

BPIFB4 protein and monocyte phenotyping: a preclinical asset for marking the frailty condition

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ABSTRACT

Keywords

Frailty; longevity; monocytes; biomarker



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Frailty is a state of increased vulnerability to stressors arising from the systemic decline in physiological reserve mechanisms with aging. Advanced age impacts on frequency and phenotype of immune cells such as monocytes and macrophages. BPIFB4, a host defense protein with immunomodulatory activity, is protective in healthy long-living individuals in whom monocytes and macrophages have a favorable redistribution and phenotype. Although we reported an inverse correlation of the homozygous LAV-BPIFB4 haplotype with frailty in elderly subjects, the role of the circulating BPIFB4 levels as a frailty biomarker has not yet been characterized. In this study we investigated the correlation between BPIFB4 levels and both the frailty assessment/health status and monocytic profile in frail subjects.

Participants (40 frail individuals and 20 age-matched healthy volunteers) were subjected to standardized questionnaires to assess frailty risk, routine clinical examinations and blood tests; monocytes were analyzed by flow cytometry.

Overall, 70% of the frailty cohort had mild frailty, 25.5% had moderate frailty, and 5% had severe frailty. Compared to healthy controls, frail subjects showed lower levels of circulating BPIFB4 that inversely correlated with the relative risk index for hypertension and cardiovascular disease. The total circulating monocyte frequency is reduced in frail subjects compared to healthy controls. CD14⁺⁺CD16⁻ classical monocytes and CD14⁺CD16⁺⁺ non-classical monocytes were significantly increased in frail people compared to healthy controls, whereas intermediate CD14⁺⁺CD16⁺ monocytes were reduced. The M2/M1 monocytic balance was also altered in frailty condition. No relationship between BPIFB4 plasma levels and monocytes' subsets was found.

Our findings highlight that BPIFB4 protein has a potential prognostic value for marking the frailty condition.

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Introduction

People worldwide can expect to live longer. By 2030, about 20% world's population will be aged 60 years or over, which is why frailty is expected to reach epidemic proportions in the coming decades. Frailty represents an age-related dysregulation of the physiological functions and reserve mechanisms associated with adverse health outcomes. A state of vulnerability to stressors and dysfunctional homeostasis persists in frail subjects [1]. Frailty is recognized by clinicians, but its definition requires a complex systemic approach that

takes into account biological and psychosocial correlates, and single symptoms are not sufficient to highlight it [2, 3]. Precisely because of its syndromic nature, the research area lacks an operational assessment tool for frailty that meets international consensus [4-6]. Among the various frailty assessment tools, we particularly highlight Fried et al. [7] frailty phenotype and Rockwood's [8] cumulative deficit model, which have achieved an international reputation. The frailty phenotype ranges from not-frail to pre-frail and frail, [7], and the different frail states are gradually strongly associated with a higher risk of

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developing adverse geriatric outcomes. In agreement with Wleklík et al., frailty develops in 25% to 62% of patients with cardiovascular diseases and, meanwhile, the presence of CVDs implies the increased risk of developing frailty in older people [9]. Furthermore, multi-morbidities (such as hypertension, diabetes, and COPD) which are common in old and frailty, pose a detrimental predictor of health outcomes in older patients [10]. In the aging context, long-living individuals constitute a model of exceptional healthy aging, considering their ability in overcoming and coping better with age-related diseases and frailty, despite their biological age. Previous work from our group identified a longevity-associated variant (LAV) of BPIFB4 associated in homozygosity with exceptional longevity in different independent populations [11, 12]. The bactericidal/permeability-increasing fold-containing family-B-member-4 (BPIFB4) is a secreted protein highly abundant in respiratory secretions, in the upper airways and proximal trachea. Besides the longevity-associated variant (LAV), which is constituted by the minor allele of rs2070325 that is part of a four SNPs haplotype, the gene BPIFB4 presents other two isoforms: the wild type (WT)-BPIFB4, which is constituted by major alleles of the four SNPs, and the rare-variant (RV)-BPIFB4, found to be a biomarker of vascular dysfunction and hypertension [11, 13]. Compared to the other two isoforms, LAV-BPIFB4 gene transfer was found to exert advantages by reducing atherosclerosis progression and inflammation in ApoE^{-/-} mice [14], by contrasting immunosenescence and aorta senescence in a murine model of advanced age, [15], by restoring the heart function in a model of diabetic cardiopathy [16]. Furthermore, the serum of long-living individuals is enriched in BPIFB4 compared to controls and frail people, thus classifying their health status [17]. Moreover, Malavolta et al identified LAV-BPIFB4 haplotype was significantly under-represented in frail subjects and the LAV-BPIFB4 gene therapy in old mice clinically attenuated the progression of frailty [18]. LAV-BPIFB4 also showed an interesting involvement with regard to the immune compartment. Indeed, the longevity-associated variant of BPIFB4 showed the ability in driving both dendritic cells toward a regulatory phenotype [19] and the macrophage-skewing toward a pro-resolving M2 phenotype in atherosclerotic subjects [14]. Considering the age-related changes in innate immune cells and that circulating BPIFB4 levels were found to associate with the abundance of pro-resolving monocytes and macrophages in long-living individuals [20], here we evaluate the profile of monocytes and macrophages in recruited frail subjects compared to healthy volunteers and the potential correlation with BPIFB4 circulating levels. Indeed, more efforts are needed to find optimal biomarkers associated with frailty capable of being valuable for the early diagnosis or prognosis of frailty in older people. From a translational point of view, the main purpose of this work was to evaluate the possible usefulness of BPIFB4 as a prognostic tool for marking frailty.

