 2023, Cardoso et al., 2018, Nascimento et al., 2018).

Interestingly, some of these cytokines have the potential to interact with relevant receptors on different cell types, eliciting intracellular reactive oxygen species (ROS) formation (Dhillion et al., 2012, Park, Song, and Ha, 2012, Liu and Desai, 2015, Forcina et al., 2019). Recent studies revealed that individuals living with frailty had increased oxidative stress and DNA damage in hematopoietic stem/progenitor cells and peripheral blood mononuclear cells (PBMCs), with a significant increase in γ-H2AX-positive cells not only in PBMCs but also in T-lymphocytes (Grasselli et al., 2022). In another recent study, the analysis of plasma from patients living with frailty revealed mitochondrial functional impairment associated with the activation of oxidative stress and a unique inflammatory mediator profile of frailty (Bombelli et al., 2024). According to the "mitochondrial theory of aging", mitochondria experience changes in their function, structure, distribution, and dynamics with advancing age. Since mitochondria are both a source and a target of ROS (Harman, 1981, El Assar, Angulo, and Rodríguez-Mañas, 2020), these changes may lead to progressive cellular damage and promote the multiple organ dysfunction typical of frailty (Bratic and Larsson, 2013). For instance, evaluating the effect of plasma from patients with frailty on allogenic renal stem/progenitor cells, significant functional impairment and nuclear DNA damages were revealed, and these could likely promote kidney dysfunctions frequent in frailty (Bombelli et al., 2024).

A further issue to be considered in this context concerns the cellular metabolism alterations that may contribute to frailty development. So far, metabolomic approaches have been focused on peripheral blood metabolites in serum (Rattray et al., 2019, Pujos-Guillot et al., 2019), whole blood, and plasma (Kameda, Teruya, Yanagida, and Kondoh, 2020, Fazelzadeh et al., 2016), while alterations of cellular metabolism, especially those related to mitochondrial dysfunction in frailty, are still underinvestigated. For instance, nicotinamide adenine dinucleotide (NAD) regulates cellular metabolism and redox homeostasis and plays a vital role in senescence and aging (Covarrubias, Perrone, Grozio, and Verdin, 2021, Xiao, Wang, Handy, and Loscalzo, 2018). The hydrogen transfer capacity of NAD is enabled by its redox site, depends on the balance between oxidized (NAD+) and reduced (NADH) NAD, and is crucial for cellular health and especially for mitochondrial metabolism.

An additional challenge in understanding the pathophysiology of frailty is the frequent coexistence of multimorbidity, a condition defined as the presence of two or more chronic diseases in the same individual (Johnston et al., 2019). The prevalence of multimorbidity, like frailty, increases with age (ranging from 30 % to 98 % from the youngest to the oldest old), reflecting the accumulation of biological and functional deficits, which affect the individual's self-sufficiency and other health outcomes (Barnett et al., 2012). The relationship between multimorbidity and frailty is closely intertwined and bidirectional since these conditions share common pathophysiological mechanisms, such as chronic inflammation. However, they present distinct characteristics and do not entirely overlap. As revealed by a meta-analysis, 72 % of individuals living with frailty are multimorbid, whereas only 16 % of people with multimorbidity present also frailty (Vetrano et al., 2019). These data highlight the importance of considering multimorbidity when assessing frailty pathophysiology and the need to explain the biological mechanisms underlying these conditions.

Given these premises, the present project aims to delve into the mechanisms supporting frailty development and identify specific biomarkers associated with mitochondrial dysfunction, considering the interaction of this syndrome with multimorbidity. This aim will be reached by combining several approaches based on metabolomics and functional studies on PBMCs, including PBMC subpopulations such as B lymphocytes, T lymphocytes and monocytes, to highlight the potential role of mitochondrial dysfunction in frailty.

