GeroScience (2021) 43:1029–1037
https://doi.org/10.1007/s11357-020-00288-9

ORIGINAL ARTICLE

Characterization of the plasma proteomic profile of frailty phenotype

Kristina Landino · Toshiko Tanaka · Giovanna Fantoni · Julián Candia · Stefania Bandinelli · Luigi Ferrucci

Received: 5 August 2020 / Accepted: 14 October 2020 / Published online: 17 November 2020

Abstract Frailty is a risk factor for poor health outcomes in older adults. The aim of this study was to identify plasma proteomic biomarkers of frailty in 752 men and women older than 65 years of age from the InCHIANTI study. One thousand three hundred one plasma proteins were measured using an aptamer-based assay. Associations of each protein with frailty status were assessed using logistic regression and four proteins creatine kinase M-type (CKM), B-type (CKB), C-X-C motif chemokine ligand 13 (CXCL13), and thrombospondin 2 (THBS2) were associated with frailty status. Two proteins, cyclin-dependent kinase 5 (CDK5/CDK5R1) and interleukin 1 alpha (IL1A), were associated with worsening of frailty status over time in volunteers free of frailty at baseline. Using partial least squares discriminant analysis (PLS-DA), data of 1301 proteins was able to discriminate between frail and non-frail with a 2% error rate. The proteins with greater discriminatory ability represented the inflammation, blood coagulation, and cell growth pathways. The utility of these proteins as biomarkers of frailty should be further explored.

Keywords Proteomics · Aging · Frailty · Inflammation

Kristina Landino and Toshiko Tanaka contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11357-020-00288-9) contains supplementary material, which is available to authorized users.

K. Landino · T. Tanaka (✉) · L. Ferrucci
Translational Gerontology Branch, National Institute on Aging, NIH, Baltimore, MD 21224, USA
e-mail: tanakato@mail.nih.gov

G. Fantoni
National Institute on Aging, Intramural Research Program, Clinical Research Core, NIH, Baltimore, MD 21224, USA

J. Candia
Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892, USA

S. Bandinelli
Geriatric Unit, Azienda Sanitaria di Firenze, Florence, Italy

Introduction

Frailty is a geriatric syndrome that results from functional declines in multiple biological and physiological systems and that manifests itself as a vulnerable state characterized by sharp decline in health and functional status in response to minor stressors [1]. Several operational definitions of frailty have been proposed, including one by Fried and colleagues based on five criteria: poor grip strength, poor walking ability, unintentional weight loss, exhaustion, and low physical ability [2]. The presence of three or more of these criteria was defined as frailty and having one or two of these criteria was defined as "pre-frailty." Fried and collaborators demonstrated that the prevalence of both pre-frailty and frailty increases with aging, and older patients that meet such definition are frequently affected by multimorbidity and have high risk of developing

adverse health outcomes [2]. As the older portion of the population gradually expands, caring for the growing number of frail older persons is becoming a great public health concern and prevention of frailty is a research priority [3]. Identifying early biomarkers of frailty in the pre-symptomatic state may both identify individuals that should be targeted for preventive intervention and, in addition, provide clues about the biology and physiology of frailty.

Many studies have focused on single biomarkers theoretically involved in the pathogenesis of frailty. For example, high levels of circulating pro-inflammatory markers usually referred to as "inflammaging" may catabolic effect on muscle tissue and contribute to sarcopenia, one of the hallmarks of frailty [4]. High levels of C-reactive proteins (CRP) and interleukin 6 (IL-6) have been associated with frailty [5]. A few studies have implemented a more hypothesis generating agnostic proteomic approach, where larger number of proteins are examined simultaneously in relation to frailty status to identify novel biomarkers rather than exploring a specific biomarker from a known biological pathway [6, 7]. An agnostic approach is justified by the fact that mechanisms that converge to frailty are likely multiple and complex. To identify novel plasma proteomic biomarkers of frailty, we conducted an analyses of 1301 proteins, and examined their association with frailty status in a community-dwelling sample of older Italian volunteers form the InCHIANTI study.

