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Original article

Proteomics of the phase angle: Results from the population-based KORA S4 study

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SUMMARY

Background & aims: The phase angle (PhA) measured with bioelectrical impedance analysis is considered to reflect the interrelated components body cell mass and fluid distribution based on technical and physical aspects of the PhA measurement. However, the biomedical meaning of the PhA remains vague. Previous studies mainly assessed associations of the PhA with numerous diseases and health outcomes, but few connected protein markers to the PhA. To broaden our understanding of the biomedical background of the PhA, we aimed to explore a proteomics profile associated with the PhA and related biological factors.

Methods: The study sample encompassed 1484 participants (725 women and 759 men) aged 55–74 years from the population-based Cooperative Health Research in the Region of Augsburg (KORA) S4 study. Proteomics measurements were performed with a proximity extension assay. We employed boosting with stability selection to establish a set of markers that was strongly associated with the PhA from a group of 233 plasma protein markers. We integrated the selected protein markers into a network and enrichment analysis to identify gene ontology (GO) terms significantly overrepresented for the selected PhA protein markers.

Results: Boosting with stability selection identified seven protein markers that were strongly and independently associated with the PhA: N-terminal prohormone brain natriuretic peptide (NT-proBNP), insulin-like growth factor-binding protein 2 (IGFBP2), adrenomedullin (ADM), myoglobin (MB), matrix metalloproteinase-9 (MMP9), protein-glutamine gamma-glutamyltransferase 2 (TGM2), and fractalkine (CX3CL1) [beta coefficient per 1 standard deviation increase in normalized protein expression values on a log 2 scale (95% confidence interval): −0.12 (−0.15, −0.08), −0.13 (−0.17, −0.09), −0.14 (−0.18, −0.10), 0.10 (0.07, 0.14), 0.07 (0.04, 0.10), 0.08 (0.05, 0.11), −0.06 (−0.10, −0.03), respectively]. According to the enrichment analysis, this protein profile was significantly overrepresented in the following top five GO terms: positive regulation of cell population proliferation (p-value: 1.32E-04), extracellular space (p-value: 1.34E-04), anatomical structure formation involved in morphogenesis (p-value: 2.92E-04), regulation of multicellular organismal development (p-value: 5.72E-04), and metal ion homeostasis (p-value: 8.86E-04).

Conclusion: Implementing a proteomics approach, we identified six new protein markers strongly associated with the PhA and confirmed that NT-proBNP is a key PhA marker. The main biological processes that were related to this PhA's protein profile are involved in regulating the amount and growth of

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cells, reinforcing, from a biomedical perspective, the current technical-based consensus of the PhA to reflect body cell mass.

1. Introduction

The phase angle (PhA) measured with bioelectrical impedance analysis (BIA) is considered to generally reflect the interrelated components body cell mass (BCM) and fluid distribution, i.e. ratio of extracellular to intracellular fluid volume (ECF/ICF) [1]. Thereby, an increased PhA is related to an increased body cell mass (BCM) (which includes ICF [1]) with concurrently decreased ECF/ICF [2]. Generally, the PhA does not encompass a standardized assignment to a specific disease nor a distinct biomedical meaning. Among numerous outcomes that have been examined, the PhA has been observed to be a prognostic factor for mortality [3] and falls [4] and participants with a low PhA have been observed to encompass a higher prevalence of sarcopenia [5]. Primarily though, the PhA has been described as an indicator of malnutrition based on the assumption that an impaired nutritional status affects the fluid distribution displayed in the displacement of water from intracellular to extracellular space [2]. Consequently, BCM decreases with concurrently increased ECF, leading to lower PhA values [2].

In contrast to the vaguely described biological and medical meaning, the technical aspects of the PhA measurement can be clearly described as follows. The opposition of conductors in the body to an alternating electric current during BIA measurements is expressed as the impedance, which is influenced by the resistance (resistive components: fluid and electrolytes) and reactance (capacitive components: tissue interfaces and cell membranes) [1,6]. In resistive components, an alternating current passes consistently, while in capacitive components, the current flow is delayed as capacitors can temporarily store electrical charge [1]. These detaining properties evoke a time delay of the current waveform behind the voltage waveform, which is expressed in degrees as the PhA [4,5]. In over-hydration, characterized as increased ECF (resistive component) relative to BCM [1], PhA values decrease [2]. As the PhA reflects the amount of current passing through capacitive components (reactance), which is relative to the current's frequency, PhA values are frequency-dependent [1]. At low frequencies, the largest part of the current passes through ECF to avoid cells due to their large capacitive reactance, whereas at higher frequencies, current penetration through cells augments [7]. This is relevant when comparing PhA values of different device technologies such as phase-sensitive single-frequency (50 kHz) BIA and bioelectrical impedance spectroscopy (BIS), which measures the impedance for a range of frequencies [1].