2. Experimental design

This multicenter observational cross-sectional study will involve individuals aged 65 years or older in clinically stable conditions. Recruitment will take place at the Geriatrics and Orthogeriatrics Units of the University Hospital S. Anna in Ferrara (Emilia-Romagna region, Italy; coordinating center) and Fondazione IRCCS San Gerardo dei Tintori in Monza (Lombardy region, Italy; collaborating center). The local Ethical Committees of both centers approved the study protocol (approval code 653/2023/Oss/AOUFe; Oct 19, 2023, for the coordinating center; approval code: 4268_22.05.2024_M bis, May 22, 2024, for the collaborating center). Written informed consent will be obtained from each individual participating in the study.

Participants will be recruited from geriatric outpatient clinics or before discharge from the Geriatric and Orthogeriatric departments at recruiting centers. The extension of the study recruitment to both the inpatient and outpatient settings was due to the possibility of facilitating the enrolment of patients with different characteristics in terms of presence of frailty and multimorbidity. In particular, the following inclusion criteria will be considered to evaluate patients' eligibility: age ≥65 years, access to geriatric outpatient clinics or discharge from Geriatric/Orthogeriatric Units, clinically stable conditions, provision of written informed consent to participate in the study, and proficiency in

the Italian language.

Each enrolled participant will undergo a preliminary assessment of both frailty and multimorbidity and will be categorized into three distinct groups:

I. No Frailty without Multimorbidity (NFWoM) group, including individuals aged 65 years or older who do not exhibit signs of frailty and do not suffer from multimorbidity. This group will be the reference, including individuals who are not frail and have no multiple chronic conditions.
II. Frailty without Multimorbidity (FWoM) group, including frail individuals aged 65 years or older who do not have multimorbidity. This category will enable examining frailty biomarkers in the absence of multiple chronic conditions.
III. Frailty with Multimorbidity (FWM) group, including individuals aged 65 years or older who have frailty and two or more chronic diseases. The presence of both frailty and multimorbidity in this group will allow for studying the combined effects of these two conditions.

The preliminary assessment aims to ensure an equitable distribution of participants across the three groups to facilitate a comprehensive comparative analysis of the results.

Patients who have no frail but present multimorbidity will be excluded from the study.

2.1. Population and sample size

Regarding the sample size, considering the expected number of recruitable eligible patients accessing the inpatient and outpatient clinics, we have planned to enroll at least 75 individuals (25 participants for the three groups by frailty and multimorbidity). This sample will allow to reach a power of 80 % to detect a difference in the group means of a biomarker of mitochondrial dysfunction with an effect size (ratio between standard deviation on the means and within-group standard deviation) of 0.366 considering a one-way ANOVA with a type I error rate of 5 %.

3. Procedures

For each participant, data collection will be performed by physicians trained for the study activities through personal interviews, administration of validated scales and questionnaires, physical tests, and blood samples. The study procedures are graphically reported in Fig. 1, and the assessments are briefly described below.

Sociodemographic data and risk behaviours. For each participant, we will collect information on the date of birth, sex, living setting (home, long-term care, family home, hospital), and smoking habits.

Comprehensive Geriatric Assessment. Anthropometric parameters, including body weight, height, and calf circumference, will be measured for each participant. Functional status will be evaluated through the Basic Activities of Daily Living (BADL) and Instrumental Activities of Daily Living (IADL) scales (Katz et al., 1963, Lawton and Brody, 1969), and cognitive performance will be assessed using the Short Portable Mental Status Questionnaire (SPMSQ) (Pfeiffer, 1975). Mobility level will be categorized based on the ability to walk with or without aids or being bedridden. Depressive symptoms will be assessed through the Center for Epidemiological Studies-Depression (CES-D) scale. This is a 20-item measure that asks participants to rate how often, over the past week, they experienced symptoms associated with depression, such as restless sleep, poor appetite, and feeling lonely. Response options range from 0 to 3 for each item (0 = Rarely or None of the Time, 1 = Some or Little of the Time, 2 = Moderately or Much of the Time, 3 = Most or Almost All the Time). Scores range from 0 to 60, with higher scores indicating greater depressive symptoms (Radloff, 1977). Moreover, the number and type of chronically used medications will be collected from