Method

Study subjects and methods

The Invecchiare in Chianti (InCHIANTI) Study is a community-based cohort study based in the Tuscan region of Italy. A detailed description of the study has been previously reported [8]. Briefly, 1453 residents were selected for interviews from two cities (Greve in Chianti and Bagno a Ripoli) with an age range from 21 to 102. Of these, plasma proteomics were measured for 997 (69%) volunteers. The baseline visit was conducted between May 1998 and March 2000, and volunteers had a follow-up visit every 2–3 years. In this study, data from follow-up 1 (November 2001–March 2003), follow-up 2 (December 2004–March2006), and follow-up 3 (November 2007—May 2009) are used. At each visit, sociodemographic information (age, sex, education, smoking, physical activity) was obtained during a structured interview. The study protocol was approved by the Italian National Institute of Research and Care of Aging Institutional Review and Internal Review Board of the National Institute for Environmental Health Sciences (NIEHS). All participants provided written informed consent.

Proteomic assessment

Proteomic assessment was conducted using an aptamer-based assessment method. Aptamers (or SOMAmers) are short single-stranded oligonucleotides probes that are chemically modified to bind with high affinity to proteins in its native form [9]. Proteomic profiles for 1322 SOMAmers were assessed using the 1.3 k SOMAscan Assay at the Trans-NIH Center for Human Immunology, Autoimmunity, and Inflammation (CHI), National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD, USA) using the same methods as previously published [10]. Out of the 1322 SOMAmer reagents, 12 hybridization controls, 4 viral proteins (HPV type 16, HPV type 18, isolate BEN, isolate LW123), and 5 SOMAmers that were reported to be non-specific (P05186 [ALPL], P09871 [C1S], Q14126 [DSG2], Q93038 [TNFRSF25], Q9NQC3 [RTN4]) were removed, thus leaving 1301 SOMAmer reagents in the final analysis. The experimental process for proteomic assessment and data normalization has been previously described [11, 12]. The data reported are SOMAmer reagent abundance in relative fluorescence units (RFU). The abundance of the SOMAmer reagent represents a surrogate of protein concentration in the plasma sample. Data normalization was conducted in three stages. First, hybridization control normalization removes individual sample variance on the basis of signaling differences between the microarray or Agilent scanner. Second, median signal normalization removes inter-sample differences within a plate due to technical differences such as pipetting variation. Finally, calibration normalization removes variance across assay runs. Furthermore, there is an additional inter-plate normalization process that utilizes CHI calibrators that allows normalization across all experiments conducted at the CHI laboratory [11, 12].

Assessment of frailty

Frailty was defined based on five criteria "unintentional weight loss," "weakness," "lack of energy," "slowness," and "sedentariness" proposed by Fried and colleagues [2]. These criteria were operationalized as follows: (1) Unintentional weight loss assessed as self-reported unintentional weight loss of greater than 4.5 kg in the previous year, (2) weakness was assessed as being in the bottom sex-specific quintile of grip strength measured with a handheld dynamometer (Nicholas Muscle Tester; Sammon Preston, Inc., Chicago, IL), (3) lack of energy was based on self-reported feeling of exhaustion based on two questions from the Center of Epidemiology Studies-Depression scale (CES-D) [13], "I felt that everything I did was an effort" and "I could not get going," (4) slowness was based on gender and height and specific quintiles of usual walking speed assessed during a 4-m walking test, and (5) sedentariness was defined on self-reported physical activity of the past year with seven response levels: (i) Sedentary, hardly any physical activity, (ii) mostly sitting/some walking, (iii) light exercise 2-4 h/week, (iv) moderate 1–2 h/week, (v) moderate exercise > 3 h/week, (vi) intensive exercise many times/week, and (vii) walk over 5 km/day for 5 days for at least 5 years. These reposes were categorized into sedentary (i–ii), low (iii), moderate (iv), or high (v–vii). Assessment of lack and energy and sedentariness was assessed during an in-home interview by trained personnel, and assessment of unintentional weight loss, weakness, and slowness was performed during an outpatient visit at the study clinic within 3 weeks of the home interview. Volunteers with three or more of the five criteria were considered "frailty," and "pre-frailty" for frailty was defined as having one or two components, and "robust" for those reporting none of the components. Of the 997 volunteers with plasma proteomic data, frailty status was assessed in 752 (75%) volunteers that were 65 years of age and older.