Approaches to explore the biological and medical meaning as well as potential applications of the PhA beyond body composition include the identification of associations to health outcomes, diseases, and protein markers. While associations to health outcomes and diseases have been studied extensively, only few studies have yet connected protein levels to the PhA. Based on inverse associations of the PhA with the proteins interleukin-6 (IL-6), tumor necrosis factor alpha, and C-reactive protein (CRP), a Brazilian study recognized a possible link to inflammation in elderly women [8]. Of note, inflammation has been described as a crucial link between the PhA and its main application malnutrition. This is indicated by the impact of inflammation on the fluid status as well as the presence of inflammation in diseases affecting malnutrition [2]. Moreover, the proinflammatory marker CRP was inversely related to the PhA in various other populations [9–11]. IL-6 was inversely related to the PhA in obese women [12] and an increase in IL-6 was associated with a decrease in PhA over two years in patients on maintenance hemodialysis after controlling for fat mass and extracellular water [13]. The consistent findings of the inverse association between inflammation markers and the PhA have been explained with the characteristic of inflammation processes to induce cellular and tissue damage [10]. Previous results regarding the relation of the PhA with the obesity marker leptin have been inconsistent [11,12,14].

Through novel proteomics technologies, a large number of protein markers is accessible to expand and accelerate the identification of markers associated with the PhA; thereby contributing to a larger, long-term goal to broaden the understanding of the PhA's biomedical meaning and possible applications. Our goal was, therefore, to employ proteomics to explore biomedical factors of the PhA through first, the identification of protein markers associated with the PhA and second, the identification of biological processes, molecular functions, and cellular components related to the PhA's protein profile.

2. Material & methods

2.1. Study population

We analyzed data from the population-based Cooperative Health Research in the Region of Augsburg (KORA) S4 study encompassing 4261 residents from Southern Germany [15]. Of the KORA S4 study cohort, 4178 participants had complete BIA measurement data. Proteomics measurements were only planned for the age group 55–74 years (n = 1653). After exclusion of participants with missing proteomics values, 1566 participants remained. We further excluded participants, who indicated that they had cancer within the last 12 months before the S4 study as well as those, who indicated that they did not know it or did not answer the respective question [16], because we observed cancer to be strongly associated with the PhA in the pre-analysis. We did not exclude further participants based on disease status. From the remaining 1520 participants, we further excluded participants that entailed missing values for any of the covariates. Afterwards, the final data set comprised of 1484 participants (725 women and 759 men).

2.2. Proteomics

The proteomics data set used within this article has been used before for analyzing proteomics of muscle and fat mass [16]. The plasma samples for the proteomics measurements were collected from the participants during the KORA S4 study in 1999–2001 and were analyzed in 2019–2020. Blood sampling and BIA measurements were performed on the same day. We performed analyses with cardiovascular disease (CVD)- and inflammation-related plasma protein markers measured with proximity extension assay (PEA) technology, a targeted proteomics assay. With PEA, the

proteins are identified by employing pairs of antibodies, which attach to the proteins. The antibodies are labeled with deoxyribonucleic acid oligonucleotides, which are specific for each protein marker. If the oligonucleotides are in close proximity and the pair is a correct match, they bind together, followed by polymerization. The product is then quantified by microfluidic real-time polymerase chain reaction [17].

The panels we assessed for protein marker measurement were Olink® CVDII, CVDIII, and Inflammation (Olink Proteomics, Uppsala, Sweden). All three panels each covered 92 markers as log 2-normalized protein expression (NPX) values divided by their respective standard deviation, calculated in the complete data set prior to exclusions [16]. Out of these 276 protein markers, 233 remained for the final analysis according to the following criteria: We excluded protein markers due to values below the limit of detection (LOD) in >25% of the complete data set prior to exclusions, duplicate marker measurements in two of the three panels, and missing values (only observed in the panel CVDIII). We maintained the values < LOD of the remaining 233 markers and did not exchange these values [16]. Further details regarding the proteomics data set and a complete list of all protein markers can be found in our previous manuscript and the respective supporting information [16].

We assessed the inflammation and CVD protein marker panels for associations with the PhA as inflammatory markers have been frequently associated with the PhA as highlighted in the introduction. Furthermore, the PhA is considered to indicate fluid distribution, which plays an important role in CVD entities such as heart failure (HF) and hypertension. Additionally, a number of previous studies have suggested a link between the PhA and CVD [18]. However, the allocation of a protein to these marker panels does not preclude strong relations of these proteins to other disease entities.