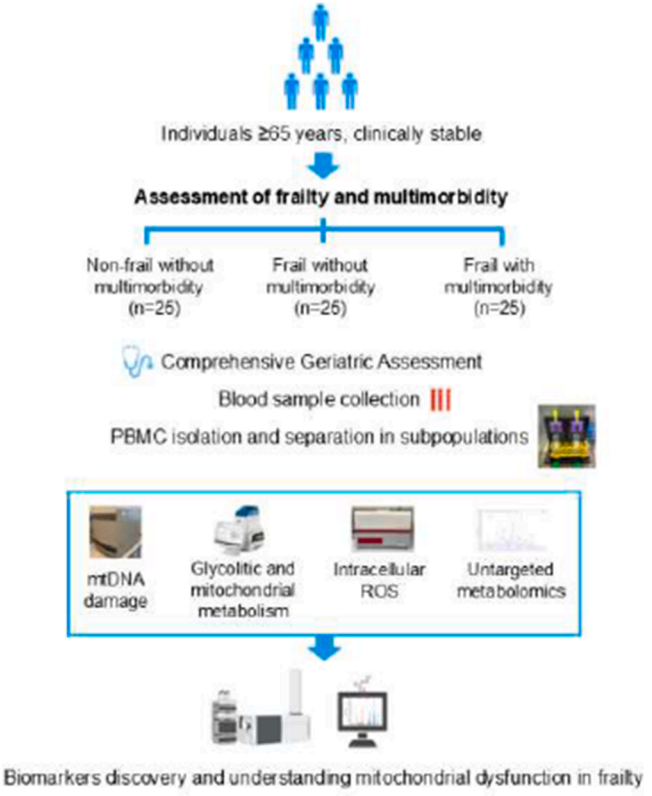

Fig. 1. Flow-chart of the FRAMITO study. Biomarkers discovery and understanding mitochondrial dysfunction in frailty.
Abbreviations. PBMC, peripheral blood mononuclear cells; ROS, Reactive Oxygen Species.

the patient's medical history. Only for participants recruited during the hospitalization, we will also record whether delirium occurred over the hospital stay and, in affirmative cases, which type of delirium.

Frailty status. Frailty will be defined according to the criteria by Fried et al. (Fried et al., 2001) as the presence of at least three of the following criteria: involuntary weight loss (≥4.5 kg) over the past year, muscle weakness measured by handgrip strength using a calibrated dynamometer, self-reported exhaustion, low physical activity, and reduced walking speed measured by the 4-m walking test.

The handgrip and walking speed values will be defined as low based on cut-offs derived from existing data of the Progetto Veneto Anziani (Pro.V.A) (Corti et al., 2002), which involved a similar older population in a close geographical area to the FRAMITO sites. In particular, the lowest 20th percentiles of sex- and BMI-specific categories for handgrip, and sex- and height-specific categories for walking speed will be considered (for details, please see **Supplementary Table 1**).

Exhaustion will be ascertained based on the CES-D, if the participant would report a frequency ≥3 days/week on at least one of the two items: "I felt that everything I did was an effort" and "I could not get going".

Physical activity will be assessed with the International Physical Activity Questionnaire (IPAQ) (Hagströmer, Oja, and Sjöström, 2006), and, according to the current literature, low physical activity will be defined as 1) not exercising, or 2) getting <3 days/week of intense physical activity or 30-min walking, or 3) getting <5 days/week of moderate physical activity or walking for >30 min, or 4) getting <5 days/week of any combination of moderate/intense physical activity or walking (Craig et al., 2003).

Moreover, from clinical data and the participants' medical history, we will derive the Primary Care Frailty Index (Vetrano et al., 2023).

Multimorbidity. Multimorbidity will be defined as the presence of at least two concurrent chronic diseases (Johnston et al., 2019), evaluated

through a review of the patient's medical history and clinical records. The complete list of chronic conditions considered is reported in **Supplementary Table 2**.

As an additional measure of multimorbidity, for each participant, we will compute the Charlson Comorbidity Index, which is a weighted measure that has been associated with one-year mortality and other negative health-related outcomes (Charlson, Pompei, Ales, and MacKenzie, 1987).