Materials and methods

Study design and sample characteristics

The study is a single-center, cross-sectional survey conducted between January 2016 and January 2017 among a group of older patients recruited from a random sample stratified by age and gender, at the Department of Medicine, Surgery and Dentistry “Scuola Medica Salernitana”, University of Salerno and the University Hospital “San Giovanni di Dio e Ruggi d’Aragona”, Salerno Italy. The primary objective was to assess, through a validated questionnaire and clinical examinations, the health status and frailty index of the young old and the old/great old, respectively. The secondary objective was to understand, through blood tests, whether the immunophenotype

and genotypic characterization had a possible correlation with frailty. It is important to use screening and assessment tools to investigate the different dimensions of health and identify the frailty condition earlier to help patients recover function and prevent adverse outcomes.

The study was performed on a group of 67 individuals, n=47 frail patients and n=20 aged-matched healthy volunteers free from risk factors for, and clinical evidence of clinical signs and symptoms of relevant communicable disease agents and chronic diseases, and treatments related to medical conditions.

For each patient, venous blood (10 mL) was withdrawn for analyses and detailed anamnesis was collected. All participants signed an informed consent for the management of personal anamnestic data and blood samples. The study was approved by the Campania Sud ethical committee and conducted in accordance with the ethical principles deriving from the Declaration of Helsinki (N.78 _r.p.s.o. del 04/07/2018.” *Studio per la valutazione della correlazione tra le isoforme del gene BPIFB4 e il rischio di fragilità umana*”).

Of the forty-seven frail patients recruited, 40 were selected and completed the study, as the eligibility criterion was that the patients’ phenotype fell within the threshold value of frailty [7], that the patients were aged 65 to 90 years or older and that there were no obvious disabilities; seven fell within the exclusion criteria as they did not have the above characteristics and belonged to the robust subjects [1]. The patients met the criteria outlined in international clinical practice guidelines for the identification and management of frailty in older adults [21].

Data collection for baseline evaluation

Standardized questionnaires ascertained self-rated health status, health habits, weight loss, and self-reported medical diagnoses of cardiovascular events (hypertension, angina pectoris, chronic heart failure, stroke), diabetes, chronic pulmonary disease, and cancer.

The multidimensional procedure “Comprehensive Geriatric Assessment” was used to assess the functional ability [22, 23], physical, cognitive, and mental health [24, 25], and socio-environmental status [26, 27] of older patients. Functional status was ascertained by asking old patients whether they had difficulty performing 12 tasks of daily living, tasks included in instrumental activities of daily living (IADLs) and activities of daily living (ADLs) [28]. Physical function was assessed with several questions from the Physical Activity Scale for Elderly (PASE) [29], which includes standardized performance-based measures of physical function, such as time (seconds) taken to walk 4 meters [25] and grip strength (kilograms) of the dominant hand (2 measures on mean), using a Smedley handheld dynamometer. Cognitive and mental health was assessed with the Mini-Mental State Examination (MMSE) [30] and the Geriatric Depression Scale (GDS) [24]. The Social Support Assessment (SSA) was used to assess whether older patients had social relationships and, if so, whether the level of support was high, fair or low [27]. Through standardized clinical examinations, such as electrocardiogram, echocardiography, and pressure report, and subsequent evaluation of the data by physicians, cardiovascular diseases (hypertension, angina pectoris, chronic heart failure, stroke) were validated [31].

Further examinations ascertained: body weight (kg) and height (cm) to calculate body mass index (BMI); blood test to determine fasting glucose level; and M1/M2 immunophenotypic analysis and genotype characterization.

Rockwood frailty index data

Rockwood’s Frailty Index was calculated using information collected during various routine health assessments of older adults,

specifically 38 variables were considered to have an accurate index [32, 33]. Issues related to functional difficulties, such as difficulty in washing, dressing, sitting or getting up from a chair, walking, eating, taking care of the house, using the toilet, climbing or descending stairs, grocery shopping, household chores, preparing meals, taking medication, managing money, staying in bed at least half the day due to the health, and reducing habitual activity, were coded as binary variables, using the convention that “0” indicates no deficit and “1” indicates the presence of a deficit. For the self-rated health question “How do you rate your health?” a six-point Likert scale was used for responses, where the endpoints are labeled 0 = excellent, 0.25 = very good, 0.5 = good, 0.75 = poor, and 1 = poor. For the question “Has your health changed in the last year”, the response includes 0 = better/same, 1 = worse.

Standardized measures, to define physical health, including time taken to walk 4 meters, the cutoff of which was coded as a binary variable, where 1 \geq 10 frailty index criterion and 0 \leq 10 non-frailty index criterion; and grip strength, which was stratified into quartiles based on gender and body mass index (BMI) [7] and then recoded into binary as follows:

1 male = presence of grip strength if BMI \leq 24 and kg \leq 29; BMI 24.1-26 and kg \leq 30; BMI 26.1-28 and kg \leq 30; BMI $>$ 28 and kg \leq 32.