2.3. PhA

The PhA, used as the outcome of the present study, was assessed by BIA with the phase-sensitive device BIA 2000-S (DATA-INPUT GmbH, Frankfurt, Germany), which employs a measurement frequency of 50 kHz and a current of 800 μA to measure the resistance and reactance. The PhA is then derived by the device using the resistance and reactance. PhA values were gathered from the BIA directly and were not calculated by us.

Before the measurement, participants were asked to empty their bladder. The measurement was performed in supine position and the participants were asked to relax, avoid movement, spread their hands in flat position, and spread their arms and legs apart to avoid contact to other body parts. The participants were connected to the BIA through attaching four skin electrodes to their hand (two electrodes) and foot (two electrodes) of their dominant side. Thereafter, the BIA generated a weak, alternating current conducting through the participants' bodies. The accuracy of the BIA measurement was tested daily before the first and after the last use of the BIA with a test resistor. In line with the recommendation of the manufacturer according to the instruction manual, deviations of ±4 Ω were within the tolerated range [resistance (R) = 500 (±4) Ω and reactance (Xc) = 144 (±4) Ω]. The BIA measurement was performed two times for each participant and the mean value of the PhA was used for analysis. Occurrence of potential technical error for intra-rater repeated measurements was assessed directly after the two measurements for each participant. If the R or Xc values of the two measurements differed substantially (R > 5 Ω and Xc > 2 Ω), measurements were repeated (two new measurements) with a prior check for accuracy using the test resistor as described above.

2.4. Covariates

In a standardized face-to-face interview, trained medial staff assessed the sociodemographic and lifestyle variables [15]. The covariates of this analysis included age, high-density lipoprotein (HDL) cholesterol, triglycerides, glycated hemoglobin (HbA1c), estimated glomerular filtration rate (eGFR), albumin, body mass index (BMI) (all continuous), sex (female/male), smoking status (never/former/current smoker), and fasting status of more than 8 h (yes/no). We transformed triglycerides with natural logarithmic transformation due large discrepancies from normal distribution. In a sensitivity analysis, we further included the variables hypertension (no/yes), myocardial infarction (no/yes), and intake of antihypertensive medication (no/yes). We described details regarding the measurements of HDL cholesterol, triglycerides, HbA1c, eGFR, albumin, smoking status, and hypertension in the supporting information elsewhere [16]. Myocardial infarction (hospitalized) and intake of antihypertensive medication were self-reported by participants during the standardized interview.

2.5. Statistical analysis

The aim of the analysis consisted of the identification of protein markers that were strongly associated with the PhA as well as incorporating these markers into a network and enrichment analysis to determine biological processes, molecular functions, and cellular components related to the PhA.

We performed boosting with stability selection [19] with the R package mboost [20] for boosting and the R package stabs [21] for stability selection using R, V.4.0.5 [22]. This method encompasses the merging of a component-wise functional gradient descent boosting on a linear regression model together with stability selection, which implements a resampling method and allows to control for false discoveries [19]. The selection of the variables is based on a cut point for the selection frequency of each variable. The cut point is determined by the algorithm parameters comprising of the number of variables that were available to be selected (here, n = 233), number of variables selected within each iteration (here, n = 15), and the maximum number of tolerable false positives (here, n = 2) [16,19]. In our analysis, the cut point was 63%, which lies within the suggested range [19]. We conducted the stability selection with the assumption "unimodal" and the sampling type complementary pairs. We calculated the boosting with an offset that included a model of all 10 covariates (model 2), which enabled us to select protein markers that were independently associated with the PhA. Afterwards, we assessed a linear regression model including all protein markers that were selected by boosting with stability selection plus the 10 covariates to identify beta coefficients and directions of association [16]. In a sensitivity analysis, we assessed the influence of hypertension (n = 1482, 2 missing values), myocardial infarction (n = 1484), and intake of antihypertensive medication (n = 1481, 3 missing values) on the association between NT-proBNP (selected marker by boosting with stability selection) and the PhA by further adjusting the linear regression model 2 separately for the three variables.

In a second step, we integrated the protein markers selected by boosting with stability selection into an enrichment analysis and created a functionally grouped network with ClueGo v2.5.8 [23] and Cluepedia v1.5.8 [24] in Cytoscape v3.8.2 [25]. In this regard, we employed the hypergeometric test with Bonferroni step down correction to identify gene ontology (GO) terms for which the protein markers were significantly (p-value $\leq$ 0.05) over-represented. The data sources consisted of GO biological process, cellular component, and molecular function, all retrieved on July 14, 2021 in ClueGo v2.5.8 [23]. We allowed GO term fusion and GO

tree levels 3 to 20. We only permitted selection of GO terms that were associated with at least four of our selected protein markers. The required proportion of selected markers in relation to all existing proteins that were associated with a GO term was set to 0%, due to the low number of protein markers included in the enrichment analysis. All other parameters remained in the default settings. We focused our main results on the top five most significant GO terms to obtain a clear visualization of the results.