Mitochondrial dysfunction parameters. Blood samples will be collected to isolate PBMCs concurrently with routine blood tests performed during clinical activities**.** Biological material will be stored and analyzed at the Laboratory of Oncology and Molecular Pathology of the Department of Medicine and Surgery at the University of Milano-Bicocca**.** Each participant will have 15 ml of blood drawn for PBMCs isolation by density gradient centrifugation with Ficoll in Leucosep tubes. Isolation of PBMCs will be performed from peripheral blood with anticoagulants (sodium heparin). Blood will be diluted with 1 volume of phosphate-buffered saline (PBS) pH 7.2. 15 ml of Ficoll density gradient medium will be placed in a 50 ml tube, 30 ml of diluted blood will be added to the tube before centrifugation at 1370 g for 20 min at 18 °C in a swinging bucket rotor without brake. Carefully the ring of mononuclear cells will be transferred to a new 50 ml tube, the tube will be filled with PBS, mixed, and centrifuged at 420 g for 15 min at 16 °C. Red blood cell lysis will be performed by RBC Lysis Buffer for 10 min at RT (Biolegend). After washing cells with PBS, PBMCs will be resuspended with complete RPMI medium (10 % FBS, 1 % Pen/Strep, 1 % Amphotericin, and 1 % Glutamine) pre-heated at 37 °C and incubated overnight.

Since PBMCs are a heterogeneous population of cells with specific metabolic behaviours, dissecting the single subpopulations' contributions is important. The obtained PBMCs will be separated into cell subpopulations, including B lymphocytes, monocytes, and T lymphocytes, using MACS Columns for magnetic cell separation, following the manufacturer's procedures (Miltenyi Biotec) and the following antibodies: anti-CD19, anti-CD14, and anti-CD3, respectively. Flow cytometry analysis will be performed on PBMCs and their subpopulations to verify their morphology and purity using anti-CD19 (clone REA675), anti-CD14 (clone Tuk4), and anti-CD3 (clone BW264/56) antibodies (Miltenyi Biotec) by Attune Nxt Cytometer (Thermo Fisher). The analysis will be performed using Kaluza 2.1 software (Beckman Coulter).

Mitochondrial DNA damage. A comprehensive assessment of mitochondrial dysfunction will be conducted in PBMCs and their respective subpopulations by assessing the mitochondrial DNA (mtDNA) damage. Total DNA extracted from PBMCs, T and B-lymphocytes, and monocytes will undergo the presence of alterations in mtDNA copy number using Real-Time PCR to assess mtDNA integrity and potential damage. Total DNA will be extracted using the QIAamp DNA Micro Kit (Qiagen). A total of 10 ng of DNA will be analyzed using a QuantumStudio 7 Real-Time PCR system (Applied Biosystems). The mtDNA copy number will be determined by multiplex analysis normalizing the level of the mitochondrial gene ND1 (mtND1) with that of RNase P nuclear gene (Thermo Fisher).

Intracellular ROS evaluation. The evaluation of intracellular ROS will be performed using fluorescent 2′,7′-dichlorofluorescein (DCF) technique. The cell-permeable fluorescent indicator 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma) undergoes deacetylation by cellular esterase into a non-fluorescent compound, which is subsequently oxidized by ROS into fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescent signal intensity correlates with intracellular ROS level. Briefly, 2×10^5 PBMCs, CD19+, CD14+, CD3+ cells will be centrifuged at 300 g for 10 min, and the pellet will be resuspended with a solution containing 10 μM of DCF-DA and incubated at 37 °C for 30 min, as described (De Marco et al., 2023, M. et al., 2024, Pasquale et al., 2020). Fluorescent intensity will be analyzed by FLUOstar Omega (BMG LabTech).