Statistical analysis

Differences in baseline characteristics by frailty status were determined using *t* test for continuous variables and chi-square tests for categorical variables. Each protein RFU was logged then z-scored before analysis. Multiple logistic regressions were used to test the association between protein values and frailty status at baseline. For each protein, two logistic regressions were performed where the outcomes were either "frailty" vs "robust" or "pre-frail" vs "robust". All models were adjusted for age at baseline, sex, and study site (Greve vs Ripoli). For longitudinal analysis of frailty, we utilized follow-up assessment of frailty status in 397 volunteers who were free of frailty at baseline. Cox proportional hazards models were conducted to assess time to development of frailty. A second set of analyses were conducted assessing the worsening of frailty status by testing time to progression from robust to either pre-frailty or frailty status. For both logistic regression and Cox proportional hazard regressions, p values were adjusted using Benjamini-Hochberg False Discovery Rate (FDR), and FDR adjusted p values ≤ 0.05 were considered significant.

Next, we used partial least squares discriminant analysis (PLS-DA) to find sets of proteins associated with frailty. First, Synthetic Minority Over-sampling Technique (SMOTE) was applied to help correct imbalanced data. Because positive (frailty) cases are less prevalent than negative (no frailty) cases, the data for this study are imbalanced, which leads to inaccurate results. SMOTE creates new positive cases by perturbing positive cases (two at a time) to create a new positive case. PLS-DA is then performed in order to sharpen the separation between groups of observations, by rotating principal components analysis (PCA) components such that a maximum separation among classes is obtained, and to understand which variables carry the class separating information. The result of a PLS-DA algorithm is made of different components, each containing a subset of the entire set of 1301 proteins measured in each subject. Each component will have an accompanying set of weights for each of the proteins. A negative weight means that higher levels of that protein are associated with lower risk of frailty, and positive protein weights indicate that higher levels of that protein are associated with higher risk of frailty. The larger the absolute value of the protein's weight, the larger the impact in either direction depending on the sign of the weight. The components can be used to explain the variance between sets. The error rate for the PLS-DA analysis indicates the proportion of volunteers which have not been correctly assigned to their frailty status. All analyses were performed using R Statistical Software version 3.5.0.

Results

Out of 1453 volunteers interviewed at baseline, proteomic profiles were available for 997 volunteers (69%) and, out of these, 752 volunteers (75%) older than 65 years were assessed for frailty status. The demographic characteristics of these volunteers with proteomic and frailty status are described in Table 1. The prevalence of volunteers with frailty or pre-frailty was 6% and 40%, respectively, at baseline. On average, volunteers with frailty were older, had fewer years of education, were female, and more likely to be sedentary ($p < 0.05$). Volunteers who were pre-frailty were in between frail and robust volunteers with respect to age and physical activity (Table 1). All subsequent analyses were conducted in 752 volunteers with data on plasma proteomic profile and frailty status at baseline.

Proteins associated with frailty status

Plasma proteins associated with frailty status at baseline were first identified using logistic regression. There were four proteins, creatine kinase type M (CKM), creatine kinase B (CKB CKM), chemokine (C-X-C motif) ligand 13 (CXCL 13), and thrombospondin-2 (THBS 2), significantly associated with frailty (FDR-$p \leq 0.05$; Supplemental Table 1; Fig. 1). Two of these proteins, CKM and CKB/CKM, are associated with decreased odds of frailty. A one standard deviation (SD) increase in CKM was associated with 60% less frailty (OR = 0.40, 95% CI 0.25–0.64). Similarly, for every one-SD increase in creatine kinase B (CKB CKM), volunteers were 60% less likely to be frail (OR = 0.44, 95% CI 0.29–0.67). In this dataset, there was high correlation between CKM and CKM/CKB ($r = 0.92$). As creatine kinases are marker of muscle, we adjusted for muscle mass, height, and fat mass to explore the effect of these variables on the model. While there was a slight change in the odds ratio towards the null, the effect was not greatly effected by inclusions of these variables in the model (CKM OR = 0.44, 95% CI 0.27–0.72). Two proteins, CXCL13 and THBS2, were positively associated with frailty status. A one-SD increase in CXCL13 was twice as likely to be frail (OR = 2.00, 95% CI 1.68–2.30) and, lastly, a subject having a one-SD increase in THBS2 was twice as likely to be frail (OR = 2.06, 95% CI 1.71–2.41). There were no significant proteins associated with being "pre-frailty" (Supplemental Fig. 1, Supplemental Table 1).