Our analytical approach was guided by recent publications analyzing proteomics data in breast cancer and HF [26–28].

We additionally performed the complete analysis again, this time, with adjustment for the covariates age and sex only (model 1), and compared the results to the main analysis (model with all 10 covariates, model 2).

We employed a sparse selection method with error control (boosting with stability selection) to identify protein markers associated with the PhA in order to obtain a specific marker profile and to minimize false-positive marker selection. In addition, selecting a high percentage of markers from the original proteomics data set could have led to the identification of GO terms that generally reflect the pattern of markers in the data set. Our proteomics data set specifically comprised markers of inflammation and CVD. As the aim of this analysis was to identify GO terms related to the PhA and not the specific proteomics pattern of the data set, a sparse and accurate marker selection was required.

3. Results

The characteristics of the study population (n = 1484) are listed in Table 1.

3.1. Selected protein markers associated with the PhA

Boosting with stability selection analysis selected seven protein markers that were strongly associated with the PhA after adjusting for all 10 covariates (model 2). N-terminal prohormone brain natriuretic peptide (NT-proBNP), insulin-like growth factor-binding protein 2 (IGFBP2), adrenomedullin (ADM), and fractalkine (CX3CL1) were inversely associated with the PhA, whereas myoglobin (MB), matrix metalloproteinase-9 (MMP9), and protein-glutamine gamma-glutamyltransferase 2 (TGM2) demonstrated positive associations with the PhA. The results of the boosting with stability selection as well as of the linear regression analysis are listed in Table 2. Adjusting for age and sex only (model 1), boosting with stability selection again selected seven protein markers, five of which (NT-proBNP, IGFBP2, MB, MMP9, and TGM2) were equivalent to the main analysis that included all 10 covariates (model 2).

Results of the sensitivity analysis, further adjusting the association between NT-proBNP and the PhA in the linear regression model 2 separately for hypertension, myocardial infarction, and intake of antihypertensive medication, yielded the following beta coefficients (95% confidence interval) for NT-proBNP: −0.12 (−0.15, −0.08), −0.12 (−0.16, −0.08), −0.11 (−0.15, −0.08), respectively.

3.2. Biological factors of the PhA's protein profile

The enrichment analysis identified that the set of selected protein markers associated with the PhA (Table 2, model 2) was significantly overrepresented in 20 GO terms, ranked by their p-values corrected with Bonferroni step down in Table 3. Figure 1 illustrates the functionally grouped network of the top five most significant GO terms and their associated protein markers. Positive regulation of cell population proliferation was the most significant GO term of the PhA-associated protein marker set (Table 3).

Table 1
Characteristics of the study population.

Characteristic	N = 1484
Age (years) [a]	63.9 ± 5.4
Sex, n (%)	
Female	725 (49)
Male	759 (51)
Triglycerides (mmol/L) [b]	1.40 (1.01)
HDL cholesterol (mmol/L) [a]	1.49 ± 0.42
HbA1c (mmol/mol) [a]	39.5 ± 7.9
HbA1c (%) [a]	5.8 ± 0.7
eGFR (ml/min/1.73 m^2) [a]	82.4 ± 13.3
Albumin (g/L) [a]	38.2 ± 3.9
BMI (kg/m^2) [a]	28.5 ± 4.3
Hypertension, n (%) [c]	
No	647 (44)
Yes	835 (56)
Myocardial infarction, n (%) [d]	
No	1418 (96)
Yes	66 (4)
Stroke, n (%) [e]	
No	1444 (97)
Yes	40 (3)
Intake of antihypertensive medication, n (%) [f]	
No	942 (64)
Yes	539 (36)
Smoking status, n (%)	
Never	711 (48)
Former	563 (38)
Current	210 (14)
Fasting state >8 h, n (%)	
Yes	1321 (89)
No	163 (11)
PhA (°) [a]	5.8 ± 0.8

BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; PhA, phase angle.
[a] Continuous variables are listed as arithmetic mean ± standard deviation.
[b] The natural logarithmic transformed variable is listed as geometric mean (antilog of standard error).
[c] N = 1482, current hypertension based on ISH-WHO 1999 (≥140/90 mm Hg) or medically controlled, known hypertension.
[d] Hospitalized myocardial infarction (self-reported).
[e] Hospitalized stroke (self-reported).
[f] N = 1481.