Glycolytic and mitochondrial metabolism. The evaluation of glycolytic and mitochondrial metabolism will be carried out using the Agilent Seahorse XFe96 extracellular flux analyzer, as previously described (Campioni et al., 2022). The Seahorse technology employs non-invasive methods to monitor cellular bioenergetics in real-time, measuring parameters such as basal, maximum, and spare glycolytic and respiratory capacities, metabolic plasticity, ATP production-linked glycolysis, and proton leak. This is achieved by quantifying the rates of oxygen consumption (OCR), extracellular acidification rate (ECAR), and proton efflux rate (PER) in living cells under basal conditions and in response to pharmacological or nutritional perturbations. PBMCs and their subpopulations from at least 14 individuals per group will be analyzed using the Agilent Seahorse XF T Cell Metabolic Profiling protocol (Agilent). This protocol involves measurements under basal conditions and following sequential injection of ATP synthase inhibitor oligomycin A, uncoupler BAM15, and electron transport chain (ETC) inhibitors rotenone + antimycin A. Preliminary Seahorse tests will determine the optimal cell seeding density. Bioenergetic parameters will be calculated using formulas reported in Table 1.

Following Seahorse analysis, the Operetta CLS^{TM} software Harmony will be used to count nuclei in each well, and Seahorse parameters will be normalized per cell number.

Metabolomic data. Untargeted metabolomics on PBMCs and PBMC subpopulations will be performed using a Liquid Chromatography-Mass Spectrometry (LC-MS) platform developed to resolve the polar metabolome (Burtscher et al., 2024). Cellular samples will be quenched and extracted using cold methanol, followed by protein precipitation by centrifugation. Hydrophilic interaction liquid chromatography (HILIC), will be utilized to separate the polar metabolome before MS detection using an Agilent 6546 LC-Q-TOF instrument. To ensure accuracy, the total protein content will be quantified with NanoDrop™ and used to normalize the metabolic profile of each sample. Data preprocessing will be conducted using MZmine, and univariate and multivariate statistical analyses will be employed, along with enrichment analysis, to study the metabolomic signature associated with frailty. In particular, the focus will be on the polar metabolome to obtain a comprehensive assessment of central and energy metabolism, aiming to identify specific metabolites and metabolic mechanisms associated with mitochondrial dysregulation. Untargeted metabolomics will encompass PBMCs and PBMC subpopulations to evaluate whether mitochondrial dysregulation occurs universally across all cell types or if different subpopulations exhibit characteristic metabolic alterations.

Table 1
Bioenergetics parameters measured with Seahorse Technology.

Respiratory parameters	Glycolytic parameters	ATP production parameters
Basal Respiration (mitoOCR) = OCR_{basal} – $OCR_{rot/antA}$ **Maximal Respiration** = OCR_{BAM15} – $OCR_{rot/antA}$ **Spare respiratory capacity** = OCR_{BAM15} - OCR_{basal} **ATP-linked Respiration** = OCR_{basal} - OCR_{oligo} **Proton Leak** = OCR_{oligo} - $OCR_{rot/antA}$ **Non-mitochondrial respiration** = $OCR_{rot/antA}$	**Basal glycolysis** = PER_{basal} - mitoPER **mitoPER** = mitoOCR x CCF ***CCF (CO_2 contribution factor)*** = 0.61 **Compensatory glycolysis** = PER_{oligo} **Glycolytic reserve** = Compensatory glycolysis - Basal glycolysis	mitoATP production rate = ATP-linked Respiration x 2 x P/O **P/O (pmol ATP/pmol O)** = 2.75 **glycoATP production rate** = Basal glycolysis **ATP production rate (total)** = mitoATP production rate + glycoATP production rate

Notes. Abbreviations: **mitoOCR**, mitochondrial oxygen consumption rate; **OCR_{basal}**, third of three measurements of oxygen consumption rate before any injection of drugs; **$OCR_{rot/antA}$**, lower value of oxygen consumption rate after the injection of drugs Rotenone and Antimycin A; **OCR_{BAM15}**, higher value of oxygen consumption rate after the injection of drug BAM15; **OCR_{oligo}**, lower value of oxygen consumption rate after the injection of drug Oligomycin A; **PER_{basal}**, third of three measurements of proton efflux rate before any injection of drugs; **mitoPER**, mitochondrial proton efflux rate (mitochondria-derived acidification); **CCF**, CO_2 contribution factor; **PER_{oligo}**, higher value of proton efflux rate after the injection of drug Oligomycin A; **P/O**, pmol ATP/pmol O.