We tested whether these four proteins were associated with development of frailty in volunteers who were robust at baseline. We tested development of frailty in 397 volunteers who were free of frailty at baseline. Fifty-two volunteers developed frailty during the with average follow-up of 8.3 years (min = 2.8 years, max = 10.5 years; Supplemental Fig. 2). The four proteins (CXC13, THBS2, CKB, CKB/CKM) were not associated with development of frailty ($p > 0.05$; Supplemental Table 1). In fact, none of the 1301 proteins assayed were associated with development of frailty. In a second analysis, we tested whether these proteins were associated with transitioning to either "pre-frailty" or frailty. There were 278 volunteers who progressed to either "pre-frailty" or "frailty" status (Supplemental Table 1; Supplemental Fig. 3). The four proteins were not significantly associated with worsening of frailty status. Two proteins, cyclin-dependent kinase 5/cyclin-dependent kinase 5 regulatory subunit 1 (CDK5/CDK5R1; RR = 1.26 [1.12–1.41]), and interleukin 1 alpha (IL1A; RR = 1.25 [1.12–1.39]) were both associated with increased risk of worsening of frailty status over time.

Partial least squares discriminant analysis of frailty status

To better understand the association between all the measured proteins and frailty levels, partial least squares discriminant analysis (PLS-DA) was performed to identify components that best discriminate the two groups. Two components that accounted for 6% (component 1) and 17% (component 2) of the variance successfully classified "robust" volunteers from volunteers with frailty with an error rate of 0.02 (Fig. 2a). The most important proteins, with variable importance in projection value (VIP) greater than 3, were two probes targeting coagulation factor X (F10), coagulation factor XI (F11), TNF receptor superfamily member 6b (TNFRSF6B), kallikrein B1 (KLKB1), insulin-like growth factor binding protein 2 (IGFBP2), and C-C motif chemokine ligand 18 (CCL18) (Supplemental Table 2, Fig. 2b). Pathway enrichment analysis of 91 proteins with VIP greater than 2 shows that there is an enrichment in inflammation, blood coagulation, and cell growth (Supplemental Table 3).

Table 1 Characteristics of 752 volunteers from the InCHIANTI study

	All		Robust		Pre-frailty		Frailty		
n	752		405		302		45		
Age (years)	73.62	(6.46)	72.04	(9.5)	74.86	(6.85)	79.4	(6.82)	< 0.001
Study site (%Bagno a Ripoli)	396	(52.7%)	185	(45.7%)	185	(61.3%)	26	(57.8%)	< 0.001
Women n (%)	420	(55.9%)	192	(47.4%)	198	(65.6%)	30	(66.7%)	< 0.001
Education (years)	5.52	(3.35)	5.81	(3.35)	5.29	(3.34)	4.38	(3.04)	0.008
Smokers n (%)	107	(14.2%)	61	(15.1%)	39	(12.9%)	7	(15.6%)	0.697
Height (cm)	158.7	(9.4)	160.2	(9.4)	157.4	(9.1)	153.9	(8.5)	< 0.001
Muscle area (mm^2)	6357	(1257)	6553	(1223)	6187	(1227)	5602	(1354)	< 0.001
Fat area (mm^2)	1912	(1164)	1721	(1063)	2164	(1211)	2057	(1437)	< 0.001
Physical activity n (%)	< 0.001								
Sedentary	128	(17.1%)	0	(0%)	93	(30.9%)	35	(77.8%)	
Light	336	(44.9%)	200	(49.6%)	129	(42.9%)	7	(15.6%)	
Moderate	242	(32.3%)	176	(43.7%)	63	(20.9%)	3	(6.7%)	
High	43	(5.7%)	27	(6.7%)	16	(5.3%)	0	(0%)	

Discussion

This study explored the plasma proteomic signature of frailty phenotype in community-based samples of aging Italians. Using univariate models, we found that higher abundances of CKM and CKB/CKM were associated with reduced odds of experiencing frailty, while higher abundances of CXCL13 and THBS2 were associated with increased odds of frailty. Further, we showed that proteomic data can be used to discriminate between volunteers with and without frailty with high degree of accuracy. The proteins that most contributed to the separation of different frailty states reflected inflammation, cell growth, and blood coagulation pathways.