By conducting the enrichment analysis with the markers selected by boosting with stability selection, this time adjusted for age and sex only (model 1), the GO terms extracellular space, positive regulation of cell population proliferation, and anatomical structure formation involved in morphogenesis were again selected as the top three GO terms (Table 4). All eight selected GO terms are listed in Table 4.

4. Discussion

To our knowledge, this is the first study to explore a proteomic profile of the PhA. We identified four protein markers that were inversely (NT-proBNP, IGFBP2, ADM, and CX3CL1) and three markers that were positively (MB, MMP9, and TGM2) associated with the PhA. To our knowledge, all protein markers except NT-proBNP have been identified as markers of the PhA for the first time. Positive regulation of cell population proliferation was the most significant biological process associated with the PhA marker set.

4.1. Selected protein markers associated with the PhA

We identified NT-proBNP along with IGFBP2 as the most important protein markers of the PhA. Both markers were inversely

Table 2
Protein markers associated with the PhA selected by boosting with stability selection.

Boosting with stability selection		Linear regression model	
Selected variables	Selection frequency	β (95% CI)	p-value
Model 1			
NT-proBNP	100%	−0.12 (−0.15, −0.08)	5.69e-10
IGFBP2	100%	−0.16 (−0.20, −0.13)	<2e-16
MB	100%	0.14 (0.10, 0.18)	1.78e-13
TGM2	93%	0.07 (0.04, 0.10)	2.20e-05
MMP2	77%	−0.10 (−0.14, −0.06)	1.80e-07
DLK1	77%	0.05 (0.02, 0.09)	0.00452
MMP9	76%	0.07 (0.04, 0.10)	4.93e-05
Model 2			
NT-proBNP	100%	−0.12 (−0.15, −0.08)	3.10e-10
IGFBP2	100%	−0.13 (−0.17, −0.09)	5.08e-10
ADM	99%	−0.14 (−0.18, −0.10)	2.18e-10
MB	83%	0.10 (0.07, 0.14)	1.06e-08
MMP9	80%	0.07 (0.04, 0.10)	5.72e-05
TGM2	73%	0.08 (0.05, 0.11)	3.53e-06
CX3CL1	71%	−0.06 (−0.10, −0.03)	0.000874

Beta coefficients are listed per 1 standard deviation increase in normalized protein expression values on a log 2 scale.
Bold font indicates markers that were selected for both, model 1 and model 2.
We adjusted the boosting with stability selection and linear regression for covariates in two models:
Model 1: age and sex.
Model 2: model 1 + high-density lipoprotein cholesterol, triglycerides, glycated hemoglobin, estimated glomerular filtration rate, albumin, smoking status, body mass index, and fasting status.
The linear regression models were in addition to the covariates adjusted for all other protein markers listed in the table for the specific model, i.e. all markers that were selected by boosting with stability selection for model 1 and model 2, respectively.
ADM, adrenomedullin; β, beta coefficient; CI, confidence interval; CX3CL1, fractalkine; DLK1, protein delta homolog 1; IGFBP2, insulin-like growth factor-binding protein 2; MB, myoglobin; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; NT-proBNP, N-terminal prohormone brain natriuretic peptide; TGM2, protein-glutamine gamma-glutamyltransferase 2.

Table 3
GO terms of selected PhA-associated protein markers after full adjustment (model 2).

Rank	GO term	GO category	p-value corrected with Bonferroni step down	Associated protein markers
1	Positive regulation of cell population proliferation	Biological process	1.32E-04	ADM, CX3CL1, IGFBP2, MMP9, TGM2
2	Extracellular space	Cellular component	1.34E-04	ADM, CX3CL1, IGFBP2, MB, MMP9, NPPB, TGM2
3	Anatomical structure formation involved in morphogenesis	Biological process	2.92E-04	ADM, CX3CL1, MMP9, NPPB, TGM2
4	Regulation of multicellular organismal development	Biological process	5.72E-04	ADM, CX3CL1, MMP9, NPPB, TGM2
5	Metal ion homeostasis	Biological process	8.86E-04	ADM, CX3CL1, NPPB, TGM2
6	Inflammatory response	Biological process	1.51E-03	ADM, CX3CL1, MMP9, TGM2
7	Response to organic substance	Biological process	1.70E-03	ADM, CX3CL1, IGFBP2, MB, MMP9, TGM2
8	Homeostatic process	Biological process	2.17E-03	ADM, CX3CL1, MB, NPPB, TGM2
9	Response to organonitrogen compound	Biological process	4.24E-03	ADM, IGFBP2, MMP9, TGM2
10	Circulatory system development	Biological process	4.83E-03	ADM, CX3CL1, MB, NPPB
11	G protein-coupled receptor signaling pathway	Biological process	6.93E-03	ADM, CX3CL1, NPPB, TGM2
12	Regulation of apoptotic process	Biological process	1.09E-02	ADM, CX3CL1, MMP9, TGM2
13	System development	Biological process	1.21E-02	ADM, CX3CL1, MB, MMP9, NPPB, TGM2
14	Regulation of signal transduction	Biological process	1.33E-02	ADM, CX3CL1, IGFBP2, MMP9, TGM2
15	Regulation of transport	Biological process	1.61E-02	CX3CL1, MMP9, NPPB, TGM2
16	Apoptotic process	Biological process	1.97E-02	ADM, CX3CL1, MMP9, TGM2
17	Positive regulation of biological process	Biological process	2.16E-02	ADM, CX3CL1, IGFBP2, MMP9, NPPB, TGM2
18	Signal transduction	Biological process	2.35E-02	ADM, CX3CL1, IGFBP2, MMP9, NPPB, TGM2
19	Extracellular exosome	Cellular component	2.77E-02	IGFBP2, MB, MMP9, TGM2
20	Cell surface receptor signaling pathway	Biological process	3.51E-02	CX3CL1, IGFBP2, MMP9, NPPB