0 male = absence of grip strength if BMI \leq 24 and kg $>$ 29; BMI 24.1-26 and kg $>$ 30; BMI 26.1-28 and kg $>$ 30; BMI $>$ 28 and kg $>$ 32.

1 female = presence of grip strength if BMI \leq 23 and kg \leq 17; BMI 23.1-26 and kg \leq 17.3; BMI 26.1-29 and kg \leq 18; BMI $>$ 29 and kg \leq 21.

0 female = absence of grip strength if BMI \leq 23 and kg $>$ 17; BMI 23.1-26 and kg $>$ 17; BMI 26.1-29 and kg $>$ 18; BMI $>$ 29 and kg $>$ 21.

Variables related to cognitive health (GDS), such as “feeling that everything is an effort”, “feeling depressed” and “feeling happy”, were coded through a three-point Likert scale, 0 = rarely, 0.5 = sometimes, 1 = most of the time. Regarding the Mini-Mental State Examination (continuous variable), recoding was done according to the severity of impairment [31], assigning 1 for scores $<$ 10 defined as “severe dementia”, 0.75 for scores \geq 10 and \leq 17 classified as “moderate dementia”, 0.5 for scores \geq 18 and \leq 20 defined as “mild dementia”, 0.25 for scores $>$ 20 and $<$ 24 “mild cognitive impairment” (MCI), and 0 for scores \geq 24 “no cognitive impairment” [33].

The comorbidities were assessed both as a cumulative total and as a single disease, such as hypertension, diabetes, chronic obstructive pulmonary disease (COPD), chronic heart failure, angina pectoris, stroke, and cancer, labeled with a three-point Likert scale with endpoints such as 0 = no disease, 0.5 = suspected presence, 1 = presence of disease.

By dividing weight (kg) by height squared (m^2), body mass index (BMI) was calculated in old patients. The BMI (variable continuous) was recoded, according to the criteria established by the WHO, considering 0 = normal weight, 0.5 = overweight, and 1 = obese. The mini-nutritional assessment (MNA) was coded into three-point Likert scales labeled as 0 if the score is between 24 and 30 and shows “normal nutritional status”, 0.5 if the score is between 17 and 23.5 “risk of malnutrition”, and 1 if the score is $<$ 17 “malnutrition” [17].

Social Support Assessment (SSA) has been coded to 0 “low social support” if the range is 0 to 2.9; 1 “moderate support” if the range is 3 to 5.9; and 2 “high support” if the range is 6 to 10 [27].

The frailty index was calculated based on the score of the deficits present in the patients in relation to the total number of deficits considered. Based on severity, the frailty index was divided into “mild” if the score was between 0 to 13.9, “moderate” if it was between 14 to 24.9, and “severe” if it was between 25 to 38.

Flow cytometry and immune phenotypical analysis

Peripheral blood mononuclear cells (PBMC) were extracted from whole blood by density gradient (Ficoll). After separation, PBMC were collected and washed for the subsequent experiments.

Conjugated monoclonal antibodies against CD14, CD16, CD86, and CD163 were purchased from BD Biosciences. After 20 minutes of incubation at room temperature in the dark, cells were washed with staining buffer and resuspended for the FACS analysis. For each test, cells were analyzed using a FACS Verse Flow Cytometer (BD Biosciences).

ELISA assay

Plasma levels of BPIFB4 were measured using an ELISA Kit (Cusabio CSB-YP003694HU) following the manufacturer’s protocol. Concentration values were subjected to statistical analysis by using GraphPad Prism 6.0 software for Windows (GraphPad software).

Genotyping

Genetic analysis for the SNP rs2070235 (p.Ile229Val) on BPIFB4 was assessed in all subjects. From all samples collected, leucocytes were used to extract their genomic DNA (DNeasy kit, Qiagen®). Then, the DNA was quantified to normalize concentrations run on quantitative polymerase chain reaction (PCR)-Taqman-based method.

Statistical analysis

Descriptive statistics were used to summarize patients’ characteristics considering perception of current and last-year health status, diseases at baseline, and frailty indexes; responses to all items were shown with absolute and relative frequency values for categorical variables and mean and standard deviation for continuous variables. Multivariate analysis plots were used to show changes in values of the multidimensional comprehensive geriatric assessment (for better understanding, CGA data were recoded into 5-point Likert with endpoints labeled as -2 worst health status and 2 best health status). Poisson regression analysis was performed to calculate significant predictors of BPIFB4 protein and Rockwood frailty index on the measured variable. The incidence ratios (IRRs) and their 95% confidence intervals (CIs) were used in the Poisson regression models to measure the independent associations between the different variables and the outcomes of interest. Pairwise correlation analysis was performed between BPIFB4 protein and patrolling. For all analyses, values of 0.05 or less were considered statistically significant. Data analyses were conducted using STATA software (Release 16.1, StataCorp LLC, College Station, TX, USA, 2019).