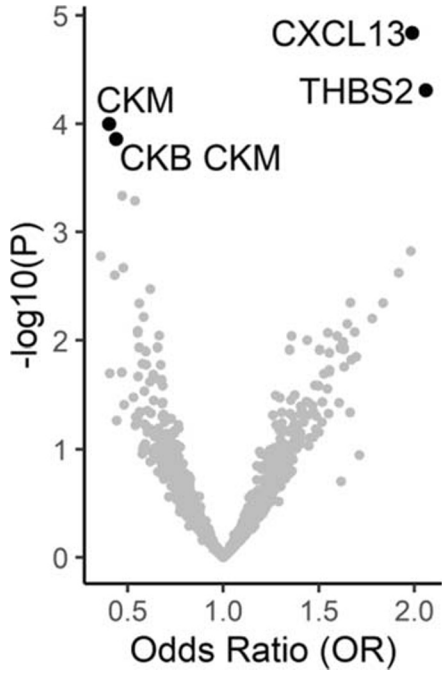

Fig. 1 Association of 1301 plasma proteins with frailty status. We tested the association of 1301 plasma proteins with frailty using logistic regression adjusted for age at baseline and sex. The volcano plot displays the odds ratio against the significance presented as -log10 (p value). Proteins were considered significantly associated at FDR adjusted p values < 0.05 (black circles)

Two isoforms of creatine kinase (CK) were found to be negatively associated with frailty. CK are enzymes that are essential in the formation of energy and are crucial in tissues that require high energy input [2]. The active enzyme is a dimer comprised of two proteins CKM and CKB [2]. In animal models of frailty, there is an impairment of ATP kinetics in muscles of frail animals compared to controls [14], and concurrently gene expression of CK is elevated in the skeletal muscle [15]. In humans, circulating levels of CK has been used as a biomarker of muscle damage [16] and in older individuals, lower levels of CK have been found in volunteers with sarcopenia [17]. In our study, adjustment for muscle mass did not have a great affect on the point estimates. These observations suggest that the negative association of CK with frailty found in this study may be due to other mediating factors than muscle composition.

The two proteins THBS2 and CXCL13 were associated with increased risk of frailty. Thrombospondins are a family of thrombin-sensitive glycoproteins secreted by