3.1. Statistical analysis

Data processing will be performed by integrating clinical data with quantitative characteristics related to mitochondrial dysfunction analysis and metabolomics. Quality control will be conducted to assess the overall consistency of the data. Descriptive statistics will be computed using mean and standard deviation (or median and first-third quartile range for variables with non-normal distribution) for continuous variables and absolute and relative frequencies for categorical variables. The distribution of biomarkers of mitochondrial dysfunction will be compared among the three study groups (NFWoM, FWoM, and FWM) using ANOVA or Kruskal-Wallis's test. Post-hoc pairwise comparisons will be performed using the Tukey HSD test or Mann-Whitney test with Holm correction. Finally, the association between the selected biomarkers of mitochondrial dysfunction and frailty status will be assessed using multivariable ordinal logistic regression adjusted for relevant potential confounders. All analyses will be performed on the overall sample and after stratifying by center, enrolment setting (especially to compare those recruited from the orthogeriatric unit vs other settings), age and sex (in order to explore possible differences between male and female participants).

Statistical analyses will be performed using R software version 4.4.2 or higher.

4. Expected results

The results of this project may provide a substantial contribution to understanding the role of mitochondrial dysfunctions in frailty development, distinguishing those presenting in individuals with frailty alone or in association with multimorbidity. This aspect will allow us to untangle the complex physiopathological mechanisms underlying both frailty and multimorbidity, shedding light on potential synergistic effects resulting from their coexistence. Consequently, these findings may provide valuable insights into identifying targets for preventive and therapeutic interventions to mitigate frailty development and prevent the onset of disability and adverse health outcomes.

The scientific implications of this project are profound, as it advances our understanding of frailty syndrome, particularly at the cellular level, involving inflammatory and metabolic pathways. Developing biomarkers for this condition remains a significant challenge; by integrating datasets generated in this project - clinical phenotype, mitochondrial functional phenotype, and metabolic phenotype – it could be possible to explore associations that may lead to the identification of potential biomarkers.

Institutional review board statement

The local Ethical Committees of both centers approved the study protocol (approval code 653/2023/Oss/AOUFe; Oct 19, 2023, for the coordinating center; approval code: 4268_22.05.2024_M bis, May 22, 2024, for the collaborating center).

Informed consent statement

Informed consent will be obtained from all subjects involved in the study.

Data availability statement

At the study's completion, data will be available upon reasonable request to the study team.

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CRediT authorship contribution statement

Edoardo Locatelli: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Barbara Torsello:** Writing – review & editing, Methodology, Investigation. **Sofia De Marco:** Writing – review & editing, Methodology, Investigation. **Martina Lombardi:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Francesca Remelli:** Writing – review & editing, Methodology. **Giulia Pampolini:** Writing – review & editing, Methodology, Investigation. **Elena Ferrighi:** Writing – review & editing, Methodology, Investigation. **Marialucia Bursi:** Writing – review & editing, Methodology, Investigation. **Andrea Bellotti:** Writing – review & editing, Methodology, Investigation. **Valentina Pasquale:** Writing – review & editing, Investigation. **Giacomo Ducci:** Writing – review & editing, Methodology, Investigation. **Ouldouz Navaei:** Writing – review & editing, Methodology, Investigation. **Raffaella Candeloro:** Writing – review & editing, Methodology, Investigation. **Maria Cristina Ferrara:** Writing – review & editing, Methodology, Investigation. **Wenxiang Guo:** Writing – review & editing, Methodology, Investigation. **Eleonora Cucini:** Writing – review & editing, Methodology, Investigation. **Giuseppe Bellelli:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Massimiliano Castellazzi:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Elena Sacco:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Giuseppe Paglia:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Paolo Mazzola:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Davide Paolo Bernasconi:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Cristina Bianchi:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Caterina Trevisan:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.archger.2025.105803.

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