Results

Demographic characteristics, perceived health status, and frailty indexes

Forty people, 35% female and 65% male, with a mean age of 73.5 ± 5.4 (range 65-86 years), with different health conditions and frailty were evaluated (**Table 1**). Overall, 70% of the cohort had mild frailty, 25.5% had moderate frailty, and 5% had severe frailty. 77.5% of patients rated perceived health as fair to good, while perceived health in the last year was rated worse for 65% of the old.

Among chronic diseases, the highest rates are evident for hypertension, cardiovascular heart failure, and diabetes (**Table 1**).

Comprehensive geriatric assessment of patients

Figure 1 shows the results of the comprehensive geriatric assessment. Physical health, assessed through standardized measures of

Table 1 | Baseline patient and disease characteristics of the 40 analyzed patients and 20 healthy controls enrolled in the study.

Patients characteristics (Tot sample = 40)	N	%	Healthy controls (Tot sample = 20)	%
Gender				
Female	14	35	7	35
Male	26	65	13	65
Age	73.5 ± 5.4 (65-86)		70 ± 4.9 (65-75)	
Perception of current health status				
Poor	2	5	–	–
Fair	11	27.5	–	–
Good	20	50	12	60
Very good	4	10	7	30
Excellent	3	7.5	1	10
Perceived health status in the last year				
Worse	26	65	N/A	N/A
Better	14	35	N/A	N/A
Prevalent Disease at Baseline				
Hypertension	32	82.5		
Diabetes	15	38.5		
COPD	14	35.9	N/A	N/A
CHF	28	71.8		
Angina pectoris	10	25.6		
Stroke	4	10.2		
Cancer	1	2.6		
Rockwood frailty index (RFI)				
Mild	28	70		
Moderate	10	25.5	N/A	N/A
Severe	2	5		

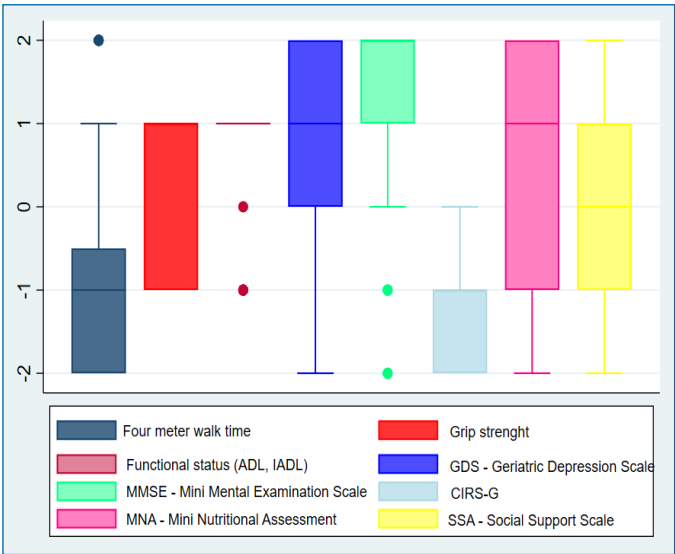


Figure 1 | Comprehensive geriatric assessment of patients. *Note:* CIRS-G Cumulative Illness Rating Scale in Geriatrics; ADL Activity of Daily Living; IADL Instrument Activity Daily Living.

some performance, including time taken to walk 4 meters, showed that 75% of the patients took longer than the established standard; while for grip strength, 2/4 of the older people presented grip ability. Regarding functional status, delineated through activities of daily living (ADL, IADL), it was inferred that 1/4 of the cohort had functional deficits.

Cognitive and mental health was good, 2/3 of the old did not suffer from depression, and only 17.5% had moderate or mild dementia. As for comorbidities, they were particularly evident in each patient. Regarding the screening of nutritional status, 1/3 of the patients had a risk of malnutrition. Finally, the social support need assessment showed that patients were equally divided between those who had high support and those who had low support.

Analysis of BPIFB4 blood levels in frail patients

The bactericidal/permeability-increasing fold containing family B member 4 (BPIFB4), characterized as both a longevity-associated and a host defense protein with a proven immunomodulatory activity (19, 20, 34), displayed prognostic relevance and inversely correlated with disease severity in COVID-19 and atherosclerosis. Furthermore, circulating levels of BPIFB4 are increased in healthy Long Living Individuals LLIs as compared to old controls [20] as a putative biomarker of life-long expectancy.

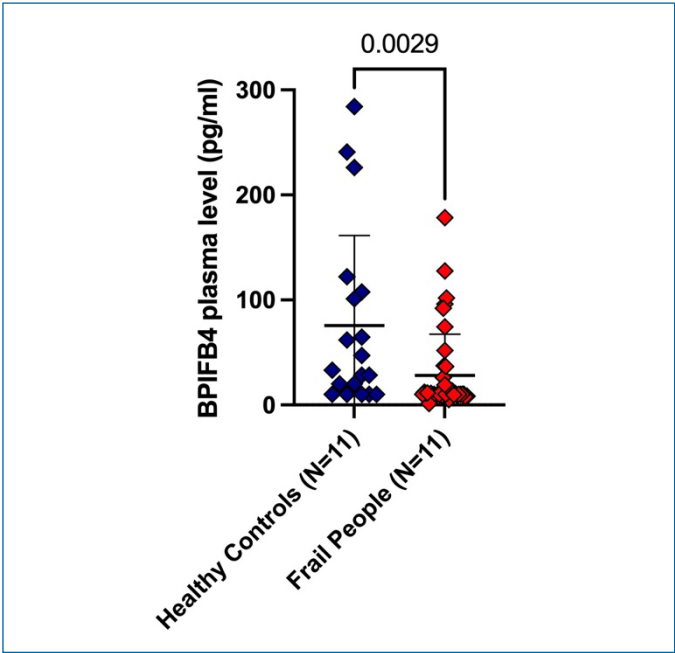


Figure 2 | ELISA quantification of BPIFB4 plasma levels in Healthy control and Frail People (non parametric Mann-Whitney U test).