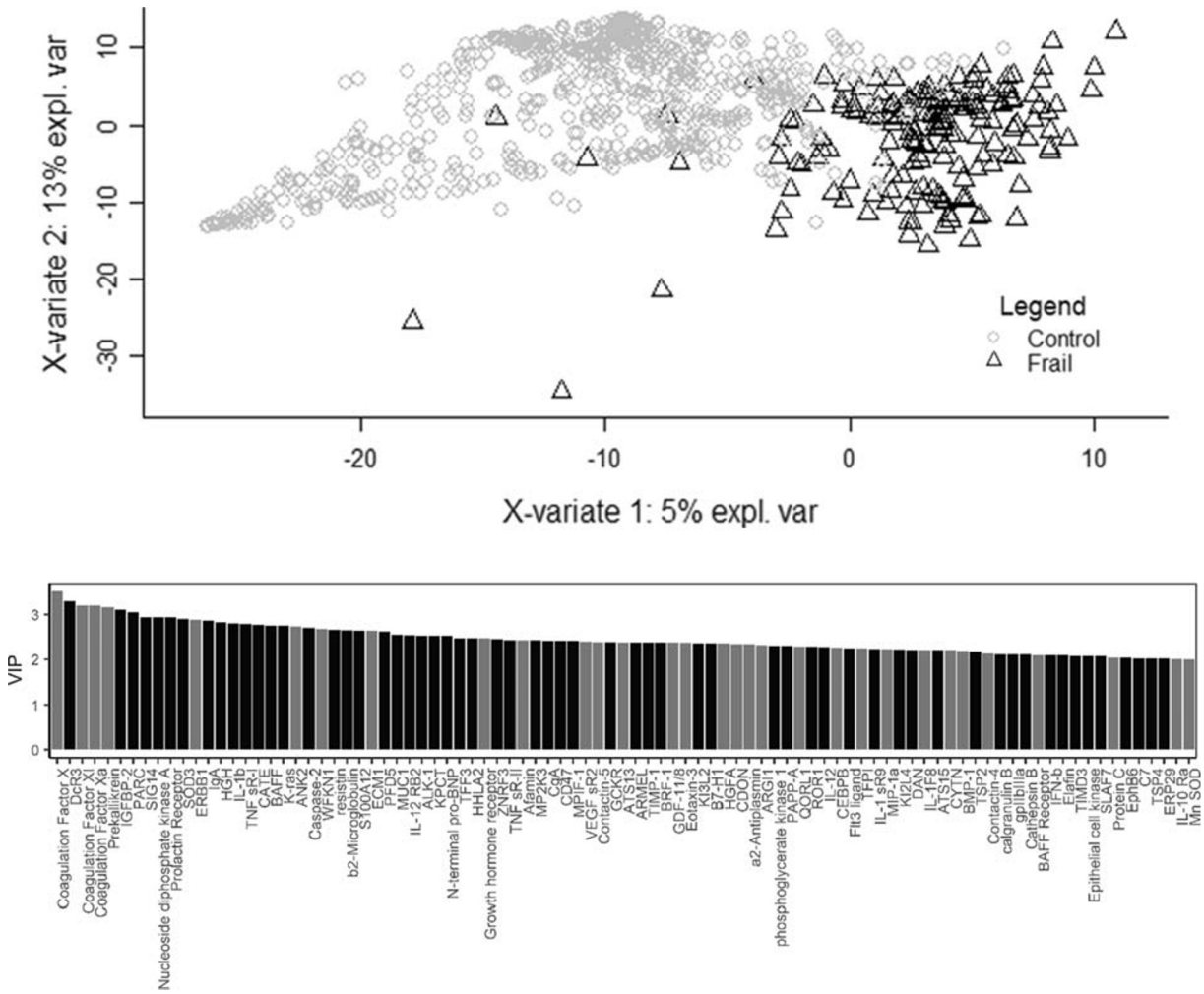

Fig. 2 PLS-DA analysis using proteomic data. **a** The scatter plot displays the volunteers by two components from the PLS-DA analysis, and shows discrimination based on their frailty status with an error rate of 2%. Volunteers with frailty are represented with triangles, and those without frailty with circles. **b** Ordered list of 91 proteins with higest discrimination ability based on VIP of greater than 2 are displayed. The gray bars represent proteins that are associated with reduced odds of prevalent frailty, while black bars represent proteins that are associated with increased odds of frailty based on the logistic regression models

activated platelets [18]. THBS2 is an endogenous inhibitor of angiogenesis and inflammation through its effects on endothelial cell migration, proliferation, survival, and apoptosis [19, 20]. In humans, circulating THBS2 has been associated with aging diseases such as chronic kidney disease, rheumatoid arthritis, and heart failure [20, 21]. The chemokine CXCL13, coupled with its G protein receptor, CXCR5, was first identified in gene expression studies of malignant B cell and shown to be important in development of differentiated B-cells [22]. CXCL13 has multiple effects on inflammation, infection, and immune response through activation of PI3K/Akt, MEK/ERK, and Rac pathways. The CXCL13/CXCR5 axis has been extensively studied in relation to cancer, infectious diseases such as HIV, and auto-immune diseases [23]. As chronic inflammation is hypothesized to be one of the underlying causes of frailty, THBS2 and CXCL13 are good candidate biomarkers of frailty through their role in inflammation.

Two proteins were associated with progression of frailty in volunteers who were free of frailty at baseline. Interleukin 1 alpha (IL1A) is a member of the IL-1 family involved in inflammation [24]. As with THBS2 and CXCL13, the association of IL1A with progression of frailty is consistent with inflammation being one of the central drivers of frailty. Cyclin-dependent kinase 5

(CDK5) is a serine/threonine kinase expressed predominantly in the brain that can be activated by p35 (or cyclin-dependent kinase 5 regulatory subunit 1 (CDK5R1)) or p39 [25]. CDK5 activity plays an important role in brain development, and has been shown to effect spatial learning and memory impairments [26] and dopamine signaling in animal models [27]. CDK5 is also a target protein in Alzheimer's disease, as this kinase has also been implicated in b-amyloid and tau pathology [28, 29]. Higher burden of brain pathology has been implicated in progression of frailty [30]. Further, there is evidence from epidemiological studies that cognitive impairment and frailty are associated, and each increases the risk for the other. Specifically, cognitive impairment increases the risk for developing frailty [31, 32] and conversely, having frailty can exacerbate cognitive decline [33]. Taken together, CDK5 may influence development of frailty through its effect in the brain.

Using PLS-DA, the proteomic data were able to accurately discriminate between frail and non-frail volunteers. The most important proteins with high VIP values included two aptamers for coagulation Factor X (F10), TNFRSF6B, and Coagulation Factor XI. The link between coagulation and frailty has been previously explored [34]. In particular, coagulation factors such as coagulation factor VIII have been shown to increase with age [35]. Heightened levels of coagulation factor X and XI may be responsible for an increased risk of developing cardiovascular disease, which is more common among frail patients compared to control patients [35]. In addition to cardiovascular health, elevation in coagulation has been associated with increased risk for developing mobility disability even after adjustment for cardiovascular health [36]. This observation supports that elevated blood coagulation is an important underlying mechanism of frailty, consistent with the enrichment of coagulation proteins among the frailty discriminating factors.