NT-proBNP is represented by NPPB, because there is no unique UniProt ID of NT-proBNP that could be included in the analysis. Instead, NT-proBNP belongs to the NPPBs in this analysis.
ADM, adrenomedullin; CX3CL1, fractalkine; GO, gene ontology; IGFBP2, insulin-like growth factor-binding protein 2; MB, myoglobin; MMP9, matrix metalloproteinase-9; NPPB, natriuretic peptides B; NT-proBNP, N-terminal prohormone brain natriuretic peptide; TGM2, protein-glutamine gamma-glutamyltransferase 2.

associated with the PhA. In line with our results, a Korean study observed in patients with stage 5 chronic kidney disease not undergoing dialysis that NT-proBNP inversely correlated with BIS-measured PhA using a device that measures the impedance of 50 frequencies (5–1000 kHz). PhA was described as the lag between voltage waveform at 50 kHz and current waveform [11]. Multifrequency BIA-measured PhA (frequency not indicated) was also inversely associated with NT-proBNP in hemodialysis patients in a longitudinal Dutch study [30]. From a clinical perspective, increased NT-proBNP is a marker of HF. In line with this, an article investigating PEA proteomics of HF detected NT-proBNP as one of the key markers for HF with a reduced ejection fraction (HFrEF) and

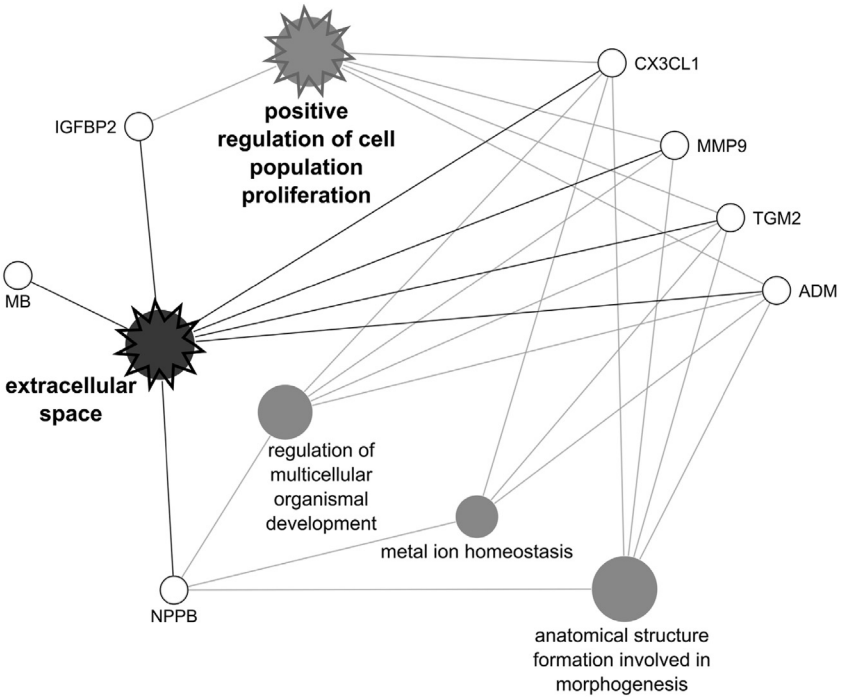

Fig. 1. Functionally grouped network of selected PhA-associated protein markers and their top five GO terms. GO terms are clustered in functional groups based on the kappa score, which considers the number of protein markers associated with two GO terms [29]. GO terms belonging to the same functional group are illustrated in the same color (black or grey). A star around the node (i.e. color-filled circle) and bold description indicate the GO term of each functional group with the highest significance. The size of the GO term node corresponds to the GO term p-value corrected with Bonferroni step down. A larger node indicates a higher significance. The white nodes represent the protein markers. NT-proBNP is represented by NPPB, because there is no unique UniProt ID of NT-proBNP that could be included in the analysis. Instead, NT-proBNP belongs to the NPPBs in this analysis. ADM, adrenomedullin; CX3CL1, fractalkine; GO, gene ontology; IGFBP2, insulin-like growth factor-binding protein 2; MB, myoglobin; MMP9, matrix metalloproteinase-9; NPPB, natriuretic peptides B; NT-proBNP, N-terminal prohormone brain natriuretic peptide; TGM2, protein-glutamine gamma-glutamyltransferase 2.

Table 4
GO terms of selected PhA-associated protein markers after adjustment for age and sex (model 1).

Rank	GO term	GO category	p-value corrected with Bonferroni step down	Associated protein markers
1	Extracellular space	Cellular component	8.31E-05	DLK1, IGFBP2, MB, MMP2, MMP9, NPPB, TGM2
2	Positive regulation of cell population proliferation	Biological process	2.31E-03	IGFBP2, MMP2, MMP9, TGM2