We examined the plasma BPIFB4 levels in N=40 frail people and N=20 healthy controls, for comparison (**Figure 2**). Notably, BPIFB4 values were significantly lower in frail individuals as compared with old controls pointing to BPIFB4 as a bona fide biomarker inversely related to frailty condition.

Significant predictors of BPIFB4 protein, genotype, and Rockwood frailty index

The Poisson regression model constructed to study the relationship between BPIFB4 protein and patients' comorbidities showed a significant protective effect for hypertension (IRR = 0.32; 95% CI 0.12-0.844; p = 0.02) and cardiovascular disease (IRR = 0.50; 95% CI 0.26-0.97; p = 0.04), while there were no significant relationships with

diabetes, COPD and stroke (Model 1 **Table 2**). Furthermore, Poisson regression results revealed that statistically significant predictors of RFI were homo/hetero genotype (IRR = 0.78; 95% CI 0.64-0.95; p = 0.01) (Model 2 **Table 2**).

Characterization of monocytic dynamics in frail elderly

The frequency and phenotype of different immune cell populations are severely affected by the advanced age and its related comorbidities. Our data demonstrate that total circulating monocyte frequency is significantly reduced in N=11 immunophenotyped frail subjects as compared with healthy controls (**Figure 3**).

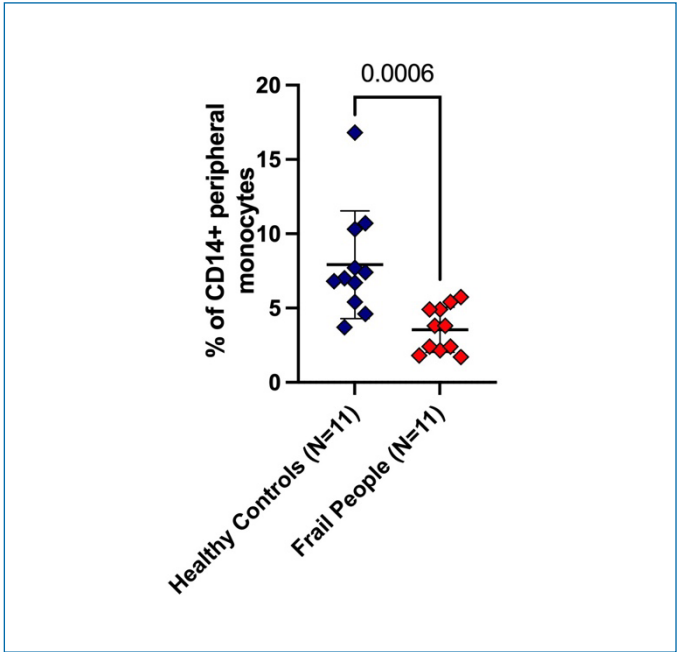


Figure 3 | Frequency of monocytes in Healthy Controls (N=11) and Frail People (N=11) expressed by percentage of total CD14+ positive cells in cytofluorimetric analysis. (non parametric Mann-Whitney U test).

Table 2 | Correlation analysis between BPIFB4 protein and patients' comorbidities.

Model 1. BPIFB4 (sample size = 39)			
Log likelihood = -50.03, x² = 15.42 (6 df), p = 0.017			
	IRR	95% CI	p
Hypertension	0.32	0.12 - 0.84	0.02
Diabetes	1.03	0.55 - 1.94	0.91
COPD	0.79	0.41 - 1.53	0.49
CHF	0.50	0.26 - 0.97	0.04
Angina pectoris	0.87	0.36 - 2.12	0.77
Stroke	1.11	0.23 - 17.22	0.89
Model 2. RFI (sample size = 39)			
Log likelihood = -116.11, x² = 6.28 (2 df), p = 0.043			
BPIFB4	0.99	0.99 - 1.00	0.50
Genotype			
WT	1	1	1
homo/hetero	0.78	0.64 - 0.95	0.01