The enrichment analysis of frailty discriminatory proteins in this study points to the key role of inflammatory proteins. Chronic inflammation is one of the most important underlying mechanisms of aging, and consequently of frailty [37]. Studies have consistently shown elevation of inflammatory markers such as white blood cell count, IL-6, and CRP in subjects with frailty [5]. While some of these classical inflammatory biomarkers were not among the top proteins associated with frailty in this study, our data are consistent with the notion that frailty is a pro-inflammatory state.

There has been one study examining the association of plasma proteins with frailty using a complementary aptamer-based assay [38]. In this study, the association of 4625 plasma proteins with frailty index (FI) in 880 individuals in the LonGenity cohort was reported. It was found that the fatty acid binding proteins (FABP, and FABPA), leptin, and ANTR2 were the most significant FI-associated proteins. There was also an enrichment in proteins from lipid metabolism, musculoskeletal development and function, cell-to-cell signaling and interaction, cellular assembly, and organization pathways. Finally, the authors developed a frailty prediction model with 110 plasma proteins that was correlated with observed FI ($r = 0.57$). Of the proteins associated with frailty phenotype reported in this study, only THBS2 was associated with FI in the Longenity study. The differences in the study results are not surprising. The frailty index (FI), developed by Rockwood and colleagues, is a continuous construct based on a unspecified checklist of clinical measures that reflects the accumulation of deficits [39]. This is in contrast to the frailty phenotype used in this study that is a categorical variable derived from five set criteria [2]. The FI and frailty phenotype can be thought of as complementary yet independent measures of frailty [40]. Given this, the results from our report should complement the report by Sathyan and colleagues towards a more comprehensive characterization of plasma proteins that are important in frailty states.

This study has several strengths. First, this study was conducted in a well characterized population that is representative of older Italian population. Proteomic profiles were measured in plasma, which is easily accessible and, if confirmed, could potentially serve as a biomarker for frailty in the clinic. Finally, this analysis included both cross-sectional and longitudinal analyses of frailty and shows that different proteins are associated with prevalent frailty status and future risk of frailty in healthy volunteers. These observations should be futher explored and validated in other studies. This study also has several limitations. First, the aptamer assay measured 1301 proteins, which does not reflect all proteins found in plasma. Currently, there are newer version of the panel with over 5000 proteins; thus, a study using such panel will be important. Second, the number of volunteers with frailty status in this population was small. Provided these limitation, it is important that

future studies are conducted to confirm the proteins identified in this study in an independent cohort with larger sample size and with longitudinal data to provide added confidence in our findings. Another important confirmation step is to conduct a proteomic study using different protein assays that could compliment the findings from this study. It would also be important to explore different frailty construct such as the frailty phenotype and FI to build a comprehensive list of proteins that are associated with frailty states.

In summary, we report several plasma proteins in association with frailty status in older participants of the InCHIANTI study. In addition to identifying CK and inflammatory proteins CXC13 and THBS2 that appear independently associated with frailty status, we demonstrate that proteomic data are able to discriminate individuals who have frailty from those who are robust with a high degree of accuracy. The proteins that were most discriminating of frailty status represented proteins in inflammation, blood coagulation, and cell growth pathways. Further studies are needed to confirm our findings and further determine whether these proteins can indeed be used as potential biomarkers for the development of frailty.

Funding "The InCHIANTI study baseline" (1998–2000) was supported as a "targeted project" (ICS110.1/RF97.71) by the Italian Ministry of Health and in part by the U.S. National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821336); the InCHIANTI Follow-up 1 (2001–2003) was funded by the U.S. National Institute on Aging (Contracts: N.1-AG-1-1 and N.1-AG-1-2111); the InCHIANTI Follow-ups 2 and 3 studies (2004–2010) were financed by the U.S. National Institute on Aging (Contract: N01-AG-5-0002);supported in part by the Intramural Research Program of the National Institute on Aging, National Institutes of Health, Baltimore, Maryland.Data availabilityAccess to proteomic data is available upon review and subsequent approval of proposals submitted through the InCHIANTI study website (inchiantistudy.net).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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