3	Anatomical structure formation involved in morphogenesis	Biological process	4.28E-03	MMP2, MMP9, NPPB, TGM2
4	Response to organic substance	Biological process	1.72E-02	IGFBP2, MB, MMP2, MMP9, TGM2
5	Animal organ development	Biological process	2.70E-02	MB, MMP2, MMP9, NPPB, TGM2
6	Signal transduction	Biological process	3.29E-02	DLK1, IGFBP2, MMP2, MMP9, NPPB, TGM2
7	Extracellular exosome	Cellular component	3.69E-02	IGFBP2, MB, MMP9, TGM2
8	Metal ion binding	Molecular function	4.68E-02	DLK1, MB, MMP2, MMP9, TGM2

NT-proBNP is represented by NPPB, because there is no unique UniProt ID of NT-proBNP that can be included in the analysis. Instead, NT-proBNP belongs to the NPPBs in this analysis.
DLK1, protein delta homolog 1; GO, gene ontology; IGFBP2, insulin-like growth factor-binding protein 2; MB, myoglobin; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; NPPB, natriuretic peptides B; NT-proBNP, N-terminal prohormone brain natriuretic peptide; TGM2, protein-glutamine gamma-glutamyltransferase 2.

proposed that relevant selected terms of the enrichment analysis of HFrEF relate to cell proliferation [27], resembling our results. Patients with HF are characterized by over-hydration (increased ECF relative to BCM) [1], which is associated with lower PhA [2], supporting the inverse association of NT-proBNP (i.e. HF marker) with the PhA. Additionally, besides (higher) NT-proBNP [31], (lower) PhA was employed as a marker of congestion in patients with acute decompensated HF [32] since congestion markers largely explained the data variability of PhA in patients with acute and chronic HF [33]. The inverse link between IGFBP2 and the PhA might be explained by the indirect impact of IGFBP2 on BCM due to regulating cell proliferation and growth via influencing the bioavailability of insulin-like growth factors (IGF) [34]. IGFBP2 predominantly inhibits IGF action [35], potentially resulting in lower BCM (and PhA). IGFBP2 was inversely associated with incident [36] and prevalent type 2 diabetes (T2D) [37]. As the PhA was

positively associated with T2D [38], this might support the inverse association between IGFBP2 and the PhA, potentially explained by higher skeletal muscle index in prevalent T2D [39]. In summary, lower IGFBP2 in T2D, which decreases the inhibition of IGF action, thereby increasing cell proliferation, could potentially result in increased muscle mass, BCM, and thus PhA.

Increased ADM has also been discussed as a potential marker of HF [40]. ADM might compensate fluid overload and high fluid volume could be indicated by increased ADM levels in plasma [40]. This reinforces the inverse association of ADM with the PhA, as a lower PhA is related to higher fluid overload (higher ECF/ICF) relative to BCM [1]. Next to volume overload [40], other stimuli of ADM synthesis are inflammation-related markers [41], which can induce cell damage, while cell damage can initiate inflammation [42], potentially decreasing the PhA.