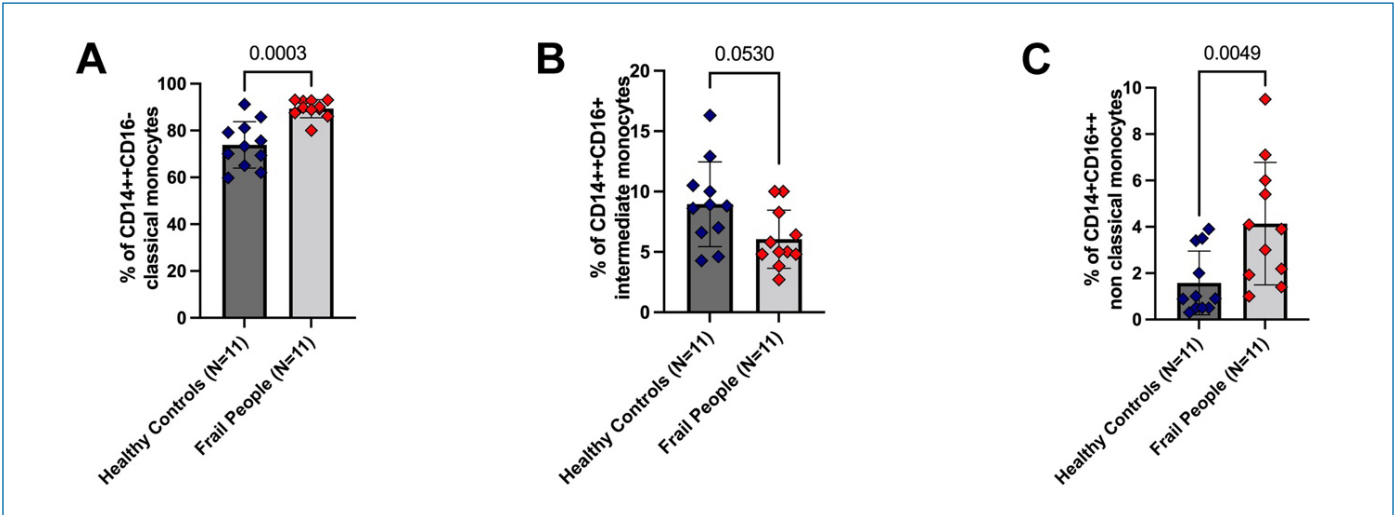


Figure 4 | FACS analysis of monocyte subpopulations in the healthy control group (N=11) and frail people group (N=11). In (A) is expressed the % of CD14++CD16- Classical monocytes, which appears to be increased in Frail People versus Healthy controls). In (B) it is expressed the % of CD14++CD16+ Intermediate monocytes that result decreased in Frail People compared to Healthy controls while in (C) it can be appreciated the difference in % of CD14+CD16++ Non-Classical monocytes subgroup that is increased in Frail People (non parametric Mann-Whitney U test).

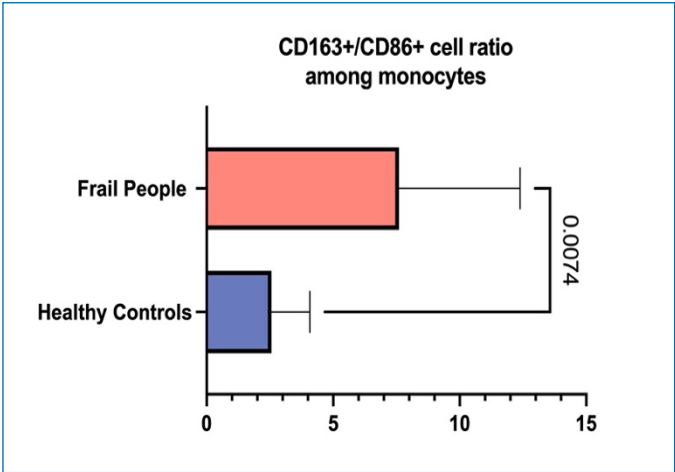


Figure 5 | CD163+/CD86+ ratio in CD14+ cells in Frail People (N=11) and Healthy Controls (N=11) (non parametric Mann-Whitney U test). In Frail People there is a higher ratio, meaning that CD14+CD163+ M2 monocytes are more abundant compared to CD14+CD86+ M1 monocytes.

Looking for differences in subsets of monocytes, CD14++CD16- classical monocytes and non-classical CD14+CD16++ monocytes were significantly increased in frail people compared to old controls, whereas intermediate CD14++CD16+ monocytes were reduced (**Figure 4**).

Moreover, when we profiled total CD14+ monocytes according to their surface levels of

CD86, a classical M1 pro-inflammatory marker, and CD163, a canonical M2 pro-resolutive marker, we described an altered balance of M2/M1 in frailty conditions compared to old volunteers (**Figure 5**).

As levels of CD163 are strongly regulated by mediators in the inflammatory response [35], its enhanced expression on monocytes from frail elderly may be a potential biomarker reflecting efforts by the immune system to resolve immune activation and inflammation (typically referred to as *inflammaging*).

Correlation analysis between BPIFB4 and circulating monocyte subsets.

As in LLIs BPIFB4 levels are associated with a favorable redistribution of monocyte compartment and macrophage polarization *in vitro*, we asked if the reduced BPIFB4 levels in frail people may dictate or contribute to the altered monocyte frequency and phenotype (**Table 3**).

Table 3 | Correlation analysis between BPIFB4 protein and patients' monocyte pool.

	BPIFB4	Non-classical monocytes	Intermediate monocytes	Classical monocytes	CD14 monocytes frequency
<i>BPIFB4</i>	1.000				
<i>Non-classical monocytes</i>	0.159	1.000			
<i>Intermediate monocytes</i>	0.021	0.179	1.000		
<i>Classical monocytes</i>	-0.100	-0.742*	-0.784*	1.000	
<i>CD14 monocytes frequency</i>	0.209	0.199	-0.184	0.019	1.000

Note: * significant correlation with p value ≤0.05.

Correlation analysis between the BPIFB4 protein and all the monocytes' subsets showed no relationship. Statistical significance was shown only between classical monocytes and non-classical monocytes ($p = 0.008$) and between classical monocytes and intermediate monocytes ($p = 0.004$).

Discussion

The main objectives of this study were to assess the health status and frailty index of a group of young old and old/great old, respectively, and to present associative clinical evidence between frailty and both frailty-specific protein biomarkers and immunophenotypically peculiar assets. Frailty is considered a complex and multidimensional syndrome influenced by both clinical features and social and environmental determinants of health. Frail people have a multisystemic reduction in normal physiological functions leading to increased vulnerability to stressful events and a reduced ability to restore homeostasis [4, 36]. The old population is normally characterized by a progressive loss of physiological reserves, but in frailty, this mechanism is even more evident [37]. Frailty is known to be associated with increased adverse sequelae [38, 39], depression [40], reduced self-sufficiency [41], fractures [42], cognitive impairment [43], hospitalization [44, 45], the need for long-term care interventions [41], reduced levels of quality of life [46, 47], and premature death [48, 49]. Therefore, in our study, using Rockwood and Mitnitski's model [33], a frailty index was constructed that provides a holistic view of different dimensions of health to identify this condition early, helping patients to prevent associated adverse outcomes. The older one gets the greater the risk of developing chronic diseases and multimorbidity (presence of two or more chronic diseases) situations [50, 51]. Accordingly, our results showed that comorbidities were particularly evident in each patient. Although the presence of multiple chronic conditions is associated with the development of frailty [52], frailty is not necessarily the result of chronic disease. On the other hand, it is also true that intensive or overtreatment of chronic diseases can increase adverse health outcomes in frail people [52, 53] as clarified by Elliot et al. [54] who claimed that frailty can hinder adherence to both pharmacological and rehabilitative therapies. Several studies have shown that a cardinal manifestation of frailty is loss of physical function [55]. Poor physical function, muscle atrophy, and dyspnea are shared conditions between the phenotypic model of frailty and sarcopenia. The approach to the diagnosis of sarcopenia involves the search for symptoms such as falls, weakness, slowness, self-reported muscle atrophy, or difficulty performing activities of daily living [56, 57]. According to the European Working Group on Sarcopenia in Older People (EWG-SOP) [56], the approach to the diagnosis of sarcopenia should be stepwise and begins with the measurement of muscle strength, usually grip strength, following which sarcopenia may be suspected. In our research, the results of standardized measures of some performances, including time taken to walk 4 meters, grip strength, and activities of daily living (ADL, IADL), showed that half of the cohort analyzed had suspected sarcopenia. In agreement with Coelho-Junior et al. [58], the risk is that a physically inactive lifestyle may lead to the progression of both conditions. In support, Landi et al. [59] evaluating this scenario, proposed sarcopenia as the biological substrate of frailty, while Marzetti et al. [60] combined the two conditions into a new clinical entity called physical frailty-and-sarcopenia. On the other hand, the pathophysiology of frailty and sarcopenia may have a multifactorial etiology involving many of the biological features of aging (e.g., genomic and epigenetic instability, loss of proteostasis, mitochondrial shortening, telomere shortening, stem cell depletion, cellular senescence) [61, 62].

In agreement with Solfrizzi et al. [63] who studied cognitive and mental components, they deduced a correlation between physical frailty and cognitive impairment, defined as cognitive frailty. The cognitive and mental health of the patients in our study was good, most of the old people examined did not suffer from depression, and only a small percentage (1/4) had moderate or mild dementia. It is important to remember, however, that cognitive frailty, like physical frailty, can be delayed at least in the early stages and that its presence can lead to an increased risk of events that negatively impact health [64], such as worsening quality of life [65], increased hospitalizations and mortality [66].

It is well known that health is a reflection of several factors in addition to biomedical factors, such as social [67] and environmental factors [68, 69]. Social determinants of health, such as work, social networks, eating habits, and internal and external living environment, can indeed decrease an individual's intrinsic and extrinsic abilities, making him or her frail. The results of our study showed that patients were equally divided between those with high support and those with low support. In agreement with Aliberti et al. [26], the old person needs the support of family and third parties, as well as cultural activities and recognition can have a significant impact in terms of personal well-being. Azzopardi et al. [67] in the context of frailty, point out that social aspects and especially environmental and personal (e.g., relational) factors of an individual are not sufficiently considered by social and health professionals.

Collectively, the results of our study showed that two-thirds of patients had mild frailty, so it was possible to intervene to help patients regain function. As changes in the proteome and the degree of peripheral immune response are also related to the progression into frailty [70], we propose protein biomarkers and immune traits related to the frailty condition and its progression. Indeed, the diagnosis of frailty is usually clinical and based on selected criteria, which are sometimes inconsistent. Therefore, there is an urgent need to identify and validate novel biomarkers.