In this article, MB, MMP9, and TGM2 were positively associated with the PhA, potentially through positive links with muscle mass and thereby BCM. Quadriceps muscle cross-sectional area (CSA) and BCM, which is closely related to the PhA [2], correlated positively with MB in healthy participants [43]. Moreover, MB, BCM, and CSA were lower in patients with cancer cachexia compared to healthy controls [43], a condition also exhibiting lower PhA values [44]. Biological functions of MB comprise oxygen storage as well as regulation of reactive oxygen species and mitochondrial function in the muscle [45]. MB appears positively related to cell mass and health, resembling the positive association with the PhA. MMP9 has been linked to the development of various diseases, in particular cancer [46] and CVD [47]. The positive association between MMP9 and the PhA might be supported by the observation that overexpressed MMP9 caused skeletal muscle hypertrophy in transgenic mice [48]. The positive association of TGM2 with the PhA could also be explained by muscle growth as TGM2 was observed to increase myotube protein synthesis and hypertrophy in mice skeletal muscle [49].

CX3CL1, inversely associated with the PhA in our study, promotes cell adhesion in transmembrane form, whereas in soluble form, the chemokine enhances cell survival. CX3CL1 was reported to encompass abilities to enhance tumors and metastasis and to promote anti-tumor immunity [50].

The PhA's protein profile encompassed markers that have been related to various disease entities and not to one specific disease or health outcome. This supports the previous broad application and research of the PhA for numerous health outcomes.

4.2. Biological factors of the PhA's protein profile

Due to a high number of selected GO terms, we focused on the five most significant and related terms. The most significant biological factor was positive regulation of cell population proliferation. In fact, the PhA is assumed to indicate BCM in relation to ECF/ICF [1], which could be affected by cell proliferation. Cell population proliferation could indicate physiological but also pathophysiological processes as for instance in cancer cells [51]. Also related to cell mass are the further top five GO terms anatomical structure formation involved in morphogenesis and regulation of multicellular organismal development. The PhA marker set was also associated with (regulation of) apoptotic process, which is again related to the amount of cell mass. The selected GO term extracellular space is somewhat contradictory to the biological processes mentioned above, as extracellular space does not contain cells. Nevertheless, higher ECF is related to lower PhA values [1]. The underlying reason for the selection of extracellular space might however, lie upon the fact that the protein markers included in the analysis might commonly be represented in extracellular space. Another top five biological process was metal ion homeostasis. Maintaining the metal ion homeostasis in the body is imperative, as metal ions are important for health, while being able to destruct proteins and DNA. Furthermore, the imbalance of the metal ion homeostasis can lead to cell death [52].

The PhA's protein profile was mainly associated with biological processes involved in influencing the amount of cell mass, supporting the consensus that the PhA is considered to indicate BCM [2]. In line with the protein marker set, the most significant biological factors are not specific to any pathophysiological area. The potential explanation could entail that factors influencing the cell growth and amount might affect various outcomes and not one specific disease.

4.3. Strengths and limitations

Limitations concern the generalizability of the data (primarily white Europeans aged 55–74 years) and the assessment of relative and not absolute values of the protein markers. The inflammation-/CVD-targeted proteomics set might not include other potentially relevant markers [16] and thus might have restricted identifying other relevant biological factors.

Strengths included the population-based design, the measurement of the PhA as a directly derived BIA parameter, and the implementation of boosting with stability selection to select markers limited the identification of false positive markers and thereby biological factors falsely related to the PhA. A strength of the data set included the simultaneously measured high number of markers.

5. Conclusion

Implementing a proteomics approach, we identified six new markers that were strongly associated with the PhA and confirmed that NT-proBNP is a key PhA marker. The main biological processes that were related to this PhA's protein profile are involved in regulating the amount and growth of cells, reinforcing, from a biomedical perspective, the current technical consensus of the PhA to reflect BCM.

Ethical statement

The ethics committee of the Bavarian Chamber of Physicians, Munich (EC No. 99186) approved the study. The ethical standards of the Declaration of Helsinki were fulfilled. All included participants signed a written informed consent.

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Author contribution

Marie-Theres Huemer: Conceptualization, Methodology, Formal analysis, Visualization, Writing - Original Draft; **Agnese Petrera:** Resources, Writing - Review & Editing; **Stefanie M. Hauck:** Resources, Writing - Review & Editing; **Michael Drey:** Writing - Review & Editing; **Annette Peters:** Resources, Funding acquisition, Writing - Review & Editing; **Barbara Thorand:**

Resources, Supervision, Writing - Review & Editing, Funding acquisition.

Data availability

The informed consent given by KORA study participants does not cover data posting in public databases. However, data are available upon request from KORA.PASST (https://helmholtz-muenchen.managed-otrs.com/external/) by means of a project agreement. Requests should be sent to kora.passt@helmholtz-muenchen.de and are subject to approval by the KORA Board.

Conflict of Interest

Marie-Theres Huemer, Agnese Petrera, Stefanie M. Hauck, Michael Drey, Annette Peters, and Barbara Thorand declare that they have no conflict of interest.

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