While most popular circulating markers are those related to the inflammatory response (eg, C-reactive protein [CRP], IL6, and tumor necrosis factor- α [TNF α]) or oxidative stress and/or hormones (insulin-like growth factor-1 [IGF1], testosterone) [71] here for the first time the peculiar asset of monocytes and BPIFB4 circulating factor may constitute new disease biomarkers and therapeutic opportunities in the complexity of the frailty condition. BPIFB4 has already been shown to serve as a biomarker of healthy aging [14, 20, 72] and display prognostic significance in vascular pathology and COVID-19 [73] mainly influencing mono-macrophage skewing. Here BPIFB4 plasma levels are inversely correlated with frailty condition even though no significant correlations were found between BPIFB4 and different monocyte subsets characterizing frail elderly. Noteworthy, we corroborated a decline in monocyte frequency in frail people compared to the non-frail group. The peripheral reduction of monocytes may suggest their robust recruitment in the damaged tissue as also suggested by the higher levels of monocyte chemoattractant protein-1 (MCP-1) characterizing frail vs non frail-group [74]. The functional changes in peripheral monocyte response deserve much attention as they reveal an expected inflammatory arm (CD14⁺⁺CD16⁻ Classical monocytes *high*) but counterbalanced by a reparative polarized activity (CD14⁺CD16⁺⁺ Non-Classical monocytes *high* and CD14⁺CD16⁺ monocytes *high*) of monocytes. Indeed, as levels of CD163 are strongly regulated by mediators in the inflammatory response [35], its enhanced expression on monocytes from frail elderly may be a potential biomarker reflecting efforts by the immune system to resolve immune activation and inflammation (typically referred to as *inflammaging*). On

the other hand, the prevalence of CD14+CD16++ Non-Classical monocytes and CD14+CD163+ monocytes failing to be activated may reflect an exhausted state of the frail vs non-frail group. This scenario also emerged from the transcriptome on the single-cell level in human-aged immune cells from frail ($n = 5$; age, 88.0 ± 5.8 years) individuals compared to healthy old individuals ($n = 6$; age, 85.8 ± 11.1 years) [70].

Even though not investigated in our report, probably a different monocyte balance in the dynamic course of the frailty (pre-frail/frail) may be useful to dissect the pathophysiological process and its clinical progression. The lack of association with BPIFB4 levels can be in part explained by the sample size, which is an obvious limitation of our study, or probably because high BPIFB4 levels correlate with pro-resolutive M2 response only when resulted protective and useful to blunt inflammatory tone [14, 15, 75, 76], while in this context the CD163+/CD86+ balanced ratio contributed to the impaired nature of frail monocytes. Likewise, monocyte variations in some frail individuals can reflect a plethora of comorbidities-associated states for some of which BPIFB4 levels do not guarantee a proper degree of protection. Indeed, here BPIFB4 levels resulted protective for hypertension and cardiovascular disease, while there were no significant relationships with diabetes, COPD, and stroke (Model 1 **Table 2**). This is consistent with the cardiovascular benefits of carrying the LAV isoform of the BPIFB4 gene associated with healthy aging and a high degree of protection from hypertension, ischemia, and atherosclerosis. Furthermore, the association of the LAV haplotype with lower frailty in elderly subjects and the reduced frailty observed in mice treated with LAV-BPIFB4 gene therapy are in perfect agreement [18]. Here we confirmed a protective incidence relationship between frailty and homo/hetero BPIFB4 genotype (Model 2 **Table 2**). This genetic association may be strengthening the diagnosis of frailty which can take advantage of biomarkers, genetic and proteomic research, and incorporation of sociodemographic variables associated with frailty.

Conclusions

The epidemiological transition has led to a longer life expectancy with an increase in chronic diseases compared to acute diseases [77]. This may predispose to a progressive, whole-organism process of decompensated homeostasis with a substantial contribution from the impaired immune responses.

However, frailty is often reversible [78] in the early stages, before the onset of functional impairment. Therefore, early identification, through protein biomarkers and immunophenotypically at the pre-frailty or mild frailty stage, is important to help patients regain function and prevent adverse outcomes associated with the syndrome [79]. Our findings highlight that BPIFB4 protein has a potential prognostic value for marking the frailty condition deserving much attention in the near future.

Author contributions

E.C. designed and conducted the study, coordinated the research team, and wrote the manuscript. and S.M.A. performed statistical analysis and data interpretation and wrote the manuscript. F.M., V.L., C.B., A.C. and P.D.P., A.M. performed laboratory activities. M.C.C. and C.V. cared for the subjects of the study and evaluation of their health status and reviewed critically the paper. M.C. reviewed critically the paper. A.A.P. performed data interpretation and reviewed the manuscript. A.A.P. supervised the project in its entirety and provided financial support. All authors approved the final version to be published.

Competing interests

All authors declare no financial or competing interests that are directly relevant to the content of this manuscript.

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Declarations

Ethics approval and consent to participate.

The study was approved by the Campania Sud ethical committee and conducted in accordance with the ethical principles deriving from the Declaration of Helsinki (N.78 _r.p.s.o. del 04/07/2018." *Studio per la valutazione della correlazione tra le isoforme del gene BPIFB4 e il rischio di fragilità umana*"). All participants signed an informed consent for the management of personal anamnestic data and blood samples.

Consent for publication

Not applicable

Availability of data and materials

Data, materials, and protocols will be available on request by emails to the corresponding authors due to privacy/ethical restrictions.

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